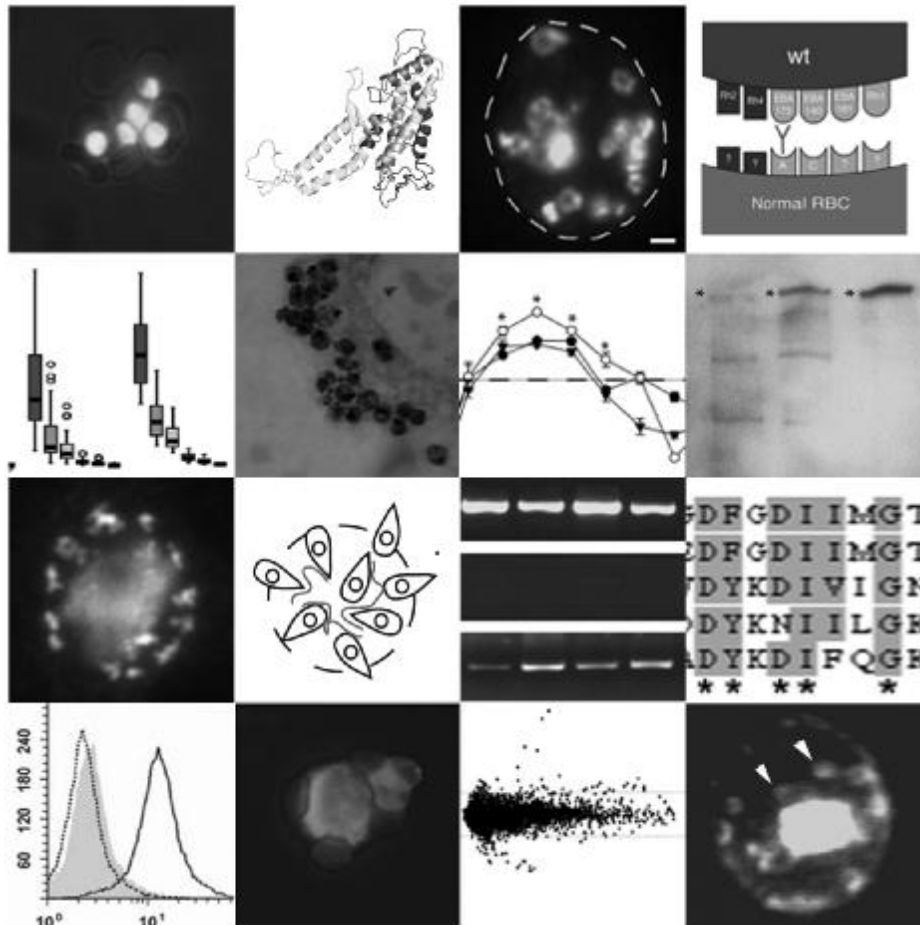


METHODS IN MALARIA RESEARCH



Fifth Edition

edited by

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Preface V

This is the fifth edition of Methods in Malaria Research. The first two were our “in-house” collections of protocols that we have found very useful in the lab. The fifth edition is a revised and expanded version of the fourth, which was produced with the help of the BioMalPar of EU and MR4 of ATCC, who also distribute the book online globally (www.biomalpar.org and www.malaria.mr4.org/publication.html). We hope you find our collection of malaria protocols useful.

The new edition contains around 30 new protocols and several of the old protocols have been updated and revised. We have kept the former layout giving a list of equipment and materials and reagents in the beginning of each chapter.

In assembling this book, we have tried to maintain a broad angle on what protocols to include that are relevant to malaria research, be it culturing of parasites, immunological assays, or molecular biological techniques. One criterion we have set is that the methods be well-tried by those sending us protocols. We have intended to give enough detail in the methodology that someone who is not an expert in the field of malaria will still be able to make use of these protocols. Naturally we hope those of you who are expert malariologists will also find this book beneficial and interesting.

We consider this book a “working document” of malaria protocols that, with the help of our readers/users, will continuously grow and evolve as new and improved methods are communicated to us. If you find mistakes or have a method you think is suitable for the next edition, please write to:

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Abbreviations

2-ME	2-mercaptoethanol (= β -mercaptoethanol)	MR4	Malaria Research & Reference Reagent Resources Center
AO	acridine orange	NDSB	nondetergent sulfobetaine
ATCC	American Type Culture Collection, Manassas, VA, USA	NHS	normal human serum
BrB	breaking buffer	NGS	normal goat serum
BSA	bovine serum albumin	NPP	nitrophenyl phosphate
CAT	Chloramphenicol acetyl-transferase	OD ₂₆₀	optical density at 260 nm
CGMRC	Centre for Geographic Medicine Research Coast	PAGE	polyacrylamide gel electrophoresis
CHEF	clamped homogeneous electric field	PARIF	parasite immunofluorescence
CLB	cell lysis buffer	PBMC	peripheral blood mononuclear cells
CMCM	complete malaria culture medium	PBS	phosphate-buffered saline
CPD	citrate phosphate dextrose	PCR	polymerase chain reaction
cpm	count per minute	RFP	red fluorescent protein
CSA	Chondroitin Sulfate A	PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
CSP	circumsporozoite protein	PFGE	pulsed-field gel electrophoresis
DAPI	4,6-diamidino-2-phenylindol	PHA	phytohaemagglutinin
DEPC	diethyl pyrocarbonate	PMSF	phenylmethylsulfonyl fluoride
DMSO	dimethyl sulfoxide	PRBC	parasitized red blood cell(s)
DTT	dithiothreitol	RBC	red blood cell(s)
EDTA	editic acid; ethylenediamine-tetraacetic acid	RESA	ring-stage erythrocyte surface antigen
EGTA	ethylene glycol-bis(β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid	RT	room temperature
ELISA	enzyme-linked immunosorbent assay	RT-PCR	reverse transcription polymerase chain reaction
EM	electron microscopy	SAP	shrimp alkaline phosphatase
EMEAS	erythrocyte membrane staining by enzyme linked antibodies	SDS	sodium dodecyl sulfate
EMIF	erythrocyte membrane immunofluorescence	SG	salivary glands
EthBr	Ethidiumbromide	SLO	StreptolysinO
FBS	fetal bovine serum	SNP	single nucleotide polymorphisms
FCS	fetal calf serum	SSC	sodium chloride/sodium citrate (buffer)
FITC	fluorescein isothiocyanate	TAE	Tris/acetate (buffer)
GDA	glutaraldehyde	TBA	transmission blocking assay
GDNA	genomic DNA	TBE	Tris/borate (buffer)
GFP	green fluorescent protein	TCM	tissue culture medium
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid	TE	Tris/EDTA (buffer)
HGG	human gamma globulin	TEMED	<i>N,N,N',N'</i> -tetramethyl-ethylene-diamine
HS	human serum	TEN	NaCl in TE buffer
IMCM	incomplete malaria culture medium immunoprecipitation	TH	Tris-buffered Hanks' solution
IP		TO	thiazole orange
IPTG	isopropyl-1-thio- β -D-galactopyranoside	Tris	tris(hydroxymethyl)amino-methane
KEMRI	Kenya Medical Research Institute	TSE	Tris/sodium chloride/EDTA solution
LB	Luria broth	USP	United States Pharmacopeia
MAP	multiple antigen peptide	WB	washing buffer
MCM	malaria culture medium	WBC	white blood cell(s)
MOPS	3-(<i>N</i> -morpholino)propane sulfonic acid	X-gal	5-bromo-4-chloro-3-indolyl- β -galactoside

PARASITES

I. Culturing of erythrocytic asexual stages of *Plasmodium falciparum* and *P. vivax*

I:A. The candle-jar technique of Trager–Jensen

by **Fingani Mphande**, **Sandra Nilsson**¹, and **Ahmed Bolad**²

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Equipment

incubator (37 °C)
glass desiccator (e.g., candle jar)
cell culture flask
candles
centrifuge
sterile pipettes
sterile tubes
glass slides and coverslips
microscope, fluorescence or light

Materials and reagents

purified erythrocytes (or human blood type O⁺ in CPD-adenine (Terumo) or S.A.G.M. ("Sagman" solution or EDTA)
MCM (see below)
Tris (Sigma)
Albumax II (Gibco)
RPMI 1640 (Gibco)
gentamicin
1 M HEPES (Gibco)
Hanks' balanced salt solution (Gibco)
acridine orange (10 µg/mL) or Giemsa 5%

optional:
human serum
glucose
hypoxanthine (Sigma)
Tris-buffered Hanks' (TH)

Preparations

Prepare malaria culture medium (MCM) and Tris-buffered Hanks' (TH) for washing cells.

Albumax complete medium:

10.43 g RPMI 1640 powder (Gibco)
25 mL 1 M HEPES solution or 6 g HEPES (Gibco)
2 g NaHCO₃
0.5 mL gentamicin (from 50 mg/mL stock)

5 g Albumax II
Add distilled water to 1 liter. Filter-sterilize.
Use within 10 days, store at -20°C .

Comment: For growing parasites from patient blood, use 10 g of Albumax for 1 liter of complete MCM. The vast majority of cultures will survive at least 2 weeks. It is also important to avoid serum in the culture for preparation of crude parasite antigen (see SEROLOGY, section III:B). Not all strains can be adopted to Albumax II medium.

Alternative MCM:

10.43 g RPMI 1640 powder (Gibco)
25 mL 1M HEPES or 6 g HEPES
2 g NaHCO_3
0.5 mL gentamicin (from 50 mg/mL stock)
Add distilled water to 1 liter. Filter-sterilize and store at -20°C in 45-mL aliquots.

For complete MCM (cMCM), add 5 mL of human blood type AB⁺ serum (inactivated at 56°C for 60 min; then stored at -20°C) to 45 mL of medium. Complete MCM can be used for up to one week if stored at 4°C .

MCM can also be made from commercial liquid RPMI with sodium bicarbonate and HEPES buffer (Gibco). Just add 5 mL of $100\times$ L-glutamine (Gibco) and 0.25 mL gentamicin (Gibco) to a 500 mL bottle of the RPMI.

TH (0.15 M Tris-buffered Hanks') (pH 7.2):

2.11 g Tris-HCl
0.2 g Tris-base
7.88 g NaCl
Dissolve in distilled water and bring volume to 1 liter.
Mix 1 volume of Tris buffer with 1 volume of Hanks'.

In vitro cultures in tissue-culture flasks

- Wash the erythrocytes 3 times in TH or RPMI 1640 to remove CPD, serum, and leukocytes if present. Dilute to 5% hematocrit with cMCM in small flasks of 25 cm^2 (0.2 mL of packed cells to 4 mL of cMCM) or in 75-cm^2 flasks (1.0 mL to 20 mL).
- Add parasites to an appropriate parasitemia (see below).
- Put the flask in a candle jar and loosen the screw cap. Produce low oxygen by burnt out candle and place the jar at 37°C .
- Replace the MCM every day (not necessary the day after subcultivation).
- Subculture the cultures 2 times/week.

Subcultivation

- Stain a drop of the culture with acridine orange ($10\ \mu\text{g}/\text{mL}$) on a glass slide and put on a coverslip or by Giemsa staining of a thin smear (see PARASITES, section III:A or B).

- Count the parasitemia (i.e., the percentage of infected cells, see PARASITES, section III:C).
- Prepare freshly washed O⁺ blood in cMCM (5% hematocrit) and add it to the culture to obtain a parasitemia of not more than 1%, preferably 0.1 to 0.5% if two cycles until next subculturing, 0.5 to 1% if one cycle. Parasitemia should never exceed 15%.

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I:B. Establishment of long-term in vitro cultures of *Plasmodium falciparum* from patient blood

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Careful adherence to the procedures described below should allow a very high success rate with immediate culture (provided parasites are not already damaged by antimalarial drugs), and even when using cryopreserved parasite stabilates, a success rate of >70% should be within reach.

Equipment

centrifuge

incubator

Materials and reagents

culture medium:

500 mL RPMI 1640 (Gibco)

25 mg gentamycin (Gibco)

91.6 mg L-glutamine (Sigma)

2.5 g Albumax II (Invitrogen) (See comments below.)

10 mL normal human serum

10 mg hypoxanthine (Sigma) (See comments below.)

washing medium:

500 mL RPMI 1640

25 mg gentamycin (Gibco)

freezing solution:

sterile, distilled water

3.0% sorbitol (Sigma)

28.0% glycerol (Sigma)

0.65% NaCl

thawing solution:

sterile, distilled water

3.5% NaCl

Giemsa stain:

10% Giemsa in phosphate buffer

gas mixture:

2% O₂, 5.5% CO₂, 92.5% N₂ (Special gas mixtures such as this one can be bought from suppliers of compressed gases.)

red blood cells (RBC) for subcultivation:

Blood type O Rh⁻ or O Rh⁺ RBC in CPD buffer, washed 3 times in washing medium to remove the Buffy coat (See comments below.)

Step-by-step manipulations

- Collect heparin or CPD anticoagulated venous blood from patient. Do not use EDTA blood, which does not support parasite survival. The collected blood can be used for cultivation immediately (“straight out of the arm”) or after cryopreservation.

Preparation for immediate cultivation (“straight out of the arm”) (optional)

- Wash the anticoagulated blood sample 3 times in prewarmed (37 °C) washing medium (centrifuged for 8 min at 600 × g). Remove buffy coat after each centrifugation.
- Continue as described under *Cultivation of fresh patient isolates* below.

Cryopreservation (optional—Do only if immediate culture is not possible or desirable.)

- Spin the anticoagulated blood sample (centrifuged for 8 min at 600 × g) and remove plasma.
- Mix equal amounts of ring-stage parasitized packed RBC and freezing solution (see comments below).
- Distribute the mixture immediately into 1.8-mL screw-cap tubes and snap-freeze in liquid nitrogen.
- Store the tubes in liquid nitrogen until use.
- On day of use, thaw the RBC suspension in a 37 °C-water bath.
- Spin the tube (8 min at 600 × g) immediately after thawing (see comments below).
- Discard the supernatant, add an equal amount of thawing solution, mix well, and incubate the cells for 1 min (see comments below).
- Fill the tube with prewarmed (37 °C) washing medium and repeat centrifugation as above.
- Wash the RBC 2 times in prewarmed (37 °C) washing medium (centrifuge for 8 min at 600 × g).
- Continue as described under *Cultivation of fresh patient isolates* below.

Cultivation of fresh patient isolates

- Suspend RBC at ~4% hematocrit in prewarmed (37 °C) culture medium.
- Add 5 mL of the RBC suspension to a 50-mL cell culture flask (T flask) or 25 mL to a 250-mL cell culture flask.
- Flush 50-mL flasks for ~30 s with gas mixture (250-mL flasks for ~90 s) at 1.5- to 2-bar pressure (see figure and comments below).
- Incubate the flasks at 37 °C for 24 h.
- Remove the culture flask gently from the incubator and remove spent supernatant.
- Remove a tiny amount of RBC with a sterile Pasteur pipette and use these RBC to make a Giemsa-stained thin smear.
- Add new (prewarmed) culture medium to the flask, gas it as above, and return it to incubation at 37 °C.
- Subcultivate the parasites when necessary (see comments below).

Comments

- The Albumax II and hypoxanthine we use cannot be considered sterile. We thus make a stock solution of 100 g of Albumax II and 400 mg of hypoxanthine in 2 liters of RPMI 1640 and filter the solution through 0.8- μ m and 0.2- μ m filters. We then use 50 mL of this stock solution per 500-mL bottle of culture medium.
- It is important to keep all media preheated to 37 °C and to minimize handling time outside the 37 °C incubator. (No coffee breaks while handling cultures!)
- When gassing the culture flasks, fit the gas hose with a 0.2- μ m filter unit, and use a sterile needle (preferably blunt to avoid accidents) (see the figure).

- If initial patient parasitemia is high (>0.4%), change the medium the next day, otherwise leave it for 48 to 72 h before the first change of culture medium.
- Subcultivate patient isolates to keep parasitemia below 1 to 2% by adding fresh uninfected RBC. Although many laboratory-adapted parasite lines tolerate 5% parasitemia or more, this is not the case for most patient isolates.
- Do not leave parasites in freezing or thawing solution longer than absolutely necessary, as these solutions are harmful to the parasites.
- Washed uninfected RBC for subculture should be kept in the refrigerator for 24 h before first usage (to discourage any remaining leukocytes), and can be kept in the refrigerator for up to 14 days.

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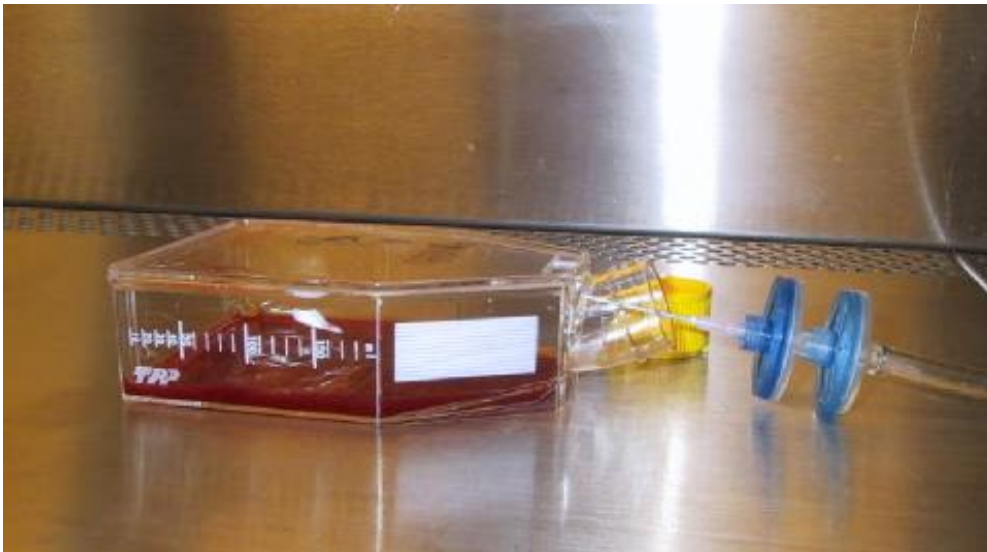
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Figure



I:C. Short-term cultivation of *Plasmodium falciparum* isolates from patient blood
by **Ulf Ribacke**

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We give here a protocol for growing *P. falciparum* from patient blood. The vast majority of cultures will survive only the first cycle (i.e., from the ring stage to trophozoites/schizonts) and will not be able to reinvade new RBC. In our hands, about 90% of patient isolates grew for at least one cycle. For establishment of long-term cultivation, see PARASITES section I:B.

Equipment

37 °C incubator
centrifuge for 500-3000 × *g*
candle-jar or gas (92% N₂, 5% CO₂, 3% O₂)

Materials and reagents

venous blood from malaria patient
heparinized or EDTA tubes
Polymorphprep (Axis-Shield)
RPMI 1640 (optimal HEPES-buffered)
O⁺ RBC (or autologous blood group)
MCM with AB⁺ serum
culture flasks or plates

Procedure

- Collect 2 to 5 mL of venous blood into heparinized/EDTA tubes and keep at 4 °C. Process the sample within 1 hour.
- Carefully layer the blood over 5 mL of Polymorphprep and centrifuge at 500 × *g* for 15 min.
- Collect the cell layer of interest. Erythrocytes are in the bottom of the tube.
- Add 10 mL of sterile RPMI 1640 (37 °C) to the cells, resuspend, and centrifuge at 500 × *g* for 5 min.
- Aspirate the supernatant and repeat the wash twice.
- Estimate the pellet volume and mix with equal amount RPMI 1640.

If you wish to keep the monocytes, transfer the cell layer containing monocytes into a sterile 15-mL tube (see the manufacturer's protocol for Polymorphprep). Wash once with RPMI 1640 by centrifugation at 3,000 to 5,000 × *g* for 2 min. Remove the supernatant and add 1 mL of 10% DMSO in fetal calf serum. Freeze the cells immediately at −70 °C and then transfer to liquid nitrogen.

Reference:

Haeggström M, Kironde F, Berzins K, Chen Q, Wahlgren M, Fernandez V. 2004. Common trafficking pathway for variant antigens destined for the surface of the *Plasmodium falciparum*-infected erythrocyte. *Mol Biochem Parasitol* 133(1):1-14.

I:D. Growing *Plasmodium falciparum* cultures at high parasitemiaby **Ulf Ribacke***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institutet for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: ulf.ribacke@smi.ki.se*

By using increasing volumes of culture medium, one can grow *P. falciparum* to higher parasitemia (above 10%). This protocol may be especially suited for maintaining high parasitemias of wild isolates, allowing maturation of a maximum number of ring stage parasites to trophozoites.

Procedure

- Set up cultures as described in PARASITES, section I:A, but use the table below as a guide for medium volumes.
- Increase the parasitemia gradually using the indicated volumes until a desired parasitemia is reached, then maintain the culture in the appropriate volume. For example, for a 200- μ L packed cell culture, use 4 times the volume of medium in the table for any given parasitemia.

% Parasitemia	Volume of medium (mL)/50 mL of packed culture
10	2.5
12	3
14	3.5
16	4
18	4.5
20	5
24	6
28	7
32	8
36	9
40	10
44	11
48	12
52	13
56	14
60	15
64	16
68	17
72	18
76	19
80	20
84	21
88	22
92	23
96	24
100	25

Reference

KEMRI, CGMRC / Wellcome Trust Research Unit, Kilifi, Kenya

I:E. Arresting *Plasmodium falciparum* growth at the trophozoite stage with aphidicolin
by **Kirsten Moll**

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This is a method to get fresh/frozen isolates or in vitro cultures to arrest in late trophozoite stages by inhibiting DNA synthesis.

Materials and reagents

malaria culture (only young stages! see PARASITES, section I:A) or a vial of frozen malaria parasites
aphidicolin (Sigma-Aldrich)
DMSO

Preparations

- Prepare a 1.5 mg/mL stock-solution of aphidicolin in DMSO.
- Dilute stock-solution 1:1000 into your malaria culture (4 μ L in a 4-mL flask).
- Let the culture continue to grow until the parasites reach trophozoite stage (around 24 h after adding the aphidicolin). See PARASITES, section I:A.

Analyse or use your culture within 12 h after trophozoite stages are reached. Even though the parasites may look fine for some more hours, it is NOT recommended to use them after a longer time in aphidicolin.

Note: If you add the aphidicolin to a culture of later stages, the cells will not arrest.

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I:F.Culturing erythrocytic stages of *Plasmodium vivax*by **Rachanee Udomsangpetch**

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Equipment

CF11-column (Cellulose powder CF11, Whatman)
syringe
centrifuge
orbital shaker (Leigh Labs)
fluorescence or light microscope

Materials and reagents

PBS

McCoy's 5A medium (Sigma–Aldrich)

reticulocyte-enriched red blood cells (may be obtained from cord blood or from hematochromatosis patients)

human AB serum

ethidium bromide (Sigma)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

brilliant cresyl blue (ALD, Sigma–Aldrich)

slides and coverslips

Procedure

- Collect 5 mL of heparinised blood from *P. vivax*-infected patients. Prepare thin and thick blood films for examination of parasite stages and parasitemia.
- Dilute the blood with 5 mL of PBS.
- Pack a sterile CF11-column having the same volume as the diluted blood (10 mL) in a syringe and equilibrate it with PBS before use. Add the diluted blood to the column and elute the red blood cells (RBC, reticulocyte enriched) with PBS. The leukocytes will stick to the CF11 cellulose.
- Collect the RBC passing through the column (reticulocyte enriched), centrifuge at 2,000 rpm, and wash once in McCoy's 5A medium at room temperature.
- Resuspend the RBC to 5% hematocrit in McCoy's 5A medium containing 25% human AB serum and maintain the culture in a candle jar or use a gas mixture containing 5% CO₂, 5% O₂, 90% N₂.
- In order to enhance growth of parasites, shake the culture gently and continuously at 75 rpm in the orbital shaker.
- Check the blood smears daily to confirm maturation of the parasite and add reticulocyte-enriched RBC when required.
- Enumerate the infected RBC using one of the following techniques:
 - Mix 1 drop of ethidium bromide (10 µg/mL) with one drop of the culture, mount the mixture on a glass slide with a coverslip, examine it under a UV-light microscope, and count the number of the parasitised RBC from 10,000 RBC.
 - Mix 1 volume of 1% brilliant cresyl blue in 5 volumes of the culture. Mount and examine as described above.

References

Chotivanich K, Silamut K, Udomsangpetch R, Stepniewska KA, Pukrittayakamee S, Looareesuwan S, White NJ. 2001. Ex-vivo short-term culture and developmental assessment of *Plasmodium vivax*. *Trans R Soc Trop Med Hyg* 95(6):677-680.

Udomsangpetch R, Socci R, Williams J, Sattabongkot J. Modified techniques to establish a continuous culture of *Plasmodium vivax* [abstract]. 2002. In: American Society of Tropical Medicine and Hygiene 51st Annual Meeting program; 2002 Nov 10-14; Denver, CO.

II. Freezing and thawing of asexual *Plasmodium* spp.

We give several methods below for cryopreserving and thawing *Plasmodium* strains and wild isolates. In general, parasites frozen according to one method can be thawed using another method's thawing protocol. For convenience, we generally freeze parasite strains using the Stockholm Sorbitol Method, while thawing using the simpler NaCl Method or the 5% & 27% Sorbitol Method. It appears that the lengthier thawing protocol of the Stockholm Sorbitol Method gives lower initial parasitemia (killing of the later stages) and a more synchronous culture during the first two cycles.

When parasites are cultured in Albumax II MCM to avoid adhering IgG, no addition of serum is required.

II:A. Stockholm Sorbitol Method

by **Kirsten Moll**

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Equipment

tabletop centrifuge (Beckman)
liquid nitrogen tank
immufuge

Materials and reagents

fetal bovine serum (Gibco)
freezing medium:
28% glycerol
3% sorbitol
0.65% NaCl
distilled water
To make 250 mL: Mix 180 mL of 4.2% sorbitol in 0.9% NaCl with 70 mL of glycerol. Filter-sterilize; store frozen.

Freezing

- Transfer a 4-mL culture into a test tube.
- Pellet erythrocytes by centrifugation (immufuge on low for 1 min) and discard the supernatant; the pellet is approximately 0.2 mL.
- Add 0.3 mL of serum (of complementary blood group) (40% hematocrit). For Albumax II cultures (see PARASITES, section I:A), add 0.3 mL of fetal bovine serum (40% hematocrit).
- Add 0.5 mL of the freezing medium, drop-by-drop, while shaking the vial gently; the addition should take approximately 1 min.
- Transfer the medium into a sterile cryovial.
- Drop the vial gently into the liquid nitrogen tank.

Thawing

- Prepare the sorbitol solutions listed below, have MCM and heat-inactivated serum ready (see PARASITES, section I:A). For Albumax II MCM, see also the comment above.

- Take a malaria culture vial from the liquid nitrogen tank.
- Thaw it quickly, approximately 1 min in a 37 °C-water bath.
- Put the vial on ice and transfer the contents into chilled 50-mL centrifuge tubes and centrifuge gently to pellet the cells (250 × g for 5 min at 4 °C in a Beckman tabletop centrifuge).

- Remove the supernatant and add the following, drop by drop, while shaking the vial gently:
 - 1 mL 17.5% sorbitol in ice-cold PBS, then
 - 2 mL 10% sorbitol, and finally
 - 2 mL 7.5% sorbitol

- Centrifuge as before, remove the supernatant, then add:
 - 1 mL 10% sorbitol, then
 - 2 mL 7.5% sorbitol, and finally
 - 2 mL 5% sorbitol

- Centrifuge, then add:
 - 1 mL 7.5% sorbitol, then
 - 2 mL 5% sorbitol, and finally
 - 2 mL 2.5% sorbitol

- Centrifuge, then add:
 - 1 mL 5% sorbitol, then
 - 2 mL 2.5% sorbitol, then
 - 2 mL MCM with 15% serum (at room temperature)

- Centrifuge, then add:
 - 1 mL 2.5% sorbitol, then
 - 2 mL MCM with 15% serumTransfer to a 5-mL tube.

- Centrifuge in the immufuge.
 - Add 4 mL of MCM containing 15% serum (see comment for Albumax II MCM above) and packed erythrocytes of a complimentary blood group (see also PARASITES, section I:A).

II:B. Freezing of patient isolates and strains with glycerolyte
by **Johan Normark**

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e-mail: johan.normark@smi.ki.se

This protocol is useful for all primate malaria parasites tested (*Plasmodium falciparum*, *P. vivax*, *P. knowlesi*, *P. coatneyi*, *P. fragile*, and *P. cynomolgi*).

Cryoprotective solution

glycerolyte:

- 57% glycerol USP
 - 16 g/liter of sodium lactate
 - 300 mg/L of KCl USP
 - 25 mM sodium phosphate (pH 6.8)
- (This solution may be purchased from Baxter/Fenwal.)

Procedure

All steps are at room temperature unless otherwise stated.

- Take heparinized blood containing very young ring stages of malaria and centrifuge it for 10 min at $700 \times g$ (approximately 3,000 rpm).
- Remove the supernatant and estimate volume of packed cells, V.
- SLOWLY add $0.33 \times V$ of glycerolyte through gentle mixing. Let the tube stand for 5 min.
- Add dropwise $1.33 \times V$ of glycerolyte, mixing gently.
- Label cryotubes with the parasite's code/name and date.
- Distribute 1 mL of the preparation per cryovial.
- Freeze at -70°C for at least 18 h.
- Transfer vials to liquid nitrogen for long-term storage.

II.C. Thawing of glycerolyte-frozen parasites with NaCl

by **Karin Blomqvist**

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Materials and reagents

sterile 1.6% NaCl (Baxter/Fenwal, 4B7870)

sterile 12% NaCl (Baxter/Fenwal, 4B7874)

malaria culture medium (MCM, see PARASITES, section I:A)

15% AB⁺ serum

Procedure

- Remove a vial from cold storage and thaw it at 37 °C for 1 to 2 min.
- Transfer blood to 50-mL centrifuge tubes with a sterile pipette. Measure blood volume, V.
- Add $0.1 \times V$ of 12% NaCl slowly, dropwise, while shaking the tube gently.
- Let the tube stand for 5 min.
- Add $10 \times V$ of 1.6% NaCl slowly, dropwise, swirling the tube.
- Centrifuge the tube at $500 \times g$ at 20 °C for 5 min.
- Aspirate the supernatant and add $10 \times V$ of MCM slowly, dropwise, while shaking the tube.
- Centrifuge the tube at 1,500 rpm at 20 °C for 5 min and aspirate the supernatant.
- Resuspend pelleted blood cells in MCM with 15% AB⁺ serum and transfer to a culture flask and desiccator. For continued culturing, see PARASITES, section I.

II:D. Thawing of cryopreserved *Plasmodium falciparum* using sorbitolby **Kirsten Moll***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: Kirsten.Moll@ki.se*Equipment

water bath (37 °C)
table-top centrifuge (Beckman)

Materials and reagents

27% sorbitol in PBS, filter-sterilized, stored at 4 °C
5% sorbitol in PBS, filter-sterilized, stored at 4 °C
15% AB⁺ serum

Procedure

- Remove a vial from liquid nitrogen and thaw it quickly in a 37 °C water bath; shake it lightly.
- Transfer thawed parasites into a round-bottomed 13-mL tube (max. 1 mL/vial).
- Slowly add 2 volumes of 27% sorbitol, the first volume over 8 min, the second over 5 min. Slowly stir the tube constantly on a vortex.
- Let it stand for 5 min.
- Add 2 volumes of 5% sorbitol over 10 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ (1,250 rpm on a table-top Beckman centrifuge) for 3 min; discard the supernatant.
- Add 2 volumes of 5% sorbitol over 8 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ for 3 min; discard the supernatant.
- Add 1 to 2 mL of MCM over 1 min. Centrifuge the tube at $400 \times g$ and discard the supernatant. The wash may be repeated once.
- Resuspend the pellet in 15% AB⁺ serum in MCM. Add washed, packed RBC's to 5% hematocrit.
- Change the medium the next day. Continue culturing as usual (see PARASITES, section I).

Reference

Procedures received from M. Hommel, modified from:

Diggs C, Joseph K, Flemmings B, Snodgrass R, Hines F. 1975. Protein synthesis in vitro by cryopreserved *Plasmodium falciparum*. Am J Trop Med Hyg 24(5):760-763.

III. Staining of parasite culture or patient blood and estimation of parasitemia

III:A. Acridine orange (AO) vital stain of cultures

by **Karin Blomqvist**

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Equipment

UV-equipped microscope

Materials and reagents

Acridine orange (AO) (Fluka)

Stock solution (10x):

Dissolve 10 mg of AO in 10 mL of distilled water. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Working solution:

Add 0.1 mL of stock solution to 9.9 mL of PBS (pH 7.2) or RPMI-1640. Store in the dark at 4 °C.

Alternate acridine orange hydrochloride solution (Fluka)

Dilute 1:5000 in PBS. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Procedure

- Mix one drop of culture with a small drop of AO on a slide.
- Put on a coverslip.
- Analyse the slide immediately or keep it in the dark.
- Count 5 to 10 fields of vision to estimate the parasitemia (40× objective, 10× ocular).

III:B. Giemsa staining of thick or thin blood films

by **Marianne Lebbad**

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Center (MTC), Karolinska Institutet SE-171 82 Solna, Sweden

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Equipment

light microscope

Materials and reagents

Giemsa stain:

For best and most reproducible results, buy ready-made Giemsa stain from, for example, Sigma–Aldrich. It is quite difficult to prepare a batch that works as well as the commercial one.

Check every new batch of Giemsa stock solution before using it. **Prepare a new working solution for each staining session.**

phosphate buffer, 6.7 mM (pH 7.1):

0.41 g KH_2PO_4

0.65 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$

Bring to 1 liter with distilled water.

methanol (reagent grade for analysis, Merck)

Giemsa staining of thick blood films from cultures

- Leave the thick film to dry properly, preferably overnight. In case of hurry, leave it to dry in an incubator at 37 °C for 1 h. Do not fix.
- Prepare a fresh 2% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Leave it to stain for 30 min. (A stronger Giemsa solution will over-stain parasites from culture.)
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

Giemsa staining of thick blood films from patients

- Leave the thick film to dry properly. In case of hurry, leave it to dry in an incubator at 37 °C for 30 min.
- Prepare a fresh 4 to 5% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Do not fix.
- Leave the slide to stain for 20 min.
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

Giemsa staining of thin blood films from patients or cultures

- Air-dry the thin film.

- Fix the film in methanol for about 30 s.
- Prepare a fresh 5 to 10% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide in a staining jar or on a staining rack and pour the Giemsa solution on the slide.
- Leave it to stain for 20 min.
- Rinse it carefully and thoroughly under running tap water.
- Leave the slide in an upright position to dry.
- Observe the film with immersion oil and objective at 100x.

Reference

Malaria Working Party of The General Haematology Task Force of the British Committee for Standards in Haematology. 1997. The laboratory diagnosis of malaria. Clin Lab Haematol 19(3):165-170.

III:C. Estimation of the percentage of erythrocytes infected with *Plasmodium falciparum* in a thin blood film

by **Marianne Lebbad**

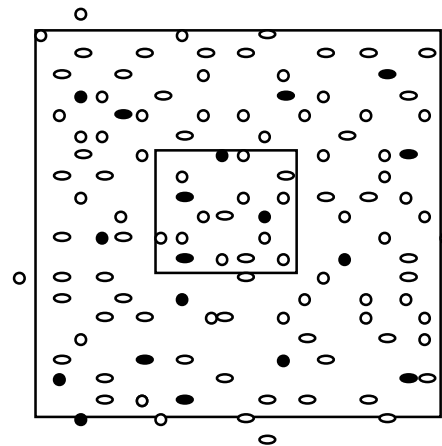
Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology Center (MTC), Karolinska Institutet SE-171 82 Solna, Sweden
e-mail: marianne.lebbad@smi.ki.se

Equipment

Use a light microscope with a special ocular (i.e., a reticulocyte ocular) which has a large and a small square where the small square is 10% of the large one (see the figure below, although proportions are not maintained).

Procedure

- Use the 100× objective to view cells under oil immersion.
- Choose an area of a Giemsa-stained thin blood film where the erythrocytes are evenly distributed.
- Count *all* erythrocytes in the small square plus two of the borders.
- Without moving the slide, also count the number of *infected erythrocytes* in the whole area of the big square plus two borders (i.e., including those in the area of the small square).
- Move the slide to “randomly adjacent” fields and continue counting as above (i.e., all erythrocytes of small fields and parasites of accompanying large fields). Continue counting fields until you reach the sum of 100 erythrocytes in the small fields. By extrapolating, the equivalent of 1000 erythrocytes have been examined (regarding parasites).
- Repeat the counting at least twice for a total examination of three different parts of the slide. Take the mean number of infected erythrocytes per 1000 erythrocytes and divide by 10 to get the percent infected erythrocytes.



Please note: If one erythrocyte contains ≥ 2 parasites it is still counted as **one** infected erythrocyte.

Reference

Brecker G, Schneiderman M. 1950. A time saving device for the counting of reticulocytes. Am J Clin Pathol 20:1079-1083.

III:D. Evaluation of parasitemia by lactate dehydrogenase assay

by **Makoto Hirai**^{1,2} and **Ingrid Faye**¹

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Evaluation of parasitemia by lactate dehydrogenase assay is a high throughput method for screening of antimalarial factors.

Equipment

microplate reader
flat-bottomed 96-well plate

Materials and reagents

Malstat reagent (Flow Incorporated)
red blood cells
nitro blue tetrazolium (NBT) stock solution:
100 mg NBT
50 mL distilled water
Dissolve NBT in distilled water. Store in the dark at 4 °C.

phenazine ethosulphate (PES) stock solution:
5 mg of PES
50 mL distilled water
Dissolve PES in distilled water. Store in the dark at –20 °C.

Procedure

- Add 100 µL of Malstat reagent to 96-well microplate.
- Add 20 µL of infected or noninfected red blood cells (hematocrit is 1 to 2%).
- Incubate the plate at room temperature and shake gently several times during incubation to solubilize red blood cells.
- During incubation, mix equal volumes of NBT and PES in the dark and add 20 µL of the mixture to the wells.
- Keep the plate in the dark.
- After 30 to 60 min, check the color development (dark purple).
- Read the plate at 650 nm. Use uninfected red blood cells as reference.
- This assay can be used in 0 to 7% of parasitemia (non-synchronized parasite in 2% of hematocrit).

If you deal with a large number of samples, 20 µL of red blood cells in 96 wells can be stored in 96-well plates at –80 °C before the enzyme assay.

References

Gomez MS, Piper RC, Hunsaker LA, Royer RE, Deck LM, Makler MT, Vander Jagt DL. 1997. Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. *Mol Biochem Parasitol* 90(1):235-246.

Makler MT, Hinrichs DJ. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg 48(2):205-210

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am J Trop Med Hyg 48(6):739-741.

IV. Purification and synchronization of erythrocytic stages

IV:A. Enrichment of knob-infected erythrocytes using gelatine sedimentation

by **Victor Fernandez**

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Equipment

immufuge blood centrifuge

Materials and reagents

mature parasite stages

gelatine (Sigma G2625)

RPMI 1640 with HEPES

Procedure

- Make 0.7% gelatine in RPMI 1640 with HEPES.
- Warm and stir the solution at 37 °C until the gelatine is dissolved.
- Filter-sterilize the solution and store it in the refrigerator. Warm it to 37 °C before use.
- Use mature parasite stages. (Ring forms, knobless late stages, and rosettes will sink to the bottom.)
- Wash the culture once using RPMI 1640.
- Resuspend the pellet, approximately 0.2 mL, in 2 mL of 0.7% gelatine solution.
- Incubate the pellet for 1 h at 37 °C.
- Carefully remove the supernatant, which contains the “knobby” parasites, without touching the pellet.
- Centrifuge the supernatant to pellet the parasitized RBC (PRBC) (1 min on high setting in the immufuge blood centrifuge).
- Wash the cells 2 or 3 times using RPMI 1640.
- Subculture the cells using fresh blood and media (see PARASITES, section I).

Reference

Goodyer ID, Johnson J, Eisenthal R, Hayes DJ. 1994. Purification of mature-stage *Plasmodium falciparum* by gelatine flotation. *Ann Trop Med Parasitol* 88(2):209-211.

IV:B. Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes
by **Victor Fernandez**
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Equipment

centrifuge

Materials and reagents

parasite culture of >5% parasitemia
sorbitol
malaria culture medium (MCM)

Procedure

- Take parasites when they are mostly at the ring stage. They must not be later than 10 to 12 h postinvasion when the sorbitol treatment is done.
- Spin down the parasite culture at $600 \times g$ to a pellet (take 4 mL of a culture of >5% parasitemia).
- Add 4 mL of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Shake 2 or 3 times.
- Centrifuge the culture at $600 \times g$, and wash it 3 times in malaria culture medium, and dilute it to 5% hematocrit.
- Count parasitemia and subculture as usual.
- Repeat the procedure after one cycle (approximately 48 h).
- To keep the parasites synchronized, the sorbitol treatment must be performed once a week.

Reference

Lambros C, Vanderberg JP. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 65(3):418-420.

IV:C. Enrichment of late-stage infected erythrocytes in 60% Percollby **Victor Fernandez**

Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden

e-mail: victor.fernandez@smi.ki.se

Equipment

centrifuge, swing-out rotor, refrigerated

Materials and reagents

mature parasite stages

malaria culture medium (MCM) with the human serum replaced by 10% FBS

60% Percoll solution:

60 mL Percoll

7 mL 10× PBS

33 mL PBS

The solution is enough for 40 tubes of 2.5 mL each, which is enough to enrich 80-mL of culture. Make fresh solutions before each use. Keep all solutions on ice throughout the preparation.

PBS

Procedure

- Wash a mature-stage *Plasmodium falciparum* culture 3 times in MCM with the human serum replaced by 10% FBS and resuspend it to 10% hematocrit in cold MCM with 10% FBS.
- Distribute the culture in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge in a swing-out rotor at $1,500 \times g$ and 4 °C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw carefully the cells at the interphases, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.

Reference

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. Clin Exp Immunol 54(1):127-134.

IV:D. Separation of *Plasmodium falciparum* mature stages in Percoll/sorbitol gradients
 by **Victor Fernandez**
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Equipment

centrifuge, fixed angle rotor (Sorvall SS-34 or Beckman JA-20)
 benchtop centrifuge

Materials and reagents

heparin
 PBS (pH 7.2)
 Percoll
 sorbitol
 RPMI 1640
 distilled water
 malaria culture medium (MCM)
 Corex tubes
 23 G 0.6-mm Ø needle
 tuberculin syringe

Preparation of gradients (for 4 gradients):

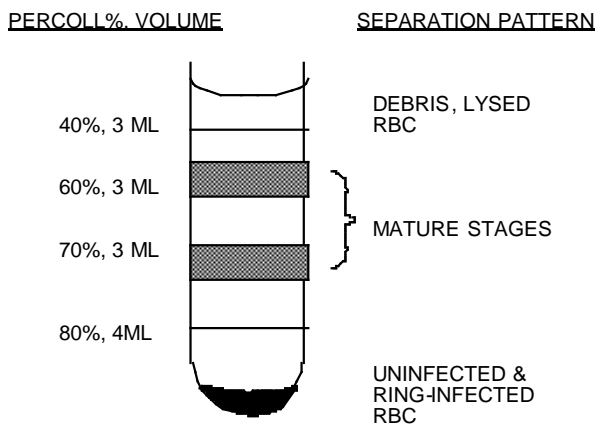
<p><u>50 mL of Solution H:</u> (90% Percoll, 5% sorbitol) 45 mL Percoll 5 mL 10× RPMI 1640 2.5 g sorbitol</p>	<p><u>30 mL of Solution L:</u> (0% Percoll, 5% sorbitol) 27 mL distilled water 3 mL 10× RPMI 1640 1.5 g sorbitol</p>
---	--

Prepare gradients:

<u>Volume</u>	<u>Percoll</u>	<u>Solution L</u>	<u>Solution H</u>
15 mL	40%	8.3 mL	6.7 mL
15 mL	60%	5.0 mL	10.0 mL
15 mL	70%	3.3 mL	11.7 mL
20 mL	80%	2.2 mL	17.8 mL

Procedure

- For each gradient, fill a 15-mL Corex tube as indicated below. Using a syringe, and starting with the lightest solution (40%), carefully layer the next denser layer under the previous one:



Separation of parasite stages

- Wash the PRBC and resuspend them in 1 mL of Solution L.
- Add 100 U/mL of heparin and pass the suspension 5 times through a 23 G 0.6-mm Ø needle using a tuberculin syringe (to disrupt rosettes).
- Very carefully overlay the cell suspension on top of the gradient.
- Centrifuge the gradients in a fixed angle rotor at 10,000 rpm for 30 min at 20 °C. Do not use a brake to decelerate.
- Discard the top of the gradient.
- Collect the parasites (floating as described in the diagram above) in a fresh centrifuge tube, no more than 5 mL/tube in a 10-mL tube. Fill the tube with PBS, mix very gently, and spin at 3,000 rpm for 1 min in a benchtop centrifuge.
- Resuspend the PRBC in MCM (see also PARASITES, section I:A).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. *Infect Immun* 66(6):2969-2975.

IV:E. Obtaining free parasitesby **Denise Mattei***Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France**e-mail: dmm@pasteur.fr*Equipment

centrifuge (15-mL tube and microfuge)

Materials and reagents

Plasmion (Fresenius France)

5% solution (w/v) of Gly-Ser (Sigma G3252)

40 mM HEPES

10 mM D-glucose in distilled water

Procedure

- Enrich parasitized red blood cells with Plasmion. Recover them by centrifugation at 2,000 rpm for 2 min. Resuspend the pellet at 37 °C in 1.5 volumes of a 5% solution (w/v) of Gly-Ser made with 40 mM HEPES (pH 7.2) and 10 mM D-glucose in distilled water.
- Incubate at 37 °C for 10 to 30 min.
- Add 5 volumes of culture medium.
- Centrifuge at 1,000 rpm for 3 min. Free parasites are in the supernatant; intact cells are in the pellet. The pellet of intact PRBC can be re-incubated with Gly-Ser.

Reference

Elford B. 1992. Generating viable parasites extra-erythrocytic forms of *P. falciparum*. TDR News 41:11.

IV:F. Obtaining semi-intact cellsby **Denise Mattei***Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France**e-mail: dmm@pasteur.fr*

In eukaryotic cells this method extracts soluble cytoplasmic components with the membranes retained.

Equipment

refrigerated microfuge

Materials and reagents

Plasmion (Fresenius France)

washing buffer (WB):

10 mM HEPES (pH 7.2)

15 mM KCl

breaking buffer (BrB):

50 mM HEPES (pH 7.2)

90 mM KCl

Procedure

- Enrich parasitized red blood cells with Plasmion. Wash 1 volume of Plasmion (without centrifugation) 3 times in PBS and resuspend it in the same volume of ice cold washing buffer, WB. For example, if you start with 250 μ L of Plasmion, resuspend the pellet in 250 μ L of WB.
- Centrifuge the cells in a refrigerated microfuge at 15,000 rpm for 10 min at 4 °C.
- Wash the pellet once in WB and resuspend it in the original volume of WB.
- Incubate it on ice for 10 min. Recover PRBC by centrifugation at 15,000 rpm for 10 min at 4 °C.
- Resuspend the pellet in the original volume of breaking buffer, BrB.
- Centrifuge as above and resuspend the pellet in one tenth of the original volume of BrB. Semipermeable parasites can be used in immunofluorescence studies or as membrane-enriched extracts on Western blots or immunoprecipitations.

Reference

Beckers CJM, Keller DS, Balch WE. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell* 50(4):523-534.

IV:G. Alanine synchronization of *Plasmodium falciparum*-infected erythrocytes
by **Catherine Braun-Breton**
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e-mail: cbb@pasteur.fr

Only early trophozoites and noninfected erythrocytes will survive this treatment.

Equipment

centrifuge

Materials and reagents

early trophozoites
noninfected erythrocytes
0.3 M alanine
10 mM HEPES (pH 7.5)
culture medium (RPMI 1640 with 10% heat-inactivated human serum)

Procedure

- Prewarm a solution of 0.3 M alanine, 10 mM HEPES (pH 7.5) at 37 °C.
- Spin down the parasite culture to a pellet (5 min at 2,000 × g at 20 °C).
- Resuspend the pellet in 5 volumes of 0.3 M alanine, 10 mM HEPES (pH 7.5).
- Incubate the solution for 3 min at 37 °C.
- Add 10 volumes of culture medium.
- Centrifuge the tube for 5 min at 2,000 × g at 20 °C and resuspend the pellet in culture medium at a 5% hematocrit.

Reference

Braun-Breton C, Rosenberry TL, Pereira da Silva L. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. Nature 332(6163):457-459.

IV:H. Selection of trophozoites by using magnetic cell sorting (MACS)

by **Anna Vogt**

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Equipment

Vario-MACS magnet (Miltenyi Biotec)
MACS CS-column (Miltenyi Biotec)
3-way stopcock (Miltenyi Biotec)
10-mL syringe
5-mL syringe
0.8-mm needle
0.6-mm needle
50-mL Falcon tubes
microscope
centrifuge

Materials and reagents

4 to 6 mL malaria culture, 5 to 10% parasitemia
PBS
150 mL 2% BSA/PBS
heparin (25000 IU/mL, Lövens)

Preparation

- Connect the 3-way stopcock and the column and place it in the magnet (fig. 1).
- Fill the 10-mL syringe with 2% BSA/PBS and connect it to the left opening of the 3-way stopcock. Open the way between the syringe and the column by closing the way between the syringe and the tap way out (fig. 2). Load the column with 2% BSA/PBS by carefully pushing the connected syringe (fig.3). Fill up to 10 mm above the column matrix. Close the 3-way stopcock totally (fig. 4). Refill the syringe with 2% BSA/PBS and reconnect it to the column. Leave the column for incubation for at least 5 min.
- Preparing the malaria culture: Centrifuge the malaria culture. Resuspend the pellet in 2 mL of 2% BSA/PBS. If using rosetting parasites, add heparin (100 U/mL) and push the culture through a 0.6-mm needle five times. Check in the microscope that all the rosettes are disrupted.
- Cut the top of the cover of a 0.8-mm needle and connect it to the 3-way stopcock (fig. 1). Open the way between the column and the needle and let the excess of loaded 2% BSA/PBS elute. Do not let any air into the column.
- Load the parasites on the top of the column. Open the way between the column and needle. Let the parasites migrate into the column. Close when 1 mm of solution is left. Add some 2% BSA/PBS and let the cells sediment following a quick opening of the tap to let the last cells immigrate. Do not let blood go through the needle. Leave for incubation for 10 min.
- Wash the column by adding 50 mL of 2% BSA/PBS to the top of the column and open between column and the needle. Leave some millimeters of the solution before closing the tap.
- Disconnect the needle from the 3-way stopcock. Disconnect the column from the magnet. Elute the bound material by holding the column upside down over a

Falcon tube and flush through 50 mL of 2% BSA/PBS using the syringe connected to the 3-way stopcock.

- Centrifuge the eluted material at 1,500 rpm for 5 min at room temperature. If using heparin, wash the pellet 5 times in 50 mL of PBS.
- Count the parasitemia.
- The column can be reused immediately. Place the column in the magnet and follow the protocol from the beginning. If reused later, please use the instructions for this procedure delivered together with the columns.

Comments

Approximately 50% of the trophozoites originally found in culture will be an enriched suspension. The following factors have been shown to affect the results:

- The culture should have a parasitemia of 6 to 10%, well synchronized trophozoites.
- The volume of culture used should not be larger than 6 mL or 10^9 cells.
- There should be no (or very few) extracellular parasites in the culture, as they seem to attach better to the matrix.
- A good looking, well-synchronized, culture without extracellular parasites seems to be the most important thing for a good result on the MACS.
- If heparin-free cultures are necessary, rosettes can be disrupted mechanically by passing the culture through the needle without heparin (see above), but make sure all rosettes are disrupted before loading the column.

Reference

Uhlemann AC, Staalsoe T, Klinkert MQ, Hviid L. 2000. Analysis of *Plasmodium falciparum*-infected red blood cells. *MACS & more* 4(2):7-8. Available from: <http://www.miltenyibiotec.com/index>.

Figures

Fig 1 – Assembly of the MACS column

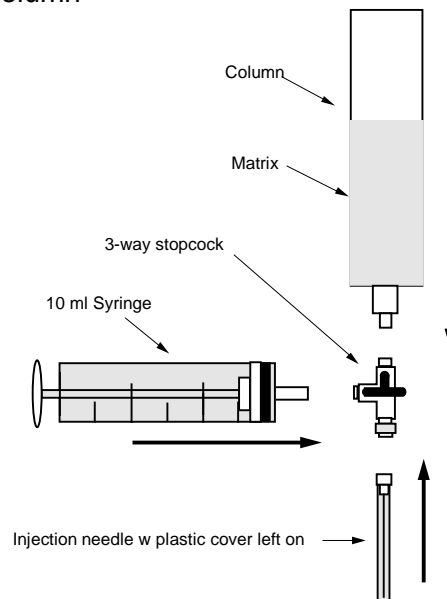


Fig 2.

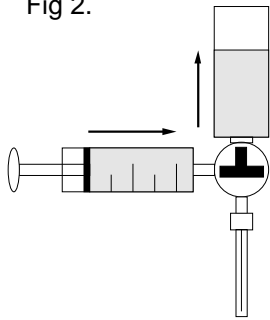


Fig 3.

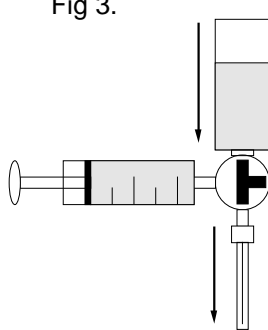
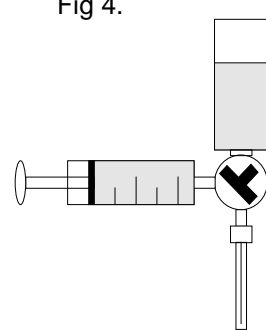


Fig 4.



Data for MACS-column:

Matrix volume	6.3 mL
Reservoir volume	6.5 mL
Maximum capacity total cells	10^9
Maximum capacity retained cells	2×10^8
Rinse volume	60 mL
Wash volume	30 mL
Elution volume	30 mL

IV:I. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

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See PARASITES, section VII:A, page 55

V. Micromanipulation cloning of *Plasmodium falciparum*

by **Victor Fernandez**

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Equipment

inverted microscope (Diaphot 300, Nikon)
micromanipulator system (NT-88, Nikon/NARISHIGE)
micropipette puller (Sutter Instrument, Model P-87)
pipette filling accessories: tubing, needle, syringe, MicroFil (World Precision Instruments)

Materials and reagents

capillaries, Boroglass, outer diameter = 1.0 mm, inner diameter = 0.5 mm (Sutter Instrument)
glass slides
12- or 24-well microplates
0.2- μ m syringe filter
culture flasks, 25 cm²
parasite culture of 5 to 10% parasitemia
malaria culture medium (MCM; see PARASITES, section I:A)
human serum, blood type AB⁺ (HS, see PARASITES, section I:A)
human erythrocytes, blood type O⁺ (see PARASITES, section I:A)
PBS (pH 7.2)
RPMI 1640
acridine orange (see PARASITES, section III:A)
Acrylease (Stratagene)

Preparation

- Pull pipettes in the micropipette puller. We use program 0 (heat: 392, vel: 100, pressure: 599). Each capillary gives two micropipettes. Break the tip with tweezers to get an inside diameter of 3 to 5 μ m. Check in the microscope that the pipette tip edge is blunt.
- Mix MCM with human serum to get 90% MCM/10% HS and filter through a 0.2- μ m syringe filter.
- Fill a pipette with filtered 90% MCM/10% HS. Attach the pipette to a tube connected to a needle/syringe. Put the tip of the pipette in filtered 90% MCM/10% HS and fill the tip by pulling the syringe. Hold for 1 to 2 min until the liquid is above the narrow part of the tip. Detach the syringe. Fill the rest of the pipette by using a MicroFil. Coat the outside of the filled micropipettes by dipping the tip in filtered 90% MCM/10% HS for at least 10 min.
- Attach the micropipette to the micromanipulator system.

V:A. Micromanipulation cloning of parasites

- Add 1 mL of filtered 90% MCM/10% HS to well A1 of a microplate. Make sure that the entire surface of the bottom is covered.
- Incubate the plate for 1 h at room temperature.
- Add 1 or 2 mL of 85% MCM/15% HS to the rest of the wells. Use the smaller volume for 24-well plates and the larger for 12-well plates.

- Add 2 to 8 μL of parasite culture to well A1. Dispense and leave it for 15 min to sediment.
- Pick individual erythrocytes with one parasite each and transfer them to the other wells of the microplate, placing only one parasite-infected erythrocyte in each well. If rosetting parasites are picked, strip off the uninfected erythrocytes and make sure that only one parasite is picked.
- Wash erythrocytes three times with RPMI 1640 and add them to all wells (except A1) to get a hematocrit of 1%; 20 μL to 12-well plates and 10 μL to 24-well plates.
- Culture the cells in candle jars as described below in section C below (see also PARASITES, section I:A).

V:B. Micromanipulation cloning of *Plasmodium falciparum* for single-cell RT-PCR

- Siliconize several slides with Acrylease.
- Put a drop of PBS on one of these slides.
- Exchange the filtered 90% MCM/10% HS in the lower part of the micropipette tip with PBS.
- Put 0.2 to 0.3 mL of PBS on another siliconized slide.
- Add 1 to 2 μL of the parasite culture and mix.
- Let the cells sediment for 5 to 10 min.
- On a siliconized slide, dispense 10 μL of PCR mix (see also MOLECULAR BIOLOGY, section V).
- Transfer 1 infected erythrocyte (with 1 parasite) from the parasite-containing drop to the PCR mix drop.
- Pipette the 10- μL drop containing the infected erythrocyte back into the PCR tube and immediately freeze the tube on dry ice.
- Pick uninfected erythrocytes as controls.

V:C. Expansion of *Plasmodium falciparum* clones

- *Day 0*: Cloning (see sections A and B above).
- *Day 3*: Change the medium. Carefully remove the supernatant and add fresh 85% MCM/15% HS. Add 1 mL to 24-well microplates and 2 mL to the 12-well plates.
- *Day 6*: Change the medium and add 1% hematocrit of washed erythrocytes (10 μL to 24-well plates, 20 μL to 12-well microplates).
- *Day 9*: Change the medium.
- *Day 12*: Change the medium and add 1% hematocrit.
- *Day 15*: Change the medium.
- *Day 17*: Change the medium and add 1% hematocrit.
- *Day 19*: Check the clones. Take a drop from the bottom of the well and put it on a slide. Add a drop of acridine orange (see PARASITES, section III:A) and use the microscope to check for parasites. Examine at least 30 fields. If the parasitemia is $>1\%$, move the clone to a 25- cm^2 culture flask with 90% MCM/10% HS and continue to incubate the culture (see PARASITES, section I:A). Change the medium in wells with very low or no detectable parasitemia.
- *Day 21*: Check wells and/or flasks and record the phenotype of the parasites. Discard empty wells and transfer clones with parasites to culture flasks as above

(see *Day 19*). When the parasitemia in the flasks is higher than 5% and the parasites are in the ring stage, they can be frozen (see PARASITES, section II).

References

Chen Q, Barragan A, Fernandez V, Sundström A, Schlichtherle M, Sahlén A, Carlson J, Datta S, Wahlgren M. 1998. Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. *J Exp Med* 187(1):15-23.

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. *Nature* 394(6691):392-395.

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. *Infect Immun* 66(6):2969-2975.

VI. Cytoadhesion and rosetting assays

VI:A. Basic cell media

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CHO and L cell medium, solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

3.0 g HEPES (Gibco)

0.4 g geneticin

450 mL distilled water

50 mL fetal calf serum

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and filter-sterilize; store at 4 °C for up to one month.

Alternative CHO and L cell medium, solution for 500 mL:

450 mL commercial RPMI 1640 (Gibco with 20 mM HEPES, without L-glutamine)

50 mL fetal calf serum

8 mL geneticin (Gibco stock concentration, 50 mg/mL)

50 mL L-glutamine (Gibco stock concentration, 20 mM, 100×)

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and store at 4 °C for up to one month.

HUVEC medium, solution for 500 mL:

5.84 g MCDB-131 (Sigma)

3.0 g HEPES (Gibco)

1.0 g NaHCO₃

10 ng/mL (final concentration) epidermal growth factor

1 µg/mL (final concentration) hydrocortisone

0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

25 mL fetal calf serum

Mix components and filter-sterilize; store at 4 °C for up to a month.

COS-7 cell medium:

Dulbecco's Modified Eagle Medium (DMEM, Gibco, 41966-029)

10% fetal calf serum

Mix components. Store the medium at 4 °C. Once the serum has been added, use this complete medium within one week.

Binding medium (pH 6.8), solution for 500 mL:

5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

3.0 g HEPES (Gibco)

450 mL distilled water

50 mL human serum, inactivated, nonimmune AB⁺ (10%)

Mix components and set pH to 6.8 immediately before use.

Alternatively, RPMI 1640 and HEPES can be mixed, filter-sterilized, and kept at 4 °C for up to a month. Immediately before use, add 10% human serum and set the pH to 6.8.

10× RPMI, solution for 100 mL:

10.43 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)

6 g HEPES (Gibco)

2 g glucose

100 mL distilled water

Mix components and filter-sterilize. Set pH to 6.8.

VI.B. Thawing melanoma and other cell lines

by **Arnaud Chene**

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Equipment

water bath (37 to 40 °C)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25-cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% CO₂)

Materials and reagents

appropriate cell medium (see PARASITES, section VI:A)
gelatine (2% gelatine in PBS, warmed for 30 min at 37 °C)

Thawing of cells

- Remove the vial with the frozen cells from the liquid nitrogen container and put it into a water bath at 37 to 40 °C. Thawing should be rapid (40 to 60 s).
- As soon as the ice is melted, remove the vial from the water bath.
- Transfer the contents of the vial to a sterile test tube and centrifuge it at 300 × *g* for 5 min.
- Discard the supernatant.
- Dilute the cell suspension with an appropriate volume of the recommended culture medium.
- Transfer the suspension to a culture flask and incubate it at 37 °C in 5% CO₂. The flask top should be loose. When cultivating HUVEC cells, the bottom of the flask should be coated with warm gelatine before the cells are added.
- In order to expedite the removal of the protective freezing additive (glycerol or dimethyl sulfoxide) from the culture medium, change the culture medium 24 h after thawing unless stated otherwise in the specific instructions for a particular cell line.

VI:C. Freezing of cell linesby **Arnaud Chêne**

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Equipment

freezing vials (Nunc Cryo Tube Vials; Nalge Nunc International)
15-mL Falcon tube (BD Labware)
Neubauer chamber
microscope (40× lens)
4 °C-refrigerator
−70 °C-freezer
liquid nitrogen tank for long-term storage

Materials and reagents

freezing medium:
7 mL RPMI 1640 medium
1 mL dimethyl sulfoxide (DMSO)
2 mL fetal calf serum (FCS)
EDTA–trypsin solution:
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
appropriate cell medium (see PARASITES, section VI:A)
trypan blue

Freezing of cells

- Prepare the freezing medium.
- Detach the cells from the cell culture flask with 2 mL of EDTA–trypsin solution and resuspend them in 10 mL of fresh medium (see PARASITES, section VI:D below).
- Count a sample of cells in trypan blue (1:2 dilution) using a Neubauer chamber.
- Centrifuge the sample at 4 °C (300 × g for 5 min) and resuspend the cells with freezing medium at 1×10^6 cells/mL.
- Aliquot the cells into freezing vials and keep them at 4 °C for 2 h.
- Place the vials in −70 °C overnight.
- Place them in liquid nitrogen for long-term storage.

VI:D. Cultivation of CHO, COS, HUVEC, melanoma, and L cells

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Equipment

centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
25-cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
incubator (37 °C, 5% CO₂)

Materials and reagents

RPMI 1640 medium
EDTA–trypsin solution
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
appropriate medium (see PARASITES, section VI:A)
0.5% gelatine in PBS for HUVEC cells

Passage of cell culture

- Aspirate the old medium from the culture.
- Wash the cells once with RPMI 1640.
- Add 2 mL of EDTA–trypsin.
- Leave the cells for approximately 5 min.
- Shake the flask sharply and check the flask in the inverted microscope to see that the cells have detached.
- Add 10 mL of fresh medium.
- Mix and transfer the suspension to a 15-mL Falcon tube and centrifuge the suspension for 5 min at 300 × *g*.
- Remove the supernatant by aspiration and resuspend the pellet in 1 mL of culture medium.
- Determine the number of cells in a Neubauer chamber.
- Add 5 mL of fresh culture medium to a new 25-cm² flask (for HUVEC cells use a gelatine-coated flask, see note below) and add approximately 10⁵ cells.
- Incubate the cells at 37 °C in 5% CO₂. The flask top should be loose.

Note: Before seeding the HUVECs, coat the culture flasks with sterile 1% gelatine in PBS for 1 h at 37 °C:

- Add 2 to 3 mL of gelatine in PBS to the cell culture flask.
- Incubate the flasks for 30 min at 37 °C.
- Remove the excess gelatine by aspiration and add cells as above.

VI:E. Formaldehyde-fixation of melanoma cellsby **Arnaud Chêne***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: arnaud.chene@ki.se*Equipment

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc International)
incubator (37 °C, 5% CO₂)
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma C32 cells in culture
appropriate medium (see PARASITES, section VI:A)
EDTA–trypsin solution:
 EDTA, 2 mg/mL
 trypsin, 2.5 mg/mL
RPMI 1640 medium
PBS
1% formaldehyde

Fixation of cells

- Place cell culture coverslip with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell cultivation medium.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding melanoma cells in cell culture bottles, add approximately 50×10^3 cells to each of the wells.
- Leave the melanoma cells to attach to the coverslip in the incubator (37 °C, 5% CO₂) overnight.
- Remove the medium from the melanoma cells and wash twice with PBS.
- Add 0.5 mL of 1% formaldehyde to each well and incubate the cells for 1 h at room temperature.
- Wash them twice with PBS.
- Add PBS and cover the wells; store at 4 °C until use. The maximum storage time is approximately 1 month.

Reference

Schmidt JA, Udeinya IJ, Leech JH, Hay RJ, Aikawa M, Barnwell J, Green I, Miller LH. 1982. *Plasmodium falciparum* malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes. *J Clin Invest* 70(2):379-386.

VI:F. Binding assays to endothelial and melanoma cells

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Equipment

24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc Int.)
centrifuge (Beckman Allegra 6R)
15-mL Falcon tube (BD Labware)
incubator (37 °C)
glass slides
inverted microscope (125×)
microscope (1000×)

Materials and reagents

melanoma or endothelial cells in culture
malaria culture of 8% parasitemia or higher with a majority of the parasites at trophozoite stage
EDTA–trypsin solution:
 EDTA, 2 mg/mL
 trypsin, 2.5 mg/mL
RPMI 1640
appropriate medium (see PARASITES, section VI:A)
5% Giemsa solution in phosphate buffer (see PARASITES, section III:B)
1% glutaraldehyde in PBS
glycerol
binding medium (see PARASITES, section VI:A).

Procedure

- Place cell culture coverslips with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell culture medium to each well.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding cells in culture bottles, add approximately 50×10^3 cells to each of the wells with coverslips.
- Leave the cells to attach to the coverslip in the incubator (37 °C, 5% CO₂) overnight. For melanoma cells, please see note at the end of the protocol.
- Inspect cells that have been plated on coverslips; each cell should be separated from other cells for easy reading of binding.
- Centrifuge the malaria culture at $500 \times g$ for 5 min at room temperature.
- Wash the culture 3 times ($500 \times g$) with binding medium and resuspend in binding medium to a hematocrit of about 2%.
- Aspirate medium from the coverslips in the 24-well plate and rinse once with binding medium. Be careful not to let the coverslip with the attached cells dry.
- Add 0.5 mL of malaria culture suspension to each coverslip.
- Incubate the cells at 37 °C for 60 min, gently rocking by hand every 15 min. Do not use a CO₂ incubator.

- Check the binding of malaria-infected erythrocytes to the cells using an inverted microscope.
- Wash away unbound red cells by dipping the coverslip carefully in each of 3 beakers of binding medium or RPMI 1640.
- Fix the coverslips in 1% glutaraldehyde for 60 min or overnight. Alternatively, fix in 1% methanol for 30 min.
- Rinse coverslips with distilled H₂O and stain with 1% Giemsa for 20 to 30 min.
- After staining, rinse the coverslips with water and let them dry.
- Mount the coverslips on glass slides with glycerol.
- Examine the slides under a microscope (oil, 1,000×) and count 300 cells.
- Calculate the average number of infected erythrocytes bound per target cell.

Note: Melanoma cells can be used fresh or fixed with 1% formaldehyde if fixation insensitive receptors are to be studied, such as CD36 (see PARASITES, section VI:E).

Reference

Marsh K, Marsh WM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum*: the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. *Exp Parasitol* 65(2):202-208.

VI:G. Selection of cytoadherent parasites by passage over C32 melanoma cells, CHO, HUVEC, or other endothelial cells

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Equipment

centrifuge (Beckman Allegra 6R)
incubator (37 °C)
15-mL Falcon tube (BD Labware)
inverted microscope (125 ´)

Materials and reagents

malaria culture of 8% parasitemia or higher (e.g., enriched) with a majority of the parasites in trophozoite stage
binding medium (see PARASITES, section VI:A above)
25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence
complete malaria culture medium (see PARASITES, section I:A)
human erythrocytes blood type O⁺ (see PARASITES, section I:A)

Selection of cytoadherent parasites

- Use malaria cultures with a parasitemia exceeding 8%; lower parasitemias are not worthwhile, because the binding will be too low. The majority of the parasites should be in the trophozoite stage.
- Transfer the malaria culture to a 15-mL Falcon tube and centrifuge at 500 × g for 5 min at room temperature.
- Wash the culture 3 times (500 × g) in binding medium and resuspend in binding medium at a hematocrit of 2 to 3%.
- Use a 25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence.
- Remove growth medium and wash the melanoma/endothelial cells once with binding medium.
- Overlay the cells with the malaria culture suspension.
- Incubate the cells at 37 °C for 1 to 1.5 h. Do not use a CO₂ incubator.
- Resuspend the cells by gently rocking the flask every 15 min.
- Wash the flask gently 3 times with binding medium to remove unbound erythrocytes.
- Check binding of erythrocytes using an inverted microscope.
- Remove binding medium and add complete malaria culture medium and washed fresh human erythrocytes (see PARASITES, section I:A) at 5% hematocrit to the culture flask.
- Transfer the culture to a new flask the next day (after merozoite reinvasion of RBC).
- Continue culturing (see PARASITES, section I).

Reference

Marsh K, Marsh VM, Brown J, Whittle HC, Greenwood BM. 1988. *Plasmodium falciparum*: the behaviour of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol 65(2):202-208.

VI:H. Binding of fluorescent receptors heparin, blood group A, and PECAM-1/CD31 to *Plasmodium falciparum*-infected erythrocytes

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Equipment

PCR tubes
centrifuge (500 × g)
microcentrifuge (500 × g)
glass slides
coverslips
UV light microscope (100× magnification)

Materials and reagents

malaria culture (with parasites in trophozoite stage)
RPMI 1640
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
for heparin:
fluorescein-conjugated heparin (2 mg/mL, Molecular Probes)
for blood group A:
fluorescein-conjugated blood group A (1 mg/mL A_{tri}-PAA-flu, Syntesome)
for CD31:
recombinant PECAM-1/CD31 (800 µg/mL, R&D Systems)
Alexa Fluor 488 Protein Labeling Kit (Molecular Probes)

Binding of fluorescent receptors

PECAM-1/CD31 is bought unlabelled and must therefore be labelled with a fluorophore before the experiment. Use the Alexa Fluor 488 Protein Labeling Kit to label 400 µg of CD31 in 0.5 mL of PBS. Follow the protocol provided by Molecular Probes.

- Wash the culture (5% hematocrit) twice in RPMI 1640 (500 × g) and resuspend to original volume.
- Aliquot 30 µL of washed culture in PCR tubes (0.6 mL). Make one tube for each receptor to be investigated.
- Centrifuge the PCR tubes in a microcentrifuge (500 × g), remove the supernatant, and resuspend the cells to a volume of 26 µL with RPMI 1640.
- Add 4 µL of the fluorochrome-conjugated receptor to each PCR tube and mix (regarding CD31 see note below).
- Incubate the cells for 30 min in the dark with resuspension after 15 min.
- Wash the cells 3 times with RPMI 1640 (500 × g) and resuspend them to 30 µL in RPMI 1640.

When using heparin-FITC or blood group A-FITC, the fluorescence might be very weak. A possibility is to amplify the signal by using Alexa 488 labelled αFITC (antifluorescein, goat IgG fraction, Alexa Fluor 488 conjugate from Molecular Probes) in a second incubation (dilution 1:8, 30 min at room temperature in the dark with resuspension after

15 min). After this second incubation, wash the cells 3 times as before and continue as described below.

- Resuspend the cells in 30 μL of RPMI 1640 and mix 10 μL of cells with 0.5 to 1 μL of ethidium bromide (1 $\mu\text{L}/\text{mL}$) on a glass slide. Alternatively, to reduce the amount of background, incubate the cells in the PCR tube with ethidium bromide (1 to 2 μL ; 1 $\mu\text{g}/\text{mL}$) for 5 to 10 s before the last wash.

If fluorescence is weak, ethidium bromide may be skipped altogether as the infected cells can be detected in the microscope by the presence of pigment.

- Add a coverslip and count the fluorescence rate at 100 \times magnification with oil in a UV-light microscope.
- Calculate the fluorescence rate as =
$$\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$$

References

Heddini A, Chen Q, Obiero J, Kai O, Fernandez V, Marsh K, Muller WA, Wahlgren M. 2001. Binding of *Plasmodium falciparum*-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): frequent recognition by clinical isolates. *Am J Trop Med Hyg* 65(1):47-51.

Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M, Marsh K. 2001. Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors. *Infect Immun* 69(9):5849-5856.

VI.I. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized receptors

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Equipment

microscope
class 2 safety cabinet (laminar flow)
centrifuge

Materials and reagents

marking pens, indelible and suitable for plastic
150 mm plastic (polystyrene) tissue culture (petri) dishes
Dako Pen for Immunohistochemistry (Dako Corp) or similar, OR plastic sealing film (eg Parafilm, Nescofilm)
liquid paraffin oil
glass coverslips
phosphate-buffered saline, pH 7.2 OR Tris-buffered saline, pH 7.4
RPMI-HEPES, pH 6.8 - 7.4 (no bicarbonate added)
bovine serum albumin
pooled non-immune human serum, OR non-immune AB serum
glutaraldehyde 2% in PBS
Giemsa stain
purified receptors
adhesion medium: RPMI-HEPES pH 6.8 - 7.4, plus EITHER 10% human serum (pooled or AB serum from non-exposed donors) OR 1% BSA

Procedure

- On the under-side of a plastic petri dish, mark approximately 10mm diameter circles for the required number of test spots (usually 3 spots for each receptor being tested) Number or label the circles accordingly.
- Inside the petri dish, on the base, use a Dako pen for immunohistochemistry to copy circles over those drawn above – this creates a circle of plastic that will prevent the parasite suspension being tested (see step 8) from spreading. Alternatively, cut holes in a layer of plastic sealing film using a hole punch and carefully press the plastic film onto the base of the petri dish (see Beeson et al., 1998). Use a blunt instrument (eg. top of a plastic pen) to press the film on completely, taking care around holes to form a good seal with the plastic dish.
- Place 5-10 μ L of each receptor, diluted in PBS or TBS, in the centre of each circle.
- Incubate overnight at 4 °C in a humid box – ensure the receptor spots do not dry out.
- Before performing the adhesion assay, block non-specific binding by placing a drop or two (50 – 100 μ L) of 1% BSA in PBS over each receptor spot. Incubate for at least 30 mins at room temperature.
- Prepare the parasites (in Class 2 cabinet):

- Spin down parasites from culture or patient sample at low speed.
- Wash parasite-RBC pellet once with RPMI-HEPES.
- Remove supernatant and re-suspend pellet in cytoadherence medium (RPMI-HEPES plus either 10% human serum OR 1% BSA) to the required haematocrit, usually 1-5%. We typically use mature pigmented trophozoite-infected RBC at a parasitemia of 1% or higher.
- Wash the plate with either PBS OR RPMI-HEPES by adding 25 mL of buffer, agitating and discarding. Repeat once.
- Aspirate, using suction, the remaining buffer from each spot and then add 40 μ L of parasite suspension to each well (performed in Class 2 safety cabinet).
- Incubate at 37 °C for 30 min (no agitation).
- Gently wash off unbound cells with RPMI-HEPES pH 6.8 – 7.4, or PBS pH 7.2-7.4.
- Add 25 mL of buffer carefully to one side of the dish using a pipette. Do not add the buffer directly over receptor spot.
- Rock gently to remove unbound cells and aspirate supernatant.
- Wash 4-6 times in total.
- Fix bound cells with 2% glutaraldehyde (in PBS) for at least two hours.
- Stain with 10% Giemsa for 10 mins, wash off stain with water, and allow to dry. Do not over stain as it becomes difficult to distinguish infected from uninfected RBC.
- Once dry, cover spots with liquid paraffin oil (or clear vegetable oil) and a glass coverslip.
- Count bound infected-RBC microscopically, expressing counts as bound parasitized RBC/mm². Compare adhesion to receptors to that of BSA-coated control spots to account for any non-specific adhesion.

Comments

- Steps 6 – 10 should be performed in a Class 2 safety cabinet.
- Purified chondroitin sulfate A – we used CSA from bovine trachea at 10 μ g/mL, or CSA from porcine rib at 50 μ g/mL. This works well for most isolates. CSA can be coupled to phosphatidylethanolamine to improve immobilization on plastic (Rogerson et al., 1995).
- Different plastic culture dishes can be used in these assays. We generally use Falcon 1058, but others also work.
- Various compounds, serum, or antibodies can be tested for inhibitory activity by incubating parasites with the inhibitor for 5 - 45 minutes prior to step 7 (see Beeson et al., 1998, Reeder et al., 1999)

References

- Beeson JG, et al, 1998, *Infect. Immun.* 66:3397-3402
Beeson JG, et al., 1999, *J. Infect. Dis.* 180:464-72
Beeson JG et al, 2000, *Nature Med.* 6:86-90
Chai W, et al., 2001, *Infect. Immun.* 69:420-5
Reeder JC, et al., 1999, *Proc. Natl. Acad. Sci. USA* 96:5198-202
Rogerson et al., 1995, *J. Exp. Med.* 182:15-20

VI:J. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized hyaluronic acid

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Hyaluronic acid (HA) preparations

HA from bovine vitreous humor (Sigma) is isolated by strong anion exchange chromatography and generally has little or no detectable chondroitin sulfates (Chai et al. 2001). Other sources of HA, but not all, also support adhesion. Some preparations of HA may contain significant amounts of copurified chondroitin sulfates.

Immobilization of HA onto plastic

Immobilization is generally effective with PBS or TBS (pH of 7.2 to 7.4) as the coating buffer, and parasite adhesion is enhanced by including calcium in the coating buffer (and in all other steps). HA effectively adsorbs to the surface of Falcon 1058 plastic dishes (tissue culture treated, Becton–Dickinson).

Optimum conditions for adhesion

- Adhesion of parasites to HA is highest at pH 7.4.
- Include calcium in all steps.
- Use mature pigmented trophozoites in assays rather than schizonts.
- Fixation of bound parasites with glutaraldehyde (see PARASITES, section VI:A) may decrease observed adhesion to HA. It may be necessary to count numbers of cells bound to HA prior to fixation.

Materials and reagents

hyaluronate lyase (hyaluronidase) from *Streptomyces hyalurolyticus*

hyaluronic acid isolated from bovine vitreous humor

phosphate-buffered saline

Step-by-step procedure for preparation and use of lyase-treated hyaluronic acid as a control in adhesion assays

- Incubate HA (1 mg/mL) with and without hyaluronate lyase (10 to 20 TRU/mL) in PBS, at 60 °C for 4 h or overnight.
- Stop incubation by boiling HA samples for 10 min at 100 °C. Cool samples and centrifuge them at high speed to remove any insoluble aggregates.
- Make aliquots of treated and untreated HA samples and store them at –20 °C.
- To measure parasite adhesion to HA, dilute lyase-treated and untreated HA samples 1:10 (i.e., a final HA concentration of 100 µg/mL) in PBS and coat plastic surfaces with it as described above (see PARASITES, section VI:A).
- Perform adhesion assay as described above (see PARASITES, section VI:A).
- Interpretation: Adhesion of parasites to nonlyase-treated HA but not to lyase-treated HA indicates specific adhesion to HA.

References

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* 6(1):86-90. [see comments.].

Chai W, Beeson JG, Kogelberg H, Brown GV, Lawson AM. 2001. Inhibition of adhesion of *Plasmodium falciparum*-infected erythrocytes by structurally defined hyaluronic acid dodecasaccharides. *Infect Immun* 69(1):420-425.

VI:K. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

by **Nilofar Rasti** and **Kirsten Moll**

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See PARASITES, section VII:B, page 56

VI:L. Enrichment of rosetting parasites using Ficoll–Isopaque (Pharmacia)

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Equipment

immufuge blood centrifuge

Materials and reagents

Ficoll–Isopaque (FIP) (Pharmacia)

RPMI 1640

MCM with 10% heat-inactivated serum

Protocol

- Use 2 mL of culture per tube, mature and rosetting parasites, with a 5 to 10% parasitemia and layer it very carefully on 2 mL of sterile, ice-cold Ficoll–Isopaque (FIP).
- Centrifuge the tube in the immufuge blood centrifuge for 12 s on the high setting at room temperature.
- Quickly suck up the pellet using a Pasteur pipette.
- Wash the pellet 3 times using RPMI 1640.
- Subculture (see PARASITES, section I) using fresh, washed erythrocytes and MCM with 10% heat-inactivated serum.

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

VI:M. Reversion of rosettesby **Anna Vogt**

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Equipment

UV-light microscope (40×)

Materials and reagents

96-well microtiter plate

Parafilm

acridine orange

Procedure

- Keep the *Plasmodium falciparum* culture under standard conditions with 10% normal serum added to the buffered medium (see PARASITES, section I:A).
- Perform the studies on cultures with 5% hematocrit, 4 to 8% parasitemia at late stage, and >50% rosetting rate.
- Mix an aliquot of 25 to 50 μ L of the rosetting *P. falciparum* culture (1:1) with various dilutions (e.g., 1:5) of sera to be tested. Also prepare a control using nonimmune human sera or MCM (see PARASITES, section I:A).
- Incubate the cultures at 37 °C for 60 min in a 96-well microtiter plate. Cover the plate with Parafilm.
- Use two separate aliquots from each well, mix each with a small amount of acridine orange, and mount them on glass slides. Count 25 consecutive fields of vision per slide using a 40× lens and incident UV light (2 slides/well, approximately 50 fields). Count fields of vision diagonally over the slide, from one corner to the other, in order to compensate for a possible uneven distribution of rosettes on the slide. If the cell concentration is low, it may be necessary to count in both diagonal directions.

Comments

Infected erythrocytes (except ring forms) within a rosette and infected erythrocytes not connected to a rosette are scored separately.

Infected erythrocytes that have bound two or more noninfected cells are scored as rosettes. If the rosette contains more than one infected erythrocyte, the cells are scored separately.

Ring forms within a rosette are not counted.

Late-stage (trophozoite and schizont, but not ring forms) infected erythrocytes are scored and added to the total number of late infected erythrocytes.

Since it can be hard to tell the difference between a ring form and an early trophozoite, only parasites with a diameter that covers at least one-third of the diameter of the erythrocyte are counted.

The number of infected erythrocytes in large rosettes (where it can be difficult to determine the exact number of infected cells) are counted as 3.

If it is difficult to determine the number of cells (usually 2 or 3) bound to an infected erythrocyte, the coverslip can be pressed lightly (using a pencil) to induce a slight movement. This is important, because quite often it is found that only 1 erythrocyte is actually bound to the infected cell, when others that may appear to be are not.

Use two controls, one before counting the samples and one after.

Reference

Carlson J, Holmquist G, Taylor DW, Perlmann P, Wahlgren M. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. Proc Natl Acad Sci USA 87(7):2511-2515.

VII. Placental malaria

VII:A. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta by **James G. Beeson¹** and **Stephen J. Rogerson²**

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Materials and reagents

50 mL plastic tubes
scalpel or scissors, forceps
phosphate buffered saline with 50mM EDTA

Equipment

microscope
class 2 safety cabinet (laminar flow)
centrifuge
rotating wheel or tube roller
protective eye wear and face mask

Procedure (Beeson et al., 1999)

- Collect placenta as soon as possible after delivery, and determine whether it is infected by performing a blood smear. Orientate the placenta so the maternal side is facing up. Clean the surface with PBS if necessary.
- From apparently healthy areas of the maternal surface, cut several pieces of placental tissue (around 1.5-2 cm in each dimension, up to 8 cm³) from different areas using either a scalpel or scissors and forceps. Immediately put cut pieces of placental tissue into 50 mL tubes containing PBS with 50 mM EDTA. Several pieces can be placed into a single tube, but the placental tissue should occupy no more than one third of the volume. Protective eyewear and a face mask should be worn during this procedure.
- Mix tubes containing placental tissue on a rotating wheel or tube roller for 1 hour at room temperature. To increase yield of placental blood and parasites, placental tissue can be cut into smaller pieces or finely chopped after 30 mins, and the incubation then continued for a further 30 mins. Doing this does not appear to significantly influence the parasitemia or parasite phenotypes of the preparation.
- Stop incubation, then compress the placental tissue with a blunt instrument to further remove any blood and parasites. Let tubes stand for one minute to allow tissue pieces and debris to settle.
- Collect the supernatant then centrifuge and wash pellet 3 times with PBS.
- Prepare a thin smear and determine the parasitemia of the preparation.
- Leukocytes can be depleted by passing a suspension of the placental blood over a cellulose column, or over Percoll density gradients.
- If necessary, the parasitemia can be enriched by passage over Percoll density gradients or by other methods, as described elsewhere.

References

Beeson JG, et al., 1999, J. Infect. Dis. 180:464-72
Beeson JG et al, 2000, Nature Med. 6:86-90

VII:B. Binding of *Plasmodium falciparum*-infected erythrocytes to placental tissue sections

by **Niloofer Rasti** and **Kirsten Moll**

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Equipment

cryostat at $-20\text{ }^{\circ}\text{C}$

Materials and reagents

binding medium:

5.2 g RPMI powder

3.0 g HEPES

500 mL distilled H_2O

liquid nitrogen

Polymorphprep (Axis-shield)

methanol

distilled H_2O

hyaluronidase: 10 $\mu\text{g}/\text{mL}$ enzyme in PBS (pH 7.2)

Streptomyces hyaluronate lyase: 25-50 U/mL in PBS (pH 7.2)

chondroitinase: 0.5 units/mL in Tris-HCl (pH 8.6)

heparinase: 0.5 units/mL in PBS

PBS

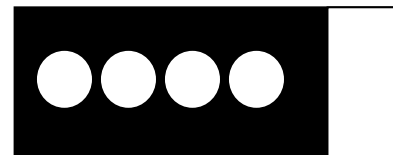
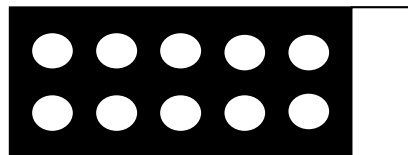
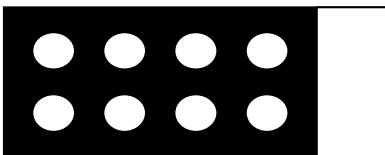
slides with 8 wells of 8 mm, 10 wells of 6 mm or 4 wells of 9 mm (Novakemi AB)

coverslips

50-mL Falcon tubes

Placenta sections:

- Cut fresh placental tissue into small cubes ($\approx 1 \times 1\text{ cm}$) and freeze immediately in liquid nitrogen. Transfer them later to $-70\text{ }^{\circ}\text{C}$ for storage.
- Adapt the placental tissue cubes to $-20\text{ }^{\circ}\text{C}$ for 1 h before use.
- Cut 5- μm thick sections with a cryostat at $-20\text{ }^{\circ}\text{C}$.
- Transfer the sections to 8- or 10- well slides (see below).
- Keep the slides with sections at $-70\text{ }^{\circ}\text{C}$. The sections should not be older than 1 week when used in experiments.
- For use, remove a slide from $-70\text{ }^{\circ}\text{C}$, transfer it immediately into cold PBS, and keep it in PBS until use.

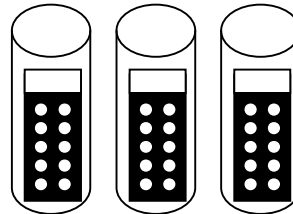


Preparation of PRBC:

- PRBC from fresh placental isolates are isolated from the placenta using the method of Beeson et al (see section VIIA) with PBS-EDTA. Leukocytes are depleted by passing the suspension of eluted material on polymorphprep.
- Enrich the infected cells for late trophozoites in a Percoll-gradient or by MACS purification (Optional for freshly eluted placental isolates).
- Keep the cells as packed cells on ice after purification.

Binding of PRBC to placenta sections:

- Resuspend 1-2 μL or 5 μL of packed cells in 23-24 μL or 45 μL of binding medium (for 6-8 mm wells versus 9 mm wells) obtaining a hematocrite of 10%.
- Add those 25 μL or 50 μL to one well containing a placental section, forming a drop on top of the section.
- Incubate the section for 1 h at 37 °C.
- Tip off the drop carefully.
- Wash the section 3 times for 5 min in binding medium: Use three 50-mL Falcon tubes with 40 mL of binding medium each; let the slide stand in each for 5 min.
- Fix the sections on a slide for 15 s in methanol.
- Let them air dry.
- Stain the sections with 5% Giemsa for 30 min (overlay sections with a drop of Giemsa stain).
- Wash away the Giemsa stain with distilled H_2O .
- Put a coverslip over the sections.

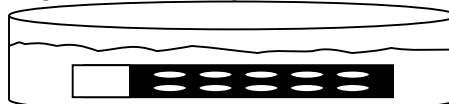
Binding of PRBC to placenta sections under the influence of inhibitor molecules (e.g. CSA, IgG):

- Incubate the placenta sections with 25 μL or 50 μL of binding medium or PBS containing the inhibitor for 1 h at 37 °C.
- Wash the sections for 3 min with PBS in a petri dish placed on a slow-moving shaker (see below).
- AND/OR: Resuspend PRBC in binding medium containing the inhibitor and incubate them for 1 h at 37 °C.

Perform the binding assay as described above.

Include at least 2 wells with a positive control (i.e., with no inhibitor present) on each slide to control the quality of the experiment.

Also assess the adhesion of each isolate in the presence of a decoy inhibitor molecule e.g BSA (1-5 mg/mL), as a negative control for inhibition.

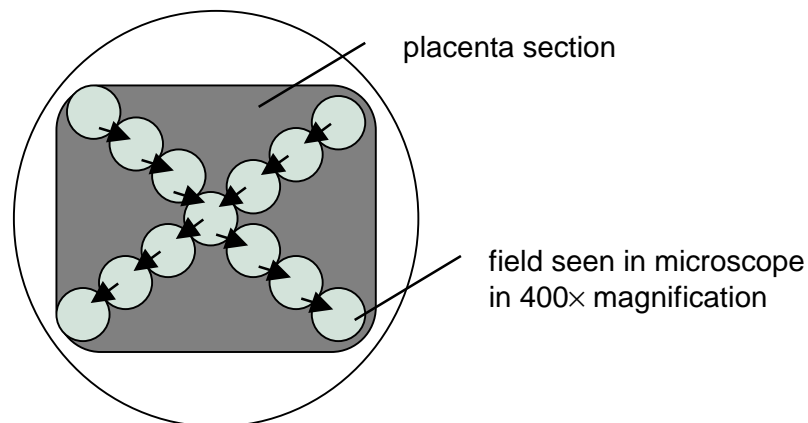


Enzyme treatment of placenta sections:

- Use a volume of 25 μL or 50 μL on each section:
 - hyaluronidase: 10 $\mu\text{g}/\text{mL}$ of enzyme in PBS (pH 7.2); incubate at 37 °C for 45 min.
 - *Streptomyces* hyaluronate lyase 25-50 U/mL in PBS (pH 7.2); incubate 1 hr at 37 °C.
 - chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6); incubate at 37 °C for 10 to 30 min.
 - heparinase: 0.5 units/mL in PBS, incubate at 37 °C for 30 min.
 - Wash the sections for 3 min with PBS in petri dishes.
 - Include at least 2 wells with a positive control (i.e., no enzyme treatment) on each slide to control the quality of the experiment.
- Perform the binding assay as described above.

Counting of bound PRBC in the microscope:

- Count from upper left corner to lower right corner of the section, field next to field at 400 \times magnification.
- Repeat from upper right corner to lower left corner.
- Determine bound PRBC/field, calculate to PRBC/ mm^2 . Count at least 30 high power fields.
- The level of adhesion or inhibition in adhesion after treatment is expressed as percentage of the positive control. Cut-off is set to the mean inhibition level obtained with the BSA controls + 2 SD.

Reference

Flick K, Scholander C, Chen Q, Fernandez V, Pouvelle B, Gysin J, Wahlgren M. 2001. Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* 293(5537): 2098-2100.

Rasti N, Namusoke F, Chene A, Chen Q, Staalsoe T, Klinkert MQ, Mirembe F, Kironde F, Wahlgren M. 2006. Nonimmune immunoglobulin binding and multiple adhesion characterize *Plasmodium falciparum*-infected erythrocytes of placental origin. *Proc Natl Acad Sci U S A.* 12;103(37):13795-800.

VIII. Detection of antibodies to the infected erythrocyte surface

VIII:A. Reversion of rosettes

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See PARASITES, section VI:M, p. 53

VIII:B. Agglutination assay using purified trophozoite-infected erythrocytes.

Measurement of antibodies to *Plasmodium falciparum* variant antigens on the surface of infected erythrocytes

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Materials and reagents

ethidium bromide

phosphate-buffered saline

microscope slide

glass coverslips

mounting medium (eg Depex)

96-well microtitre plates, Eppendorf tubes (1.5 mL), or similar

Giemsa staining (see PARASITES, section III:B)

human serum

methanol

Procedure

- Isolate trophozoite-infected RBC (not schizonts) from culture using Percoll density gradients, gelatin, or other methods, described elsewhere.
- After harvesting parasites, carefully and gently wash three times with warm PBS.
- Make a smear, stain with Geimsa, and examine parasite preparation – check that parasite-infected RBC are intact. Should aim for at least 50% parasitaemia (higher is better).
- Incubate parasites with human serum diluted 1/10, for 45 mins on a rotating wheel, room temperature. This is best done by putting 2.5 uL aliquots of each test serum into individual wells of a 96-well microtitre plate (or using Eppendorf tubes).
- Then add 22.5 uL of parasite suspension (at a 2% haematocrit) to each well containing test serum.
- Always include a negative control serum.
- Usually parasites harvested from 25 mL of culture (5-10% parasitaemia, 3% haematocrit) can be resuspended in 0.5-1.0 mL of PBS.
- Following incubation, pipette out 10 uL of the suspension and spread gently onto a glass slide making a circle of about 1.5cm diameter, using a plastic pipette tip. Make 2 'smears' for each samples (on the same or separate slides).

- [Alternatively, to examine cells as 'wet preps', place a coverslip over a 10 uL drop (do not smear out) and examine by microscopy (see last step).
- Allow to dry (5 minutes), fix smears in fresh methanol, stain for 5-10 mins with 10% Giemsa (do not over-stain). Once dry, coverslip slides using a permanent mounting medium (eg. Depex). Slides can be stored (protected from light) for long periods and examined or re-examined at any time.
 - Examine both smears for each sample, by light microscopy:
 - initially scan for agglutinates using 10x objective
 - examine any agglutinates at 40x
 - only agglutinates of 5 infected cells or more are considered positive
 - record the percentage of parasitised RBC in agglutinates and the size of the largest agglutinate observed

Comments

- Only count agglutinates of parasitised RBC – don't count agglutinates of parasites that have broken out of the RBC membrane (which frequently and easily agglutinate). Make sure you can see an intact RBC membrane around cells in agglutinates.
- Watch out for agglutinates of cells with debris – agglutinated cells must be in contact with each other.
- Do not count linear agglutinates – cells lined up in a row are rarely true agglutinates.
- Find an area where cells are evenly distributed

VIII:C. Serum micro-agglutination of infected erythrocytesby **Antonio Barragan**

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EquipmentUV light microscope
rotatorMaterials and reagentsRPMI 1640/HEPES/sodium bicarbonate/ gentamycin solution (MCM; see PARASITES, section I:A)
acridine orange (see PARASITES, section III:A)
polystyrene round-bottomed tubesProcedure

- Culture parasitized red blood cells (PRBC) to the late trophozoite and/or early schizont stage and wash the cells 3 times in MCM; resuspend the PRBC at 20% hematocrit.
- Dispense aliquots of 25 μ L in prelabelled polystyrene round-bottomed tubes.
- Add 25 μ L of serum to the parasite suspension (1:2 dilution). For a 1:5 dilution serum test, add prediluted serum at 1:2.5 in MCM.
- Incubate the PRBC and serum mixture at 37 °C for 1 h with constant rotation.
- Mount an aliquot from each polystyrene tube, mix with a small amount of acridine orange on a glass slide, and count 50 consecutive fields of vision diagonally using a 40 \times lens and incident UV-light microscopy.
- Always use negative and positive control sera along with the test sera.

Interpretation

- Score the assay as negative when no agglutinates of 4 or more PRBC are detected in the examined slide.
- Use the following semiquantitative scoring scale for agglutination analysis:
 - (-) for no agglutinate of 4 or more PRBC
 - (1+) for 1 to 5 agglutinates of 4 to 10 PRBC
 - (2+) for >5 agglutinates of 4 to 10 PRBC or 1 to 5 agglutinates of 11 to 20 PRBC
 - (3+) for >5 agglutinates of 11 to 20 PRBC or 1 to 5 agglutinates of >20 PRBC
 - (4+) for >5 agglutinates of >20 PRBC
 - (UA) for unspecific agglutination
- When using rosetting strains, note the presence or absence of rosettes.

References

Aguiar JC, Albrecht GR, Cegielski P, Greenwood BM, Jensen JB, Lallinger G, Martinez A, McGregor IA, Minjas JN, Neequaye J, et al. 1992. Agglutination of *Plasmodium falciparum*-infected erythrocytes from east and west African isolates by human sera from distant geographic regions. Am J Trop Med Hyg 47(5):621-632.

Barragan A, Kremsner PG, Weiss W, Wahlgren M, Carlson J. 1998. Age-related buildup of humoral immunity against epitopes for rosette formation and agglutination in African areas of malaria endemicity. *Infect Immun* 66(10):4783-4787.

Marsh K, Howard RJ. 1986. Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. *Science* 231(4734):150-153.

VIII:D. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected with *Plasmodium falciparum*

by **Helene Jouin**

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The indirect immunofluorescence Labeling is performed as usual on living cells in suspension, and parasitized erythrocytes are selected by gating the fluorescence intensity of either Hoechst 33 342 or thiazole orange (TO) dyes, which reflect the erythrocyte content of parasitic DNA or RNA. TO allows identification of only mature stages of the parasite. Nevertheless, since we have not observed surface immunostaining of the young stages of the parasite when using Hoechst, TO is a good reagent because it allows performing the flow cytometric analysis with a single laser analyser.

Materials and reagents

Hoechst 33 342 (Sigma)

thiazole orange (TO) (Retic-COUNT, BD Immunocytometry Systems)

FACSFlow solution (BD Immunocytometry Systems) containing 2% fetal calf serum

General procedures for DNA or RNA staining, surface immunofluorescence staining, and flow cytometry analysis

When Hoechst is used, the DNA staining is performed first, followed by surface immunofluorescence staining (using FITC or phycoerythrin conjugated second antibody). It is necessary to use two lasers because of the differing excitatory wavelengths of the two fluorochromes.

When TO is used, surface immunofluorescence staining is performed before the RNA staining. The samples can be analysed on a single laser (488 nm). Since TO fluorescence is read using the FITC channel, FITC conjugates cannot be employed for surface immunofluorescence.

Intraerythrocytic parasite DNA staining with Hoechst 33 342 (before surface immunofluorescence staining)

- Dissolve Hoechst 33 342 at 1 mg/mL of distilled water.
- Add the solution directly to the culture medium at a final concentration of 20 µg/mL.
- Incubate the culture for 30 min at 37 °C in the dark.
- Wash the culture in PBS and then perform the immunofluorescence staining.

Intraerythrocytic parasite DNA staining with TO (after immunofluorescence staining)

- Resuspend the washed immunofluorescent stained parasitized red blood cells directly in 1 mL of a solution of TO.
- Keep in the dark for 30 min prior to flow cytometric analysis.

Surface immunofluorescence staining

- Resuspend a pellet of 5×10^6 parasitized red blood cells in 100 µL of immune serum diluted 1:50 in FACSFlow solution containing 2% fetal calf serum.

- Incubate the pellet at room temperature for 30 min.
- Wash it 3 times with FACSFlow-FCS.
- Resuspend the pellet in the appropriate fluorescent-conjugated antibody.
- Incubate it at room temperature for 30 min.
- Wash it twice with FACSFlow-FCS.
- Resuspend it in 1 mL of FACSFlow.

Flow cytometric analysis

Parasitized red blood cells that have been stained with Hoechst 33 342 must be analysed on a cell sorter equipped with two lasers: a UV laser tuned to 320 nm for the excitation of the Hoechst dye and a second laser tuned to 488 nm for the excitation of FITC and phycoerythrin.

Parasitized red blood cells which have been stained with TO and phycoerythrin conjugates can be analysed on a standard flow cytometer equipped with only one laser tuned to 488 nm. The TO emission is detected in the FITC channel. The color compensation between TO and phycoerythrin fluorescences is set up using a control sample (without surface immunofluorescence staining).

Erythrocytes are gated on the basis of their forward scatter and side scatter signals using logarithmic scales. Parasitized erythrocytes are gated on the basis of their positive staining with Hoechst 33 342 or TO. All fluorescence parameters are recorded with logarithmic amplification. Process list mode data from 10,000 gated cells.

Reference

Jouin H, Goguet de la Salmoniere YO, Behr C, Huyin Qan Dat M, Michel JC, Sarthou JL, Pereira da Silva L, Dubois P. 1995. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected by *Plasmodium falciparum*. J Immunol Methods 179(1):1-12.

VIII:E Analysis by flow cytometry of antibodies to variant surface antigens expressed by *P. falciparum*-infected erythrocytes

by **Salenna R. Elliott** and **James G. Beeson**

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Materials and reagents

phosphate buffered saline (PBS) OR RPMI-HEPES

fetal calf serum (FCS) – heat-inactivated and filtered; OR Casein (filtered)

EtBr 1mg/mL

secondary antibody - Anti-human IgG (affinity purified). Eg. Goat anti-human IgG

tertiary antibody – FITC-conjugated anti-secondary Ig (affinity purified) eg. anti goat Ig; or Alexa-Fluor-conjugated tertiary antibody

U-bottom 96 well microtitre plates

Procedure (Beeson et al. 1999)

Buffers to use in the assay

For all incubations and washes, either RPMI-HEPES or PBS can be used, with either 0.1% casein or 1% FCS added

- Use parasitised red blood cells (PRBC) from in vitro culture at 1-5% parasitaemia, synchronous at mid-late stage pigmented trophozoites (not schizonts).
- Wash PRBC three times in PBS with 0.1% casein or 1% FCS prior to using in the assay.
- Pre-coat the required number of wells of a U-bottom 96 well microtitre plate with 0.1% casein or 1% FCS in PBS for 30 mins, RT, to block non-specific binding. This reduces cell loss in the assay. Drain wells after incubation.
- Aliquot serum or plasma samples (heat-inactivated) to be tested into 96 well plate.
- Resuspend PRBC to $1-2 \times 10^7$ cells/mL (0.1-0.2% haematocrit).
- Add 50 ul PRBC suspension per well containing test serum to achieve desired serum concentration (usually 1/10 or 1/20).
- Incubate 30 mins at RT, then wash 3 times.
- Add 50 uL of secondary antibody (eg. goat anti-human IgG (Fc. Sp.))
- Incubate 30 mins at RT, then wash 3 times.
- Add 50 uL of tertiary antibody (FITC-conjugated anti-secondary IgG; or Alexa-Fluor 488-conjugated antibody) diluted in buffer containing ethidium bromide 10-20 ug/mL.
- Incubate 30 mins at RT **in the dark**; wash 3 times
- Resuspend cells in 200 ul of buffer and transfer to flow cytometry tubes

Analysis by flow cytometry

- Gate RBC population

- Plot cells by fluorescence in channel 1 (for FITC or Alexa-Fluor) against fluorescence in channel 2 (for EtBr). There should be a clear separation of ethidium bromide positive cells (PRBC) from negative cells (RBC).
- Acquisition time should be sufficient to count 500-1000 ethidium bromide positive cells
- For the ethidium bromide negative and positive populations, determine the mean fluorescence intensity in channel 1 (antibody binding) of the cell population and the proportion of cells positive for antibody binding.

Antibody binding can be expressed as

- 1. mean (geometric) in fluorescence channel 1 of:
 - PRBC (ethidium bromide positive cells) minus that of RBC (EtBr negative cells), giving the absolute difference in fluorescence, or
 - PRBC divided by that of RBC, giving the fold difference in fluorescence levels
- 2. Proportion of PRBC labelled as positive relative to the mean+3SD of the fluorescence of RBC

Comments

Using anti-Ig rather than anti-IgG as the secondary antibody gives high background with some isolates.

Using low parasitemias, and no agitation during incubation of cells with antibody, reduces the problem of agglutinates forming.

A 2-step procedure (primary antibody, then labelled secondary antibody) has been used with isolates and serum from children (Kinyanjui SM et al., 2003) and monkey antisera (Baruch DI et al., 2002).

References

Beeson, J. G., Mann, E. M., Elliott, S. R., Lema, V. M., Tadesse, E., Molyneux, M. E., Brown, G. V., and Rogerson, S. J. (2004). Antibodies to variant surface antigens of *Plasmodium falciparum*-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J Infect Dis* 189, 540-551.

Mount, A. M., Mwapasa, V., Elliott, S. R., Beeson, J. G., Tadesse, E., Lema, V. M., Molyneux, M. E., Meshnick, S. R., and Rogerson, S. J. (2004). Impairment of humoral immunity to *Plasmodium falciparum* malaria in pregnancy by HIV infection. *Lancet* 363, 1860-1867.

VIII:F. Analysis of plasma antibodies to variant surface antigens by flow cytometry
by **Trine Staaloe**

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Equipment

2-color flow cytometer

Materials and reagents

PBS + 2% FCS (PBS2)

Plasmodium falciparum late-stage infected erythrocytes purified by magnetic separation (see PARASITES, section IV:H) at 2×10^6 erythrocytes/mL in PBS2

ethidium bromide (0.1 mg/mL in PBS)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

human plasma or serum

goat anti-human IgG antibody (DAKO A473)

FITC-conjugated rabbit anti-goat Ig antibody (DAKO F250)

Procedure

Antibody Labeling

- Add ethidium bromide to the erythrocyte suspension (20 μ L/mL).
- Mix 100 μ L of ethidium bromide-labelled erythrocyte suspension with 5 μ L of test plasma/serum and incubate the tube for 30 min at 5 °C.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 μ L of PBS2.
- Mix the erythrocyte suspension with anti-human antibody (diluted 1:250) and incubate it as above.
- Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 μ L of PBS2.
- Mix the erythrocyte suspension with anti-goat antibody (diluted 1:25), and incubate it as above.
- Wash the suspension once in 3 mL of PBS2 and resuspend the sample in 200 μ L of PBS2.
- After antibody Labeling, samples may be kept at 5 °C overnight before analysis.

Data acquisition by flow cytometry

- Adjust flow cytometer settings to achieve a clear separation of uninfected (ethidium bromide-negative) and infected (ethidium bromide-positive) erythrocytes in an FSC/ethidium bromide dot plot (see Fig. 1 in Staaloe et al. 1999).
- Acquire and store data on FCS, SSC, FL1 (FITC), and FL2 (ethidium bromide).

Data analysis

- Flow cytometry data files (FCS format) can be analysed in any of the many software packages available. We usually use either the freeware WinMDI program (which is good for generating overlays and other graphics for publication) or WinList (which is a very powerful analysis program with wonderful

- possibilities for creation of macros that make a breeze of the analysis of the hundreds of files you will often need).
- Set a gate around late-stage infected erythrocytes in an FSC/ethidium bromide diagram plot (similar to R3 in Fig. 1D in Staalsoe et al. 1999).
 - Use the FITC fluorescence of the cells within this gate to quantify antibody recognition of variant surface antigens. This can be expressed either as the mean/median fluorescence or as the percentage of cells with FITC fluorescence above that of a control sample incubated without plasma/serum or with control plasma/serum (*viz.*, Fig. 2 in Staalsoe et al. 1999).

Reference

Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. *Cytometry* 35(4):329-336.

IX. Immunoglobulin- or serum protein-binding to infected erythrocytes

IX:A. Stripping erythrocytes of bound serum proteins and reformation of rosettes

by **Niloofer Rasti**

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Equipment

sterile test tubes
centrifuge (500 × g)
96-well plates
microscope (400×)

Materials and reagents

500 µL of a rosetting malaria culture (5% hematocrit, rosetting phenotype with a majority of the parasites in trophozoite stage)

PBS (pH 7.2, devoid of Mg²⁺/Ca²⁺)

heparin, 25000 IU/mL (Leo Pharma)

sodium citrate or PBS-EDTA for stripping

RPMI 1640–HEPES medium (50 mL):

0.52 g RPMI 1640 medium (with glutamine, without NaHCO₃; Gibco)

0.3 g HEPES (Gibco)

45 mL distilled water

Mix and set pH to 6.8.

Disruption of rosettes with heparin

Since the rosettes of every parasite strain differ in their heparin-sensitivity, you will have to titrate the necessary heparin-concentration (e.g., FCR3S1.2: 100 U/mL).

- Transfer 500 µL of culture to an appropriate test tube and add the necessary amount of heparin.
- Incubate the culture for 15 min before the next step.

Treatment with sodium citrate or PBS-EDTA to disrupt heparin resistant rosettes

This step can be performed on its own to strip parasitized RBC of bound serum proteins such as immunoglobulins; Both sodium citrate and PBS-EDTA can be used for this purpose. Incubation with 50 mM PBS-EDTA 1 hr at RT is a sufficient alternative protocol. For stripping with sodium citrate follow the protocol below.

- Centrifuge the culture at 500 × g and remove the supernatant.
- Add 500 µL of PBS with 100 mM sodium citrate (pH 7.4) to the pellet and incubate it for 5 min.
- Wash the pellet twice with PBS; centrifuge it each time (500 × g) and remove the PBS.
- Add 0.25 mL of RPMI 1640–HEPES to the pelleted malaria culture (the hematocrit will be 10%).

You may include 10% H-Albumin (Sigma), but this can interfere or block cell interactions and the binding to solid surfaces.

Rosette reformation

- Prepare the test substance (e.g., sera or proteins) in 2× concentration of above RPMI 1640 medium.
- Mix 25 µL of the washed culture (10% hematocrit; as above) with 25 µL of the test substance in a 96-well plate. The final hematocrit will be 5% (H-Albumin concentration, if included, will be 5% as well).
- Incubation probably works as well at room temperature as it does at 37 °C for 15 min.
- Read the rosetting rate (=reformed rosettes) using acridine orange (see PARASITES, section III:A)

Reference

Treutiger CJ, Scholander C, Carlson J, McAdam KP, Raynes JG, Falksveden L, Wahlgren M. 1999. Rouleaux-forming serum proteins are involved in the rosetting of *Plasmodium falciparum*-infected erythrocytes. *Exp Parasitol* 93(4):215-224.

IX:B. Detection of serum proteins on the surface of *Plasmodium*-infected erythrocytes by *Niloofer Rasti*

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Equipment

centrifuge (500 × g)
appropriate test tubes for centrifuge
microfuge tubes
glass slides
coverslips
UV-light microscope (100×)

Materials and reagents

malaria cultures (5 to 10% trophozoites)
antibody against serum protein to be detected (e.g., αIgM)
fluorochrome conjugated secondary antibody
RPMI 1640
ethidium bromide (1 µg/mL)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

Immunofluorescence

- The cultures to be tested should have a parasitemia of 5 to 10% with a majority of parasites in trophozoite stages.
- Transfer the culture to appropriate test tubes and wash (500 × g) it twice in RPMI 1640. Add RPMI 1640 to the initial volume.
- Dilute antibody to double final concentration of desired dilution in RPMI 1640. (A dilution factor of two will be introduced during the assay because of cell suspension.)
- Aliquot 50 µL of each dilution of antibody in small tubes (e.g., microfuge tubes) and add 50 µL of resuspended culture.
- Mix by shaking the tubes gently and incubate them for 30 min in the dark at room temperature with careful resuspension after 15 min.
- Shake the tube gently after 15 min.
- Wash the cells 3 times (500 × g) in 0.5 mL of RPMI 1640.
- Remove the supernatant carefully and add 100 µL of secondary antibody diluted to the final concentration. Mix gently.
- Incubate the cells for 30 min in the dark with careful resuspension after 15 min and wash them as before.
- Resuspend the cells in 40 µL of RPMI 1640 and mix 10 µL of cells with 0.5 to 1 µL of ethidium bromide (1 µg/mL) on a glass slide.
- Add a coverslip and count the fluorescence rate at 100× magnification with oil in a UV-light microscope.
- Calculate the fluorescence rate as =
$$\frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}}$$

Note: To reduce the amount of background the cells can be incubated with ethidium bromide (1 to 2 μL ; 1 $\mu\text{g}/\text{mL}$) for 5 to 10 s before the last wash instead of being mixed with ethidium bromide on the slide.

Note: Count all nonfluorescent cells with the corresponding size of the parasite as the parasites in the fluorescent cells. This is important to achieve the correct fluorescence rate, which could otherwise be too high.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med* 2(2):204-208.

VIII:C. Formaldehyde fixation for immunofluorescence analysis (IFA) of *P. falciparum*
by **Michael J. Blackman**

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Materials and reagents

snap-top plastic bags

silica gel dessicant

Immunopen

4% formaldehyde (Formaldehyde: Agar Scientific cat # R1026 (10 x 10 mL vials
16% microfiltered solution))

0.1% (v/v) TX100 in PBSA

3% (w/v) BSA in PBSA

PBS with 0.5% (v/v) Tween 20, 1% (w/v) BSA

1 µg/mL 4,6-diamidino-2-phenylindol (DAPI)

Procedure

- Air-dry thin films in a hood for 10-15 min at room temperature, then store in the presence of silica gel dessicant at -70 °C in snap-top plastic bags.
- For IFA, begin by removing the air-dried thin films from storage at -70 °C and allow them to reach room temperature still in the snap-top plastic bags containing dessicant. Remove from the bag and draw circles for antibody application with an Immunopen.
- FIXATION: make up 4% formaldehyde fresh by adding one vial (10 mL 16% formaldehyde) to 30 mL PBSA. Place air-dried slides into the fixative and incubate 30 min at room temperature.
- PERMEABILISATION: transfer slides to 0.1% (v/v) TX100 in PBSA at room temperature, incubate 10 min.
- Wash slides twice for 5 min each in PBSA.
- BLOCKING STEP: Block slides for 1 h at room temperature (or overnight at 4 °C) with 3% (w/v) BSA in PBSA.
- Continue as usual with antibody incubations, diluting antibodies in PBS/0.5% (v/v) Tween 20/1% (w/v) BSA and washing in PBS between steps. Finally counter-stain parasite nuclei with 1 µg/mL DAPI in PBS.

IX:D. Binding and eluting antibodies from the *Plasmodium*-infected RBC surface
by **Arnaud Chêne**

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Equipment

immufuge blood centrifuge
round-bottomed test tubes (e.g., 5-mL snap-cap tubes)
microfuge tubes
high speed microcentrifuge with cooling system
high quality pH paper

Materials and reagents

P. falciparum culture with a parasitemia of >8%. (If rosetting is of interest, the rosetting rate should be >50%.)
serum or antibody solution to be investigated
RPMI 1640
PBS (optional)
acridine orange

Solution A:

0.2 M glycine / 0.2 M NaCl:
3.75 g glycine
2.9 g NaCl
Bring to 250 mL in distilled water.

Solution B: (0.2 M HCl)

Mix 1.64 mL of concentrated HCl into 98.4 mL of distilled water.

Note: Mix acid into water, never vice versa with concentrated acids.

Glycine–HCl elution solution (pH 3):

Mix 5 mL of solution A and 1.3 mL of solution B.
Adjust to pH 3 using a high quality pH paper or pH meter.
Make fresh for each day of elution.

Neutralization solution: (2 M Tris-base)

9.69 g Tris-base
Bring to 40 mL; pH will be ~11.

Regarding disruption of rosettes and stripping of serum bound proteins, see Materials and reagents in PARASITES, section IX:A..

If using Percoll/sorbitol gradients to separate trophozoites for binding antibodies to the parasite, see Materials and reagents in PARASITES, section IV:D.

Procedure

- Use at least 1 mL of culture where the parasitemia is >8 to 10%. (If rosetting is of interest, the rate should be >50%; see PARASITES, sections VI:L, M.)

- If purified trophozoites are desired for the antibody binding, use Percoll/sorbitol gradients as in PARASITES, section IV:D. Have a large prep ready with either 12 small bottles (25-cm²) or 3 large ones (75-cm²) of culture for 8 Percoll gradients. Follow the protocol for gradients in section IV:D, then resuspend purified cells in 0.5 to 1 mL of RPMI 1640 before the addition of serum/antibodies, followed by the protocol below from the incubation/elution step onward.
- If using the culture straight as it is without trophozoite purification, place the culture in a round-bottomed tube holding a few milliliters and fitting an appropriate blood centrifuge (e.g., a 5-mL snap-cap tube in an immufuge blood centrifuge). Disrupt rosettes using heparin and a syringe, then strip cells of serum-bound proteins (see PARASITES, section IX:A above).
- After the sodium citrate treatment of cells, wash them once with PBS and then with RPMI 1640 (see section IX:A above).
- Add an equal volume of serum or antibody solution to the cell pellet as the starting volume of culture (i.e., mg/mL concentrations of antibody).
- Gently mix and slant the tube so that the blood solution almost reaches the surface of the tube. Carefully roll to mix the settling RBC every 15 min or so. Incubate the tube for 2 to 3 h at room temperature.
- While the tube is incubating, prepare the glycine–HCl elution solution. At the end of the incubation, check that RBC look normal through acridine orange vital staining (see PARASITES, section III:A).
- Spin down the cells (500 × g), remove the supernatant, and wash the cells twice with PBS.
- Elute bound antibodies by adding glycine–HCl elution solution to cells in the amount of the starting culture volume. Incubate them for 1 to 1.5 min only! (It is possible that the bound antibodies can be eluted with less elution buffer for increased antibody concentration; this would have to be tested empirically.)
- Quickly transfer the cells to 2 microfuge tubes and centrifuge them at 8,000 to 10,000 rpm in a chilled centrifuge for 1 min.
- Taking care not to touch the cell pellets, remove the supernatant with the eluted antibodies and pool them into one tube.
- To each milliliter of elution, add 26 to 30 μL of 2 M Tris-base. Mix carefully and use 4 μL to spot on a high quality filter paper. Check the pH. Add more Tris-base, 1 μL at a time, until the pH reaches 7.0 to 7.2. Check the pH after each addition. If the eluate becomes too basic, lower the pH by adding 10 to 20 μL of 0.2 M HCl (= solution B).
- Remove the precipitate that may form by spinning the eluate for a minute in the blood centrifuge.
- If you wish, you may dialyze the eluate overnight against a few changes of PBS in the cold, stirring it slowly.

For storage, you may add sodium azide to 0.02% and a “tiny tad” of pure human serum albumin (e.g., Sigma A3782) if this does not interfere with your subsequent assays.

You can use these eluted antibodies in surface immunofluorescence (see PARASITES, section IX:B above) or in standard Western blotting. Typically, 1:2 or 1:4 dilution of the eluted antibodies (from serum) would be visible in surface immunofluorescence, and 1:10 to 1:20 dilutions in Westerns.

IX:D. Enrichment of immunoglobulin binding parasites using Dynabeads
by **Mats Wahlgren**

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Equipment

Magnetic Particle Concentrator (MPC-1, Dynal)
haemocytometer
cryotubes
rotator for incubating cryotubes
25-cm² cell culture flask
centrifuge for washing RBC (immunofuge II, Baxter)
microscope (400×)

Materials and reagents

uncoated Dynabeads M-450 Tosylactivated (90 mg/mL, Dynal)
appropriate antibodies for coating the Dynabeads
0.1 M borate
PBS 0.1% HSA (50 mL PBS + 50 mg albumine, filter-sterilized)
PBS 10% HSA (50 mL PBS + 5 g albumine, filter-sterilized)
acridine orange
RPMI 1640
MCM without serum (see PARASITES, section I:A)
MCM with 10% serum (see PARASITES, section I:A)

Enrichment of the parasites

- Coat uncoated Dynabeads M-450 Tosylactivated with antibodies using the protocol provided by Dynal.
- Count the parasitemia of the *Plasmodium falciparum* culture and determine the stages of the parasites using acridine orange (see PARASITES, section III:A). It should have a parasitemia of approximately 10% and the parasites should be in trophozoite stages.
- Determine the number of erythrocytes per milliliter using a haemocytometer. Calculate the number of target cells (=infected RBC) by multiplying the number of cells with the parasitemia. One or two milliliters of parasite culture (approximately 5×10^7 target cells) is usually enough to achieve a good parasitemia after enrichment.
- Calculate the volume of Dynabeads to have 6 Dynabeads per target cell in the solution.
- Wash the Dynabeads 3 times with 3 mL of RPMI 1640 using a 3.6-mL cryotube and a Magnetic Particle Concentrator.
- Wash the culture 3 times (500 × g) with MCM without serum (see PARASITES, section I:A).
- Resuspend the erythrocytes in approximately 2 mL of RPMI 1640 and add the RBC to the Dynabeads in the cryotube.
- Immediately incubate the tube on a rotator at room temperature for 1.5 to 2.0 h, during which time the trophozoites will attach to the coated Dynabeads.

- After the incubation, before the beads/erythrocytes have time to settle, use the Magnetic Particle Concentrator to remove the medium while the PRBC attached to magnetic beads are bound to the MPC-1.
- Wash the beads/erythrocytes very gently, 1 or 2 times with RPMI 1640 using the Magnetic Particle Concentrator.
- Resuspend the beads/erythrocytes in MCM with 10% serum (see PARASITES, section I:A) in a 25-cm² cell culture flask at a hematocrit of 5% (i.e., 3.8 mL of MCM with serum, 0.2 mL of packed erythrocytes, and the Dynabead-bound *Plasmodium falciparum* infected erythrocytes in a 25-cm² flask).

During the next day, remove the beads from the culture using the Magnetic Particle Concentrator. The parasitized erythrocytes should by then have infected the free erythrocytes in the culture. Continue culturing the remaining erythrocyte mixture as described in PARASITES, section I.

Reference

Heddini A, Treutiger CJ, Wahlgren M. 1998. Enrichment of immunoglobulin binding *Plasmodium falciparum*-infected erythrocytes using anti-immunoglobulin-coated magnetic beads. Am J Trop Med Hyg 59(5):663-666.

X. Fractionation of the iRBC

X:A. Plasmodium-infected erythrocytes: separation of ghosts from parasite membranes

by **Stefan Baumeister**, **Markus Winterberg** and **Klaus Lingelbach**

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Equipment

centrifuge (10.000 × g), refrigerated
appropriate reaction tubes for centrifuge

Materials and reagents

Buffer A (hypotonic lysis buffer): 5 mM KH₂PO₄, pH 7.4

Buffer B (washing buffer): 100 mM PBS, pH 7.4

buffers contain:

Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Procedure

- Use the pellet of 1×10^8 – 5×10^8 enriched trophozoite stage parasites (~ 80% parasitemia).
- Resuspend the pellet in 0.4 mL buffer A and incubate for 10 min at RT (vortex every 2 min).
- Centrifuge the sample for 10 min at 10.000 x g in a microfuge (4 °C).
- To avoid cross contamination separate about 80% of the opaque supernatant (ghost fraction).
- Discard the remaining ghosts as well as 20% of the remaining parasites containing pellet.
- Wash the parasite pellet twice with 0.4 mL of buffer B (store at -20 °C).
- Spin down the ghost fraction at 10.000 x g for 20 min at 4 °C and wash the sediment with 0.5 mL buffer B (store at -20°C).

Comments

if available use 400 µL test tubes instead of 1.5 mL tubes. Because of the smaller diameter the ghost layer is bigger and separation much easier.



1.5 0.4 mL reaction tube

X:B. Subcellular fractionation of iRBC: use of saponin and streptolysin O

by **Stefan Baumeister, Markus Winterberg and Klaus Lingelbach**

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General comments

Treatment of infected erythrocytes with Streptolysin O (SLO) results in the release of the erythrocyte cytosol. Parasites contained within an intact parasitophorous vacuole can be sedimented by centrifugation. Treatment of infected erythrocytes with saponin results in the disintegration of the erythrocyte membrane and the parasitophorous vacuolar membrane. Intact parasites can be sedimented by centrifugation.

Permeabilization of iRBC with Streptolysin O

Equipment

centrifuge, refrigerated
counting chamber

Materials and reagents

Buffers:

PBS⁺⁺: 10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 145 mM NaCl, 3 mM KCl, pH 7,2
buffer contains: Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Streptolysin O (SLO): kindly provided by S. Bhakdi, Mainz, Germany

100 µg of lyophilized protein are dissolved in 1,12 mL PBS⁺⁺ and stored on ice for determination of hemolytic units (see below)

Alternative: Preparation of SLO from SIGMA see below

Procedure

- Incubate iRBC (in aliquots of 2×10^8 cells) in 200 µL PBS with 3–4 hemolytic units of SLO at room temperature for 6 min.
- Centrifuge samples at 10,000 x g for 15 s.
- Save supernatant (containing the cytosol of the infected RBC).
- Wash the pellet (containing intact parasites, the vacuolar contents, and membranes) twice with 200 µL of PBS.
- Fractions are now ready for further analyses.

Determination of hemolytic units (HU)

1 hemolytic unit is determined as the amount of dissolved SLO necessary for the lysis of 50 % of red cells

- Take ~200 µL of packed red blood cells and wash three times with PBS.
- Resuspend in 1 mL PBS⁺⁺.
- Count cells with counting chamber.
- Aliquot 10^8 cells in 6 cups (I – VI).
- Cup I: **hemoglobin control**, adjust cell suspension to final volume of 100 µl, lyse cells by three freeze/thaw-cycles, spin at 10,000 x g, 20 min, 4 °C and dilute supernatant 1 : 500.
- Extinction at 412 nm gives total amount of haemoglobin.

- Cups (II – VI): **SLO-lysis**, adjust cell suspension to 90µL and suspend cells thoroughly, add to each cup increasing amounts of SLO (6, 8, 10, 12, 14 µL) and gently vortex each cup, incubate for 6 minutes at room temperature, spin at 10,000 x g, 20 sec, 4 °C and dilute supernatants 1 : 500.
- Extinction at 412 nm gives the released amount of haemoglobin.
- Choose amount of SLO sufficient for hemoglobin release of ~100 % (2 HU).
- Make proper aliquots of SLO-solution and store at -80°C.

Use of Sigma SLO

Sigma provides SLO as a lyophilized powder with a total activity of 25,000 units

Buffers: 10 mM PBS pH 7.4, 100 mM NaCl
1 M DTT (in water or PBS)

- Dissolve SLO in 2.25 mL PBS.
- Make aliquots of 90 µL and freeze them at -20° C.
- Thaw 90 µl SLO and add 10 µl of 1 M DTT (in water or PBS) - incubating 15 min at RT yields activated SLO (SLO*).
- Use 300 units SLO* for 1 x 10⁸ iRBC:
- 10⁸ iRBC are dissolved in 70 µL PBS
- Add 30 µL SLO*.
- Incubate 10 min at RT.

Permeabilization of iRBC with Saponin

Materials and reagents

Buffers:

PBS⁺⁺: 10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 145 mM NaCl, 3 mM KCl, pH 7,2
buffer contains: Protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Saponin from SIGMA

Procedure:

- for saponin lysis, incubate iRBC (in aliquots of 2 x 10⁸ cells) in 200 µL of 0.1% saponin in PBS pH 7.2 on ice for 5 min.
- centrifuge the samples at 2,500 x g for 5 min
- remove supernatant (containing host cytosol and vacuolar contents)
- wash the pellet (containing the intact parasite) twice with 200 mL of PBS before further processing

References

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X:C. Purification of cholesterol-rich membrane microdomains (DRM-rafts) from *Plasmodium* infected erythrocytes

by **Marta Ponzi**¹ and **Catherine Braun Breton**²

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Equipment

Eppendorf microfuge
Beckman ultracentrifuge
SW60 rotor
Dounce homogenizer

Materials and reagents

Purified infected RBC ($2-5 \times 10^8$)
Erythrocyte lysis buffer (10x stock solution):
1.5M NH₄Cl
0.1M KHCO₃
0.01M EDTA
Parasite lysis buffer:
25mM MES pH 6.5
0.15 M NaCl
1% TritonX-100
Protease inhibitors (Roche tablets)
MES buffered saline:
25mM MES pH6.5
0.15M NaCl
80% sucrose in MES buffered saline (solution A)
30% sucrose in MES buffered saline (solution B)
5% sucrose in MES buffered saline (solution C)

Procedure

Erythrocyte lysis:

- Resuspend purified infected RBC pellet in 2 mL of cold 1x erythrocyte lysis buffer.
- Incubate this suspension in ice until lysis is completed (10-15 min) and spin at 4°C in 1.5 mL Eppendorf tubes (5 min at 6000 rpm) to collect free parasites.
- Wash the pellet several times at the same speed with cold PBS to remove soluble hemoglobin.
- *Comment:* this procedure can be adopted to lyse both human and rodent infected RBC. Hemoglobin removal is less efficient than that obtained by saponin treatment; however saponin can't be used because it partially removes cholesterol from membranes thus affecting lipid rafts integrity.

DRM-rafts purification:

- Add 720 µL of cold parasite lysis buffer to the pellet kept on ice ($2-5 \times 10^8$ parasites) and homogenize cell extract with 10 strokes of a Dounce homogenizer.
- Transfer cell lysate in an ultracentrifuge tube (Beckman, 11 x 60 mm) and adjust to 40% sucrose solution by adding 750 µL of solution A.
- Thoroughly overlay with 1.5 mL of solution B and 1.5 mL of solution C.

- Centrifuge at 45,000 rpm for 16-20 h at 4°C in a SW 60 rotor (Beckman Instruments).
- Collect 370 µL fractions from the top of the gradient (12 fractions). DRM-rafts appear as an opaque band migrating at 10-20% sucrose (fractions 4 and 5).

Comments

It is important that the entire procedure is performed in the cold to avoid solubilisation of raft-associated proteins during cell extract preparation

References

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IV:E. Obtaining free parasites

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SEE: Purification and synchronization of erythrocytic stages, IV E, page 28

XI. Fixation protocols for transmission electron microscopy

X:A. Fixation of tissue samples

by **Anne von Euler**

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Please note: Use only glutaraldehyde of high purity, "EM-grade". Purchase only small amounts at any one time, store them at $-20\text{ }^{\circ}\text{C}$, and use them as soon as possible. Also, use only paraformaldehyde powder. It is important to dissolve the paraformaldehyde in alkaline buffer before adjusting the cacodylate buffer to neutral pH.

Please note: **ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.**

Solutions

For the solutions below:

- Dissolve paraformaldehyde in double-distilled water in a FUME HOOD.
- Stir and heat the solution until it reaches $60\text{ }^{\circ}\text{C}$.
- Add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.
- Cool this solution before adding cacodylate and glutaraldehyde.
- Adjust the pH with 1.0 M NaOH or 2 M HCl.
- Store solutions at $4\text{ }^{\circ}\text{C}$ in dark bottles and use within 1 month or before a white precipitate appears.

Fixation buffer, 100 mL:

0.1 M sodium cacodylate (dimethylarsinic acid sodium salt trihydrate for synthesis), 1% glutaraldehyde, 1% paraformaldehyde (pH 7.4):

1 g paraformaldehyde (> 95% pure)

2.2 g sodium cacodylate

1.4 mL 70% glutaraldehyde (1.96 mL if 50% GA stock), EM-grade

For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water

Fixation storage buffer, 100 mL:

0.1 M sodium cacodylate, 0.1% glutaraldehyde, 0.1% paraformaldehyde (pH 7.4):

0.1 g paraformaldehyde (> 95% pure)

2.2 g sodium cacodylate

0.14 mL 70% glutaraldehyde (0.2 mL if 50% GA stock), EM-grade

For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water.

Procedure

- Cut pieces of tissue to a maximum size of 0.5 cm^3 .
- Immediately immerse the tissue cube in fixation buffer and leave it for 30 min.
- Aspirate the fixation buffer and fill the tube with fixation storage buffer. Fill the tube to the top to avoid contact with oxygen which will cause formalin crystals in the sample.
- Close the tube carefully and seal it with Parafilm.
- Store the samples at $4\text{ }^{\circ}\text{C}$. When being transported, the samples do not need to be at $4\text{ }^{\circ}\text{C}$ if that is not possible.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med.* 2(2):204-208.

X:B. Embedding samples for electron microscopy

by **Anne von Euler**

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Equipment

Oven placed in a fume hood or connected to special ventilation

Materials and reagents

Durcupan (Fluka)

acetone

First Durcupan solution:

1 part Durcupan

2 parts acetone

Second Durcupan solution:

2 parts Durcupan

1 part acetone

0.1 M sodium cacodylate (EM-buffer)

1% OsO₄ in 0.1 M EM-buffer

All ethanol used should be diluted from 99.5% ethanol.

99.5% ethanol

70% ethanol

50% ethanol

95% ethanol plus 2% uranyl acetate

Cell samples should be in small tubes, which allow good mixing. Use short glass Pasteur pipettes and break off the thin tip when handling the Durcupan. Use a special waste container for the Durcupan, one that allows the acetone to evaporate in the fume hood and the Durcupan to polymerize and detoxify.

Use 0.1 M sodium cacodylate as a buffer since phosphate buffer can produce precipitates in some samples. It is referred to as EM-buffer in the protocol.

Please note: **ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.** Work in a fume hood at all times and use proper waste containers for the chemicals.

Day 1:

- Aspirate the supernatant and rinse the cells 3 times for 5 min each in 0.1 M EM-buffer.
- To give contrast, add enough 1% OsO₄ in 0.1 M EM-buffer to cover the sample.
- Incubate the sample for 45 min at 37 °C or for 1.5 h at room temperature. The OsO₄ evaporates easily, so seal the tubes properly.
- Aspirate the OsO₄ and discard it in a proper waste container for OsO₄. Rinse the sample 3 times for 5 min each in EM-buffer.
- For dehydration, add each of the reagents listed below, one at a time for 5 min each at room temperature. Aspirate and discard the supernatant after each 5-min interval. All ethanol used should be diluted from 99.5% ethanol.
 - 50% ethanol
 - 70% ethanol

95% ethanol plus 2% uranyl acetate
99.5% ethanol
acetone

- Add the first Durcupan solution and leave the sample for 2 to 5 h at room temperature.
- Aspirate the Durcupan/acetone solution and add the second Durcupan solution and leave the sample overnight at room temperature.
- *Day 2:*
- Aspirate the Durcupan/acetone solution, add pure Durcupan, and leave the sample overnight at room temperature.
- *Day 3-4 or 3-5:*
- Change Durcupan daily until polymerization.
- Put a little label in the Durcupan, for identification, quite close to the sample itself.
- Polymerize the samples in a 50 °C oven for at least 20 to 30 h. The polymerization can be done over a weekend. The oven should be placed in a fume hood or connected to special ventilation.

Reference

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat. Med.* 2(2):204-208.

X:C. Fixation of erythrocytes for immuno-electron microscopy*by Anne von Euler**Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: anne.von.euler@mtc.ki.se***Equipment**

centrifuge

Materials and reagents

parasite culture with desired parasitemia

RPMI 1640

0.1 M phosphate buffer (pH 7.4)

Fixation solution, 100 mL (1% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4):

Prepare phosphate buffer stocks (Na_2HPO_4 and NaH_2PO_4), then mix for 0.2 M (pH 7.4) according to Maniatis or "Current Protocols in Molecular Biology".

Mix 1.0 g paraformaldehyde in 50 mL of double-distilled water. In a FUME HOOD, stir and heat the solution until it reaches 60 °C, add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.

50 mL 0.2 M phosphate buffer

0.4 mL glutaraldehyde (50% stock)

This fixing solution lasts for 2 months when stored at 4 °C.

Procedure

- Grow a parasite culture until desired parasitemia is reached.
- Cool RPMI 1640, 0.1 M phosphate buffer, and fixing solution to 4 °C.
- Wash cells in RPMI 1640 twice and once in cold 0.1 M phosphate buffer (pH 7.4).
- Fix samples in cold fixation solution for 10 min at 4 °C.
- Wash the sample three times in cold 0.1 M phosphate buffer. If using microfuge tubes, spins at 2,500 rpm for 1 min at 4 °C suffice.
- If shipping samples, fill the tubes completely with 0.1 M phosphate buffer, cap them well, and ship them on ice by express.
- If shipping antibodies, add sodium azide for a final concentration of 0.01% and send them on ice by express.

References

Method developed by Dr. Masamichi Aikawa.

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XII. In vitro reinvasion and growth inhibition assays

XI:A. In vitro reinvasion and growth inhibition assay by microscopy of erythrocyte monolayers

by **Birgitta Wahlin-Flyg**

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Equipment

centrifuge

UV-microscope

Materials and reagents

human type O+ RBC

parasitized red blood cells (PRBC)

TCM:

HEPES-buffered (20 mM) RPMI 1640 medium

10% normal human serum

2 mM glutamine

gentamycin (25 µg/mL)

0.2% NaHCO₃

Tris-buffered Hanks' solution (TH)

0.06 M bicarbonate buffer (pH 9.6)

1% glutaraldehyde in PBS

acridine orange (10 µg/mL)

96-well flat-bottomed tissue culture plates

conical tubes

8-well multitest slides

coverslips

Procedure

- Using normal human type O⁺ RBC, dilute parasitized red blood cells (PRBC) from *Plasmodium falciparum* cultures infected with late trophozoites and schizonts to a parasitemia of approximately 1% and adjust the hematocrit to 2% with TCM.
- Set up quadruplicate tests in 96-well flat-bottomed tissue culture plates. Use 100 µL of parasite culture mixed with 100 µL of TCM or various dilutions of antibodies.
- Incubate the plate at 37 °C for 18 to 20 h in a candle jar (see PARASITES, section I:A).
- Transfer the quadruplicate tests separately to 4 conical tubes, wash them by centrifugation 2 times with 1 mL of Tris-buffered Hanks' solution (TH), followed by a dilution to 1% hematocrit, by adding 150 µL of TH.
- Make monolayers in duplicates from each tube on 8-well multitest slides that have been pretreated for 30 min with 0.06 M bicarbonate buffer (pH 9.6).
- After 30 min, wash the slides in TH, fix them by two treatments for 10 s each with 1% glutaraldehyde in PBS, wash them with distilled water, and air-dry them.
- Store the slides at room temperature until they are analysed in the UV-microscope.

Analysis of percent parasitemia by UV-microscopy

- To analyse the parasites, stain by adding one drop of acridine orange (10 µg/mL) per well for a few seconds, then wash the slides with distilled water and mount a coverslip.
- Screen 25 microscope fields per well (200 visual fields/slide). Since the number of RBC/microscope field has been estimated to be 200/field, the percent parasitemia can be calculated from a total of 40,000 RBC/culture:
- Calculate the percent parasitemia as:

$$\frac{(\text{percent parasitemia in control} - \text{percent parasitemia in sample}) \times 100}{\text{per cent parasitemia in control}}$$

Reference

Udomsangpetch R, Wåhlin B, Carlson J, Berzins K, Torii M, Aikawa M, Perlmann P, Wahlgren M. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. J Exp Med 169(5):1835-1840.

XI:B. In vitro reinvasion and growth inhibition assay by flow cytometric measurement of parasitemia using propidium iodide (PI) staining

by **Alice Nyakeriga**

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This method allows for quantification of parasitemia and for quantification of parasites in different stages of differentiation according to DNA content. Many similar protocols have been published using different intercalating dyes such as acridine orange, thiazole orange, hydroethidine, or YOYO-1. Each of them has advantages and disadvantages. Below we describe our variant that we find easy and reliable. Care should be taken to distinguish nucleated cells from parasitized erythrocytes when analyzing material containing nucleated cells. The protocol can be extended by additional labeling of cells using FITC-, PE-, and APC-labeled antibodies.

Equipment

centrifuge
flow cytometer
FACScan (Becton-Dickinson)
FACScalibur (Becton-Dickinson)

Materials and reagents

PBS
PBS containing 0.025% (v/v) glutaraldehyde
PBS containing 0.01% saponin
PBS containing 2% FCS
propidium iodide (PI)

Procedure

- Take 1×10^6 to 2×10^6 red blood cells (RBC) from a culture or a drop of blood from an infected individual collected into heparinized isotonic buffer (e.g., PBS or 0.9% sodium chloride).
- Wash the cells in PBS twice.
- To fix the cells, resuspend them in 1 mL of PBS containing 0.025% (v/v) glutaraldehyde, and incubate them at room temperature for 20 min. Alternatively, incubate the cells at 4 °C for 30 to 45 min. Cells may be stored in the fixative for several weeks before proceeding.
- Centrifuge the cells for 5 min at $450 \times g$. Aspirate the supernatant followed by two washes in PBS.
- Permeabilization:
 - Resuspend the cells in 0.5 mL of PBS containing 0.01% saponin.
 - Incubate them at room temperature for 5 min.
- Wash the cells in PBS twice at $450 \times g$ for 5 min.
- Resuspend the cells in 0.5 to 1.0 mL of FACS buffer. (We use PBS containing 2% FCS.)
- Staining with propidium iodide: To the above cell suspension, add PI to a final concentration of 10 $\mu\text{g/mL}$. Incubate the cells at 37 °C for 1 to 2 h.
- Analyze the cells in a flow cytometer with a 488-nm laser for excitation, and detect emission from intercalating PI in the 670-nm long pass filter (red FL3

channel). We use the FACScan and the FACScalibur from BD and detect emission in FL3.

References

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Whaun JM, Rittershaus C, Ip SH. 1983. Rapid identification and detection of parasitised human red cells by automated flow cytometry. *Cytometry* 4(2):117-122.

Wyatt CR, Goff W, Davis WC. 1991. A flow cytometric method for assessing viability of intraerythrocytic hemoparasites. *J Immunol Methods* 140(1):23-30.

XI:C. Erythrocyte invasion assay

by **Alan Cowman, Brendan Crabb, Alexander Maier, Chris Tonkin, Julie Healer, Paul Gibson and Tania De Koning-Ward**

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see: Transfection, IV: C, page 293

XI:D. *P. falciparum* growth or invasion inhibition assays using antibodies
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Materials and reagents

RPMI-HEPES with 5% pooled serum
Albumax II
glutamine
hypoxanthine
gentamicin
U-bottom 96 well tissue culture plate (Falcon 3077)
citrate phosphate dextrose (CPD) buffer

Procedure

Growth inhibition assays performed over one or two cycles of parasite replication

- Repeatedly synchronise parasite cultures in the 1-2 weeks prior to performing the assay. Recommended methods for synchronisation:

1. Sterile 5% sorbitol (in water): performed when parasites are mainly ring forms by resuspending parasite culture pellet in sorbitol solution, incubating for 5 mins, centrifuge at 1500 rpm for 5 mins, then resuspend pellet back into culture medium
2. Gelatin enrichment of pigmented trophozoites: involves flotation of mature-stage parasites in 0.75% gelatin
3. Magnet purification of mature pigmented parasitized RBC

- Use highly synchronous parasite culture in the assays (this is critical). Most PRBC must be at the late pigmented trophozoite or early schizont stage. Do not use if cultures have many ring-stage parasites (very important)

Setting up the assay:

- Starting parasitemia should be around 0.5 – 0.8% for a one-cycle assay, and around 0.1-0.3% for a two-cycle assay. Reduce the parasitaemia of starting culture by adding fresh RBC as appropriate.
- Prepare parasites: resuspend parasites at 1% haematocrit (eg 50 uL of cell pellet in 5 mL of medium) in RPMI-HEPES with 5% pooled serum and 0.25% Albumax II (0.25% equals 2.5 g in 1 L). RPMI-HEPES must be properly supplemented with glutamine (2 mM) and hypoxanthine (50 µg/mL). Good to also include gentamicin to help prevent bacterial contamination.
- Setting up the plates: Use U-bottom 96 well tissue culture plate (Falcon 3077). Test all samples in duplicate. Include positive and negative controls in every assay run (eg. inhibitory antibodies, samples from non-exposed donors). Include negative controls on every plate if setting up multiple plates (can use method A or B).

method A:

- Aliquot samples to be tested in inhibition assays into U-bottom 96 well plates. Keep plates on ice to avoid samples evaporating.
- Add parasite suspension to each well of the 96 well culture plate. Final volume should be 25 or 50 μL . For consistency, use a multi-pipettor (eg. Eppendorf) or multi-channel pipettor to add parasite suspension to wells. Cover wells with plate lid.

method B:

- Add parasite suspension (as above) to plates first.
- Add samples to each well.

- Prepare a smear from the remaining parasite sample used for the assay – use this smear later to determine the parasitemia and stage of culture (then store these smears for later reference)
- Put plate into humidified chamber, gas (with mix of 95% nitrogen, 4% CO_2 , 1% O_2), seal chamber and incubate at 37 °C. For incubation chamber, best results are obtained with specialized culture chambers. Include wet paper towels or tissues inside the chamber to create a humid environment. Be sure to sit the test plates on a blank or empty plate to avoid direct contact of the test plates with wet paper towels (to avoid possible contamination of cultures with yeast or bacteria). Be sure to gas the incubation chamber fully, from both sides if there are two ports. It may be preferable to repeat this after 15 minutes.
- For two cycle assays: at 48 hours, add fresh medium to each well (do not remove existing medium). For 25 μL cultures, add 5 μL . For 50 μL cultures, add 10 μL .
- Agitation: Twice daily gentle agitation for 1 minute (or until pellet is resuspended) of the culture chamber may help redistribute parasites and create more even growth in the wells. Our results suggest this slightly increases the sensitivity of the assay, but it is not essential.
- Keep a sample culture in incubator to monitor developmental stage of parasites and guide the timing of harvesting parasites.
- Measure parasitemia (preferably by flow cytometry), but can also be done by microscopy or pLDH-based assay
 - One-cycle assay: measure parasitemia at 36-48 hours (or microscopy at 24 hours) by flow cytometry. Best to measure parasitemia when parasites are at late ring or early trophozoite stage if measuring parasitemia by flow cytometry.
 - Two-cycle assay: measure parasitemia at 80-96 hours (this varies with the parasite line used).

Comments

Culture volumes in 96-well plates can be 25-100 μL . We routinely use 50 μL volumes. Growth rates at 50 μL are generally a little higher than 25 μL cultures. U-bottom plates are more practical, and growth rates are slightly better for U bottom plates.

Avoid using culture medium prepared with Albumax only (without serum).

Use only sterile equipment, aliquot samples and parasites in class II hood.

Always include non-immune controls (and positive controls if possible).

Clean the glass or plastic incubation chamber before and after use to avoid the build-up of yeast that may contaminate cultures.

Always use fresh RBC in the starting culture. Store RBC in CPD buffer, not PBS or RPMI-HEPES

Notes on testing human serum or plasma samples:

- Test samples at 1/10 dilution or less. Testing samples at a 1/5 dilution may lead to problems with non-specific inhibition.
- Ideally, all samples should be dialysed to equilibrate pH and remove non-specific inhibitors (eg. antimalarial drugs and antibiotics), or purification of immunoglobulins performed.
- Avoid repeated freeze-thaw cycles with serum/plasma samples.

Measuring parasitemia by flow cytometry:

- Best performed when parasites are at the late-ring to mid-pigmented trophozoite stage.
- Prepare PBS with ethidium bromide at 10 $\mu\text{g}/\text{mL}$ (use molecular biology grade ethidium bromide). Alternatively, hydroethidine or Sybr Green can be used.
- Add 100 μL of PBS-EtBr to each well of the 96-well plate.
- Incubate in darkness for 30-60 min.
- Centrifuge plate (1200 rpm, 1 minute) to pellet cells, and remove supernatant
- Resuspend parasites into 200-300 μL of PBS and transfer samples to FACS tubes (cover samples from exposure to light). When using a high throughput sampler with the flow cytometer, samples can be taken directly from the 96-well plate rather than transferring to FACS tubes.
- Measure parasitemia on flow cytometer (EthBr is detected in channel FI2).

References

Persson, K. E. M., Lee, C. T., Marsh, K., and Beeson, J. G. (2006). The development and optimization of high throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. *J. Clin. Microbiol.* *44*, 1665-1673.

XI:E. ³H-hypoxanthine incorporation assay for the study of growth inhibition by drugs
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Apparatus

speed vac (Savant Plus SC 110 A)
Harvester (Tomtec Mach III)
scintillation counter (1450 Microbeta Liquid)
Candle jar
Bag sealer

Equipment

96-well cell culture plates, flat-bottomed with lid (Costar)
Eppendorf tubes
Falcon tubes, 14-mL
pipettes, tips 5- to 200- μ L
dispenser (Eppendorf Multipette plus)
Eppendorf Combitip Plus for 0.5 mL
filter papers (Printed Filtermat A Wallac)
sample bags (Wallac)
gloves
racks

Materials and reagents

parasite strain (FCR 3 S1) in culture (unsynchronized)
complete culture medium: RPMI 1640 with 10% human serum from blood group AB+ = MCM (see PARASITES, section I:A)
human erythrocytes, blood type O⁺
test compounds dissolved in DMSO (in conc. 5 mM)
DMSO (dimethyl sulfoxide)
acridine orange (see PARASITES, section III:A)
Beta-plate scintillation fluid
chloroquine diphosphate, in two concentrations, 40 nM and 640 nM, as a control drug
stock solution for control drug 1 (64×10^{-5} M chloroquine diphosphate (MW: 515.9 g/mol):
33 mg chloroquine diphosphate
100 mL distilled water.
Prepare the stock solution. Store at 4 °C in the dark in a plastic flask.
(The drug adheres to glass.)

working solution for control drug 1:

Prepare the working solutions of control drug 1 by diluting the stock solution by 1:100 in RPMI 1640 (containing 1% DMSO); 64×10^{-7} M = control drug 1

working solution for control drug 2:

Make two-fold dilutions of the working solution for control drug 1 in 4 steps; 4×10^{-7} M = control drug 2

$8\text{-}^3\text{H}$ -hypoxanthine (Amersham), 1 mCi/mL in 50% ethanol
 Aliquot 100 μL (in 100- μL Eppendorf tubes) and store at $-20\text{ }^\circ\text{C}$.
 Prepare the working solution by evaporating the alcohol in a speed vac.
 Resuspend the isotope in 5 mL of RPMI 1640 (i.e., 20 μL isotope/mL of RPMI 1640).

Prepare a new working solution for each assay.

Preparation

Day 1

Work in a sterile hood and use sterile tips and tubes.

- Dilute the test compounds (25 per microplate)
 A: 1/1005 μL in 0.5 mL RPMI
 B: 1/100050 μL of A in 0.5 mL RPMI
 Final concentrations will be: A: 5 μM , B: 0.5 μM
- Prepare a dilution of washed, uninfected human type O⁺ erythrocytes at 2% hematocrit in MCM as background.
 Example: 20 μL O⁺ blood in 1 mL MCM. Add 1 μL DMSO (to achieve the same concentration as in the test samples).
- Estimate the parasitemia (X%) in the malaria culture and prepare a parasite suspension with 0.2% parasitemia and 2% hematocrit. A 20-mL suspension is enough for 3 plates. (Calculate the amount of parasite culture to add for a 0.2% parasitemia in 20 mL of MCM, add 0.4 mL of O⁺ blood for 2% hematocrit.)

	B 2	B 3	2 A	2 B	8 A	8 B	14A	14B	20A	20B	
	C 2	C 3	3 A	3 B	9 A	9 B	15A	15B	21A	21B	
	D 2	D 3	4 A	4 B	10A	10B	16A	16B	22A	22B	
	E 2	E 3	5 A	5 B	11A	11B	17A	17B	23A	23B	
	F 2	F 3	6 A	6 B	12A	12B	18A	18B	24A	24B	
	1 A	1 B	7 A	7 B	13A	13B	19A	19B	25A	25B	

- Fill all wells around the border of the 96-well plate with a few drops of RPMI.
- Add 10 μL of control drug I to each of the wells E2 and E3.
- Add 10 μL of control drug II to each of the wells F2 and F3.
- Add 10 μL of the test compounds in dilutions A and B above to each of the wells 1A, 1B (G2, G3) to 25 A, 25B (G10, G11), resulting in 25 compounds in two dilutions per plate.
- Add 90 μL of the uninfected erythrocytes to each of the wells B2 and B3 for background.
- Add 90 μL of the parasite suspension to each of the wells C2, C3, D2, and D3 for controls.
- Add 90 μL of the parasite suspension to each of the remaining wells with control drugs and test compounds.

- Continue in the same way with the next plate.
- Note the date, starting time, and number of the plate on the lids.
- Put the plates in a candle jar and incubate them at 37 °C for 24 h. Use a thin layer of sterile water on the bottom of the jar.

Day 2

- Add 25 µL of the ³H-hypoxanthine working solution to each well (0.5 µL/well) and continue the incubation for another 18 h.

Day 3

- Harvest the cells to labeled filter papers in a cell harvester and dry them in an incubator for 1 h.
- Put the filters in sample bags and add 3.2 mL of scintillation fluid.
- Seal the bags and see that the fluid is evenly distributed over the filters.
- Read the cpm in a MicroBeta counter.
- Estimate the inhibition:

$$\% \text{ inhibition} = 100 - \frac{[(\text{cpm in test well} - \text{cpm in B}_{2,3}) \times 100]}{\text{cpm in C}_{2,3} \text{ D}_{2,3} - \text{cpm in B}_{2,3}}$$

Control drug 1 inhibits the growth of FCR3 S1 by >90%

Control drug 2 inhibits the growth of FCR3 S1 by 10 to 30%

References

Chulay JD, Haynes JD, Diggs CL. 1983. *Plasmodium falciparum*: Assessment of in vitro growth by ³H-hypoxanthine incorporation. *Exp Parasitol* 55(1):138-146.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* 16(6):710-718.

XI:F. *Plasmodium falciparum* hemozoin formation assayby **Mats Wahlgren***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: mats.wahlgren@mtc.ki.se*Equipment

high speed Immufuge blood centrifuge
centrifuge with Beckman JA-20 rotor
vortex
spectrophotometer (560-nm)

Materials and reagents

malaria culture
4-mL Falcon tubes (BD Labware, 2058)
1.5-mL microcentrifuge tube
5% sorbitol, prepared in distilled water and filtered through a 0.22- μ m filter
MCM with 10% serum
Triton X-100
distilled water
N NaOH
pyridine
2.5 mM potassium ferricyanide, $K_3Fe(CN)_6$
sodium hydrosulfite, $Na_2O_4S_2$
ferriprotoporphyrin IX chloride (Fluka or Sigma) (optional)

Procedure

Grow *Plasmodium falciparum* in MCM medium containing 10% human type AB⁺ serum and 5% hematocrit (type O⁺ blood) according to the candle-jar method of Trager and Jensen (see PARASITES, section I:A).

Synchronization of parasites (see also PARASITES, sections IV:B, G)

- Perform 1 cycle (or more if necessary) of synchronization by sorbitol lysis. Choose a culture with a majority of rings.
- Spin the 4-mL culture for 1 min at “high” speed in an immufuge blood centrifuge using 4-mL Falcon tubes.
- Add 4 mL of 5% sorbitol to the pellet.
- Mix the culture thoroughly by inversion and let it stand for 10 min.
- Spin again for 1 min.
- Wash the pellet 3 times in MCM with 10% serum.
- Return the cells to a culture flask.
- Wait until next ring stage period. Repeat the synchronization 2 more times if necessary.

Growth of parasites with drugs

- Use cultures at the ring stage (12 to 18 h) and a parasitemia of ~5%.
- Change the cultures to fresh MCM (with 10% serum) before adding the drugs.
- Add test drugs to a final concentration of 1 or 10 μ g/mL (or concentrations in between).

- Controls:
 - untreated PRBC culture (as background)
 - PRBC culture treated with chloroquine, or other anti-malarial drugs (e.g., Fansidar), at 1 and 10 µg/mL
- Mix by inversion and split the culture into flasks or wells in a cell culture plate.
- Grow a 4-mL culture for each time point in a 25-cm² cell culture flask or a 2.5-mL culture for each time point in a 6-well plate.
- Allow the culture to mature for ~30 h with or without an inhibitory drug.

Pyridine-hemochrome method for the measurement of haem incorporation in hemozoin

- Harvest the culture at various time points spread over ~30 h and measure hemozoin content; e.g., use cultures at: 0 h, 18 h, 20 h, 25 h, and 30 h.
- Transfer contents of the flasks/wells to 10-mL centrifuge tubes which hold for the centrifugation below.
- Add Triton X-100 to a final concentration of 1% (4 mL of a 2% Triton X-100 solution to a 4-mL culture). Leave a small volume of culture for 5% Giemsa staining (see also PARASITES, section III:B) and for counting the parasitemia.
- Spin at 4 °C and 13,000 rpm for 45 min (Beckman JA-20 rotor).
- Discard the supernatant and save the pellet.
- Resuspend the pellet with 1 mL of distilled water and transfer it to a 1.5-mL microcentrifuge tube.
- Spin it at 4 °C and 13,000 rpm for 15 min in a microcentrifuge. This step can be repeated to further wash away any free haem.
- Discard the supernatant and save the pellet.

Do the following steps in a VENTILATED HOOD since pyridine and potassium ferricyanide are VERY TOXIC. Collect waste in a flask, label it appropriately, and send it as organic waste.

- If one wishes to set up a standard curve for the assay, one can use hemin which is ferriprotoporphyrin IX chloride (Fluka or Sigma). Make standard working solutions in distilled water from a stock solution of hemin prepared in DMSO, as hemin is not directly soluble in water. Then take 520 µL of these prepared hemin standard solutions and treat them exactly as the dissolved hemozoin pellet below.
- Add 520 µL of distilled water to the tube with the culture hemozoin pellet, followed by 62 µL of N NaOH and 123 µL of pyridine (for a 4-mL sample). Use half of these volumes for a 2.5-mL sample. Vortex to dissolve the pellet. Add the same volumes of NaOH and pyridine to the hemin standard tubes.
- Split the mixture into equal parts in 2 microcentrifuge tubes. Add 20 µL (or 10 µL for a 2.5-mL sample) of 2.5 mM potassium ferricyanide to one tube to oxidize haem. Add “a pinch” of sodium hydrosulfite to the other tube to reduce haem. Mix by inversion.
- Measure the absorbance in a spectrophotometer at the wavelength of 560 nm.
- Calculate the relative amount of hemozoin at each time point as well as for the hemin standards:

$$\Delta \text{ OD}_{560} = \text{OD}_{560} (\text{reduced sample}) - \text{OD}_{560} (\text{oxidized sample})$$

- Plot hemozoin content in the culture versus time and compare relative to culture without drugs and to culture with chloroquine and/or Fansidar, as well as to the standards.

References

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MOSQUITOES AND PARASITES

I. Rearing of *Anopheles stephensi* mosquitoes

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See MOSQUITOES AND PARASITES, section II:C

II. Sugar-feeding preference method

by **Jenny Lindh**¹, **Olle Terenius**², **Karolina Eriksson-Gonzales**², **Bart G.J. Knols**³ and **Ingrid Faye**²

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Equipment

assay cage
dissection microscope

Materials and reagents

food dyes
test solutions
commercially available food dyes
Kleenex paper

Procedure

- Pick pupae for two (or 4 or 6 or ...) parallel experiments at a time and place them in assay cages (or divide the adults into groups and place in assay cages).
- On the day of the experiments, starve the mosquitoes in an environment of lower humidity and higher temperature than normal rearing conditions for seven hours. This is done to increase the number of mosquitoes that feed
- Prepare the two test solutions and add the food dyes. One colour to each solution and change the colour in the two cages: i.e. solution A is green and solution B is red in the first cage and in the second cage solution A is red and solution B green. We used 10 drops of commercially available food dyes (Hushållsfärg, Ekströms, Sweden) to 5-10 mL of test solution. These dyes are bought in solutions, which contain water, glycerol, ethanol and red (E120) or green (E104, E131) dyes. It is probably ok to use other food colourings but make sure to test the colour preference first by having solution A = solution B apart from the colour.
- Place a Kleenex paper in each test solution and place the test solutions in the cage at a set distance for a period of 2-3 hours. In our experiments starvation

- and feeding were performed during the 12h photoperiod, however, the lights were turned off during the feeding period.
- Kill the mosquitoes and count the number of mosquitoes that has fed on each solution using a dissection microscope.

Preference method separating olfactory and tactile/taste responses

Performed as above but the Kleenex paper that the mosquitoes fed from is placed in an outer cup containing sugar solution and the test solution is placed in an inner cup inaccessible for the mosquitoes.

III. Procedures required to generate *Anopheles stephensi* mosquitoes infected with the human malaria parasite, *Plasmodium falciparum*

by **Martin Looker** and **Andrew W. Taylor-Robinson**

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The production of *Anopheles stephensi* mosquitoes infected with the human malaria parasite, *Plasmodium falciparum*, involves the propagation of parasite and vector life cycles in parallel. It is a technically difficult procedure which requires considerable pre-planning and attention to detail in order to be successful. In particular, the precise timing of each stage of the process is of paramount importance to ensure a consistent supply of infected mosquitoes for the purposes of research. This 'timeline' is a valuable organisational tool in that it identifies, in short-hand form, precisely what tasks need to be performed on a day-to-day basis. In our laboratory, we have found this schematic representation to be useful not only as an aide memoire for the experienced parasitologist but also as a teaching aid to researchers new to malaria.

Schematic representation of the practical procedures and chronological order required to generate *Anopheles stephensi* mosquitoes infected with the human malaria parasite, *Plasmodium falciparum*.



For ease of examination of the detail in this timeline, the image above may be magnified within the Microsoft Word document. Alternatively, click on the link below to see a printable pdf version (Adobe Acrobat Reader required) or e-mail the authors to receive a pdf or jpg file.

IV. Culturing of sexual, oocyst, and sporozoite stages

IV:A. Plasmodium falciparum gametocyte culture, purification, and gametogenesis

by **Mrinal Kanti Bhattacharyya** and **Nirbhay Kumar**

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Equipment

incubator (37 °C)
centrifuge
culture flasks (25-cm², 75-cm², and 150-cm², Corning, Canted neck flasks)

Materials and reagents

human RBC washed in incomplete medium
incomplete medium
RPMI 1640
25 mM HEPES
0.37 mM hypoxanthine (pH 7.4)
complete medium
 incomplete medium (above) containing 10% heat-inactivated pooled normal human serum
 29 mM NaHCO₃
donor culture (intra-erythrocytic parasite culture) maintained below 5% parasitemia by routine subculturing
60% Percoll:
 9 volumes of Percoll stock
 1 volume of 10× RPMI
 5 volumes of 1× RPMI
exflagellation-inducing medium:
 10 mM Tris (pH 7.6)
 170 mM NaCl
 10 mM glucose
 10% heat-inactivated normal human serum
 25 mM NaHCO₃
 50 to 100 μM xanthurenic acid (optional)
 Nycodenz (as step gradient of 6%, 11%, and 16%)

Procedure

- Spin down freshly drawn O⁺ human blood (not more than 2 to 4 days old) in 15-mL centrifuge tubes at 1,000 rpm for 10 min. Discard the supernatant including the WBC containing buffy coat. Wash the RBC pellet 3 to 4 times using incomplete medium and resuspend it with an equal volume of incomplete medium to give rise to a final suspension of 50% RBC.
- Start cultures for gametocytes at 6% hematocrit and 0.3% parasitemia. Example: In a 75-cm² flask, add 1.8 mL of 50% washed RBC, estimated volume of parasites from a donor culture, and complete medium to make up the volume to 15 mL.
- Change medium (12 mL) every day. Make sure that the temperature of the medium is 37 °C and culture temperature does not fall below 37 °C during the

medium change. A slide warmer set at 37 °C can be used inside the hood to maintain temperature of cultures during medium change.

- Monitor the parasite growth every other day and when parasitemia reaches 3 to 5%, start changing the medium by double the medium volume (25 mL). On the subsequent days keep changing 25 mL of medium on a daily basis.
- It takes around 14 to 18 days to obtain matured gametocyte cultures.

Comments

Highly synchronous stages (I to V) of *Plasmodium falciparum* gametocytes can be obtained by N-acetyl glucosamine treatment of culture (Parasitology 93(Pt 2):263-274, 1986 and Mol Biol Pharmacol 68(1):151-154, 1994).

Enrichment using Percoll centrifugation

- Prepare the 60% Percoll.
- Spin down the parasite culture and resuspend it to ~25% hematocrit in incomplete medium.
- Carefully layer 1 volume of parasite suspension over 2 to 2.5 volumes of 60% Percoll in a 15-mL (13 × 100 mm) polypropylene or Corex glass tube.
- Centrifuge the suspension at 10,000 rpm for 20 min at room temperature.
- Collect parasites at the interphase, wash them 3 or 4 times in incomplete medium, and check them by Giemsa stain.

Gametogenesis and purification of *P. falciparum* gametes/zygotes

Mature gametocytes are stimulated to undergo gametogenesis for 30 min at room temperature (25 to 26 °C) and gametes/zygotes are purified by centrifugation.

- Resuspend mature *P. falciparum* cultured parasites at 20% hematocrit in the exflagellation-inducing medium at a pH of 8.1 to 8.3 at room temperature for 30 min. Addition of 50 to 100 μM xanthurenic acid can also enhance the parasite yield.
- Layer the parasite suspension over a discontinuous step gradient of Nycodenz (6%, 11%, 16%) and spin it at 16,000 × *g* for 15 min at room temperature.
- Gametes/zygotes sediment at the interface of 6% and 11%.

References

Lobo CA, Konings RN, Kumar N. 1994. Expression of early gametocyte-stage antigens Pfg27 and Pfs16 in synchronized gametocytes and non-gametocyte producing clones of *Plasmodium falciparum*. Mol Biochem Parasitol 68(1):151-154.

Ponnudurai T, Lensen AH, Meis JF, Meuwissen JH. 1986. Synchronization of *Plasmodium falciparum* gametocytes using an automated suspension culture system. Parasitology 93(Pt 2):263-274.

IV:B. Cultivation of *Plasmodium falciparum* gametocytes for mosquito infectivity studies
by **Martin Looker** and **Andrew W. Taylor-Robinson**

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Gametocytes of *P. falciparum* are established from continuously maintained cultures of asexual blood stage parasites. Gametocytes begin to form in significant numbers in blood culture only following daily dilution with fresh RBC and medium for several days. In order for gametocytes to comprise a substantial proportion of PRBC, it is necessary for asexual PBRC to reach a high density and become stressed in vitro before conversion to gametocyte production. This occurs typically 6 to 8 d after the dilution of a culture. Gametocytes then require at least a further 8 d to reach maturity. Cultures for gametocyte production are therefore maintained for a minimum of 14 d without further dilution with fresh RBC in order to obtain a large proportion of mature and infectious gametocytes. We routinely perform gametocyte feeds to mosquitoes using cultures propagated continuously for the preceding 14 to 17 d.

Materials and reagents

Refer to **Appendix** of this protocol for details of materials and generic methods.

Propagation of asexual blood cultures

- Retrieve an isolate of *P. falciparum* cloned line 3D7A from liquid nitrogen and place it in a 25-cm² tissue culture flask along with 0.25 mL of freshly washed RBC and 6 mL of complete medium. Gas the culture for 30 s and tighten the culture flask cap. Keep it in a dedicated 37 °C dry incubator.
- At the same time every day the culture requires a medium change. Tip the flask(s) at 30 degrees to the horizontal, which results in good separation between RBC and the medium that forms the over-layer. As flasks are settled, warm the appropriate volume of complete medium to 37 °C. Settling of flasks and warming of medium takes 40 to 60 min. Remove the spent medium from each culture, discard and replace it with an equal volume (6 mL) of fresh medium, and gas the flask for 30 s. Perform all operations on a warming plate set to 37 °C in order to minimize heat loss to cultures during the time they are out of the 37 °C incubator.

Growth of blood cultures for gametocyte production

- In order to produce gametocytes on a long term basis, it is necessary to maintain a stock of 'asexual' cultures from which the gametocyte cultures are drawn. Every 5 d, flasks which have reached or exceeded a parasitemia of 1% may be subcultured ("split") by adding fresh RBC in order to reduce the parasitemia to about 0.5%. In this way, from one flask at a high parasitemia a number of 'daughter' flasks may be started at a lower parasitemia. Some of these flasks may be used for further stock asexual cultures and some may be used for gametocyte culture. The new asexual stock cultures are grown for another 5 d before being split again when a minimum parasitemia of 1% is reached. This guarantees a continuous supply of cultures for later gametocyte preparation. Existing gametocyte cultures are not subcultured but continue to have their medium changed daily.
- After 6 to 8 d post-subculture, developmental stage I and II gametocytes will start to appear (following asexual PRBC 'stress' once a threshold parasitemia is

- reached) and from 14 d post-subculture flasks should contain mature, stage V, gametocytes. By 17 d, 1 to 4% of PRBC should be stage V gametocytes and the gametocytemia of all stages of development (I-V) may be up to 12%. Progress can be checked by examining thin blood films taken periodically post-subculture (usually on *Days 8, 12, and 15*).
- Although it is possible to attain much higher gametocytemias by enriching for gametocytes by a variety of treatments (Percoll or Nycodenz density gradient centrifugation or selective drug treatment are the most commonly used), this is not recommended for mosquito infectivity studies. In the experience of ourselves and others, artificially increasing the gametocytemia is often counterproductive to satisfactory transmission of the parasite to the mosquito vector.
 - Development of male sexual stage parasites is more rapid than that of females, so mature male gametocytes may be seen from 14 d but mature females usually only from 16 d post-subculture. In order to ensure a good balance of male and female gametocytes for the purposes of transmission, it is common practice to mix cultures of different ages at the time of the blood feed (see MOSQUITOES AND PARASITES, section II:C).

References

Graves PM, Carter R, McNeill KM. 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. *Am J Trop Med Hyg* 33(6):1045-1050.

Ifediba T, Vanderberg JP. 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. *Nature* 294(5839):364-366.

Read M, Hyde JE. 1993. Simple in vitro cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations. *Methods Mol Biol* 21:43-55. [Includes useful background information on equipment, reagents, cryopreservation of parasites, preparation of thin blood films, and treatment of contamination]

Appendix

Safety precautions

- *Plasmodium falciparum* is classified as a Category 3 pathogen and all appropriate health and safety measures must be carried out when maintaining this parasite in human blood in vitro, in keeping with local regulations. Perform all work in a Class II safety cabinet. Exclude all nonessential staff from the laboratory while work is in progress. Always wear a Howie-style laboratory coat and latex gloves.
- Any isolate of *P. falciparum* that is used routinely should not be resistant to standard antimalarial drugs.
- Discard spent medium and all disposable plasticware in a hypochlorite disinfectant (e.g., Chlorox or Presept) to a concentration of 10% free chlorine before disposal. Swab all working areas with 70% ethanol at the conclusion of any work. Provide a wash bottle of 70% ethanol to flood any spillage of infective material.

- Obtain blood and serum from a reliable sources only, such as your local Blood Transfusion Service, where it is prescreened for the presence of HIV and hepatitis viruses.

Complete medium

RPMI 1640 medium is purchased as sterile 1-liter bottles (Gibco, 041-91187A). This medium contains 5.96 g/L HEPES buffer, 3.60 g/L glucose, and 50.0 g/L hypoxanthine. To each 1-liter bottle, add 42 mL of freshly prepared filter-sterilized 5% sodium bicarbonate (Sigma S-5761) (this is referred to as 'incomplete' medium). Following the further addition of 100 mL of heat-inactivated pooled human serum, the liquid is now 'complete' medium. Store the medium at 4 °C and use it within 7 d, during which time the pH should be monitored (optimum pH 7.3).

Retrieval of parasites from liquid nitrogen

- Thaw cryotube(s) in a 37 °C water bath for 2 min.
- Transfer the thawed contents to a sterile microcentrifuge tube and centrifuge it at $10,000 \times g$ for 1 min, then remove the supernatant.
- Resuspend the pellet in 1 mL of PBS + 10% sorbitol; add this slowly dropwise with continuous mixing.
- Repeat steps 2 and 3 twice more (i.e., total of 3 washes).
- Wash and resuspend the pellet in complete medium when the cells are ready for culture.

Washing human RBC

- Use group O, Rhesus group positive whole blood taken into adenine-CPD (citrate phosphate dextrose). Blood used should be less than 7 d old.
- Centrifuge the blood at $800 \times g$ for 5 min.
- Remove the white blood cells by aspiration of the buffy coat.
- Add an equal volume of incomplete medium and centrifuge it at $800 \times g$ for 5 min.
- Discard the incomplete medium.
- Repeat steps 4 and 5 twice more (i.e., total of 3 washes).
- After washing, the packed RBC may be used directly (i.e., at a 100% hematocrit) and any remainder discarded. Alternatively, the packed RBC may be resuspended in an equal volume of complete medium (i.e., at a 50% hematocrit), which should be stored at 4 °C and used within 7 d.

Gas mixture

1% oxygen, 3% carbon dioxide, and 96% nitrogen (BOC Specialty Gases, or your local specialist gas supplier). Pass the gas mixture from the cylinder via a gas-reducing valve into the safety cabinet by a silicon tube. Filter the gas through an in-line Whatman gamma 12 sterilizing unit with a 0.3- μm filter tube. Connect the filter to a sterile Millex GS 0.22- μm disc filter, the end of which is attached to a 19G \times 1.4-inch blunt-ended needle fitted to a 1-mL syringe. When gassing a culture flask, the flow rate should be moderate but sufficiently strong to ruffle the surface of the liquid.

Pooled serum

Pool human group O, Rhesus group positive serum from a minimum of 10 donors to minimize variations of quality between individuals. Packs of serum are routinely heat-inactivated at the source, but if not they should be held at 56 °C for 1 h. Test the pools

before full-scale use to ensure that they are sterile and support parasite growth, in particular that of gametocytes. Aliquots may be stored at $-20\text{ }^{\circ}\text{C}$ for up to 6 months.

Parasites

In order to maximize the likelihood of satisfactory gametocyte production, it is best to use a line or clone of *P. falciparum* that is known for its production of gametocytes. Good producers include NF54, 3D7A, and HB3A, obtainable from the MR4 repository. Restart cultures from frozen stocks every 2 months or less since gametocytogenesis in a continuously cultured line will start to wane after this period.

Parasite staining

Use improved R66 Giemsa (BDH 350864X) diluted 1:10 with Sorensen's buffer (HD Supplies; HDS 20, pH 7.2). Fix thin blood films in 100% methanol and stain for 20 min in the 10% Giemsa solution. Wash them with tap water for 20 s and allow to air dry before viewing by light microscopy using oil immersion at 1000 \times magnification.

Thin blood film

Small volume of RBC removed from culture flask and smeared across a glass slide to give a monolayer of RBC.

Sterile plasticware

25-cm² cell culture flasks (Iwaki 3100-025 or similar)

30-mL Universal containers (Sterilin 128A or similar).

plastic pipettes, singly wrapped, disposable, graduated (2-, 5- and 10-mL; Sterilin or similar)

IV:C. Gametocyte Culture Protocol: Membrane Feeding Gametocytes to MosquitoesBy **Carter R, Ranford-Cartwright LC, and Alan P.***Div of Infection & Immunity, Institute of Biomedical & Life Sciences, University of Glasgow, UK**e-mail: L.C.Ranford-Cartwright@bio.gla.ac.uk*1. Gametocyte culture protocol

Erythrocytes: Human O+ erythrocytes from banked blood (collected in CPD-adenine), as fresh as possible (not more than 7 days after the bleed).

Wash the red cells three times (or more if necessary) with incomplete MCM, removing any buffy coat, and resuspend with an equal volume of complete MCM. This 50% hematocrit blood should be stored at 4 °C.

Incomplete malaria culture medium (IMCM) as for the asexual cultures, but with the addition of 50 mg/liter hypoxanthine (Sigma) and the omission of NaHCO₃. Adjust the pH to 7.2.

Complete malaria culture medium (CMCM): Add 10 mL of human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56°C for 1 hour, and stored in aliquots at -70°C) and 4.2 mL of a sterile, freshly made 5% solution of NaHCO₃ to 100 mL IMCM, made as incomplete medium not more than 2-3 weeks ago. Incubate at 37 °C overnight to check for contamination before use. Use within one week and discard if pH increases to >7.6 (pink in color).

A gametocyte-producing stock culture between 4 and 10%: Thaw fresh stock parasites every 2-3 months because the gametocyte-producing ability (and infectiousness of gametocytes) drops after a time.

The culture *must* be in good health and growing quickly. Ideally, it will need diluting down 2-3 times a week. Do not dilute cultures below 1% as this can stop them from growing well for a few days, and do not let the culture get too high (>6-8%) before you dilute it down, as it may 'crash'. The presence of gametocytes in a stock culture is a good thing for gametocyte culture!

Method

Set up cultures in tissue culture flasks

- Small culture flasks of 25 cm² have an initial volume of 5 mL which will increase to 7.5 mL; 75-cm² flasks have an initial volume of 15 mL which will increase to 25 mL.
- Set up cultures at 0.5-0.7% parasitemia, 6% hematocrit in complete MCM. If the culture is mainly rings, set it up at 0.7%; if mainly schizonts, at 0.5%; if mixed, at 0.6%.
- Prewarm the flasks and complete MCM to 37 °C before use.
- Gas the flasks with a mixture of 1% O₂, 3% CO₂, balance N₂ (candle jars or CO₂ incubators are not recommended) for a minimum of 10 seconds (25-cm² flasks) or 20 seconds (75-cm² flasks) at a pressure of around 5 lb/in².
- Place in a 37 °C incubator with the flasks lying down. Replace complete MCM daily and agitate cultures gently after gassing.

It is very important to keep the cultures as warm as possible at all times. When removing the spent medium, do not take off any of the black pigment that comes off just before you start to take off blood.

Bulking up the gametocyte culture:

Once the culture has reached a high parasitemia it is 'bulked up'. This means that the volume of medium added is increased to 7.5 mL (or 25 mL) without the addition of more blood cells.

- Make thin blood smears on the fourth day after setting up the culture. Fix and stain with Giemsa's stain as previously described.
- Examine under 100x oil objective. You should look for a high parasitemia and 'stressed looking' parasites such as triangular ring stages and hazy or faint-looking trophozoites and schizonts.
- If these are not present, do not bulk up. Make blood smears on the following days and bulk up when appropriate (usually 4 or 5 days after set-up).
- As soon as the culture is judged ready, bulk up by adding extra medium to each flask to give a final volume of 25 mL (75-cm² flasks) or 7.5 mL (25-cm² flasks).
- Thereafter maintain the cultures on the increased volume of medium, changing daily and gassing.
- Prepare smears once or twice a week to check the state of your cultures and to look out for contamination.
- Gametocytes are mature from 14-17 days after the start of culture.

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2. Membrane feeding gametocytes to mosquitoes

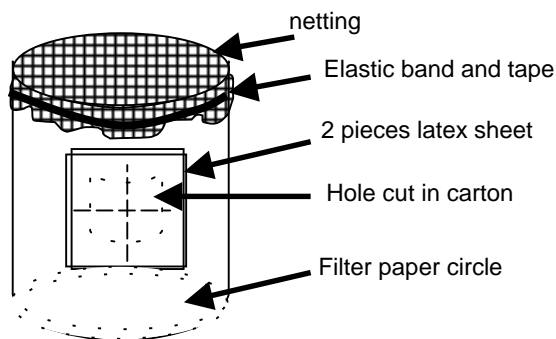
This protocol is for infection of *Anopheles* mosquitoes with *P. falciparum* gametocytes, grown according to the gametocyte culture protocol. Gametocytes should NOT be grown with gentamycin, as this can have adverse effects on the infection rates.

Note: *P. falciparum* is classified as a category 3 pathogen in some countries, which means that there are regulations regarding its safe use. Familiarize yourself with any relevant local or national safety regulations in your country before beginning work, and take steps to minimize the risk of accidental infection. This is particularly important when mosquitoes are being infected. It is necessary to have an approved secure insectary and to take every step to ensure that potentially infected mosquitoes do not escape.

Materials

- 14 - 17 day old gametocyte cultures
- Human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56 °C for 1 hour)
- Human O+ erythrocytes from banked blood (collected in CPD-adenine), washed to remove the anticoagulant before use, as fresh as possible (no more than a few days since collection)
- Glass membrane feeders and plastic tubing to connect them
- Clamps and stands to which to attach the feeders
- Circulating waterbath at 37 °C
- Baudruche membrane or Parafilm sealer and small elastic bands
- 1-pint waxed-paper cartons (e.g., ice-cream containers), filter paper circles to fit in the bottom of the cartons, latex sheeting, nylon netting, tape.
- Mosquito aspirator ('pooter')
- Anopheles* mosquitoes, 3-5 days postemergence
- 5% glucose in 0.05% PABA (4-amino benzoic acid) solution
- Absorbent cotton wool

Preparation for a membrane feed



- Prepare sufficient mosquito pots for the experiments you are planning. Cut a small hole (approximately 2 cm square) in the side of a waxed paper carton. Cover this on the outside of the carton with two squares of latex sheeting (e.g. dental dam) with a slit cut into each one, and tape securely over the hole to form the leak-proof

inlet/outlet port. Fix a filter paper circle to the base of the pot. Stretch netting over the top of the pot and secure with an elastic band and tape.

- Collect female mosquitoes two days before you are going to carry out the feed itself. These mosquitoes should be between 3 and 5 days postemergence on this day (so they will be between 5 and 7 days post emergence on the day of feed).
- Place a small piece of cotton wool soaked in distilled water on top of the netting. This should be replaced daily until the day of feed.
- Attach baudruche membrane or finely stretched Parafilm to the glass feeders with an elastic band. If using baudruche it is easier to apply it wet.
- Connect the feeders to a circulating waterbath set at 37 °C, and clamp at the correct level for the mosquito pots.

Preparing the infectious feed

Work swiftly and take steps to prevent the temperature of the parasite material from dropping below 37 °C.

- Prewarm washed uninfected fresh blood and serum to 37 °C.
- Resuspend the washed uninfected fresh blood in the serum to give a final packed cell volume (pcv) of 40%. Keep warm at 37 °C.
- Remove medium from culture flasks and resuspend the cells by gentle shaking. Transfer to tubes and centrifuge to pellet (5 minutes at 1800 x *g* at 37 °C).
- Remove supernatant, measure the volume of the pellet (approximately), and add an equal volume of serum to the pellet. Mix the pellet and serum very well (remember the gametocytes stick to the side of the tube).
- Dilute the parasite/serum mix with 3 to 9 times its volume of the fresh washed uninfected fresh blood/serum.
- Mix with a Pasteur pipette and place approximately 1 mL (depends on membrane feeder size) into each feeder.
- Place the mosquito pot underneath and allow to feed for approximately 20 minutes.
- Remove the unfed mosquitoes 2-3 hours after the feed and kill by freezing at -20 °C.

To check for exflagellation

- Place a small drop of the prepared parasite/blood/serum mixture on a microscope slide, breathe on a coverslip, and place over the drop of blood. Examine microscopically (phase or Nomarski 40x objective are best) as soon as possible (always within 10 minutes).

Maintenance and dissection

- Keep infected mosquitoes in a secure insectary at 26 °C/80% relative humidity.
- Place clean cotton wool pads soaked in 5% glucose/0.05% PABA solution on top of the netting; replace these daily.
- Kill mosquitoes with chloroform, and then dip briefly into ethanol. Dissect in 1x PBS.
- Oocysts will be visible from 7 days postfeed, but are easiest to see 9-10 days postfeed (depending on the temperature of the insectary).
- Sporozoites may be seen in the salivary glands from day 14 postfeed onwards.

References

This protocol is summarized from the reference below. Please refer to this chapter for original references.

Carter R, Ranford-Cartwright LC, and Alan P. 1993. The culture and preparation of gametocytes of *Plasmodium falciparum* for immunochemical, molecular, and mosquito infectivity studies. *In*: Hyde, JE, ed. Methods in molecular biology, Vol. 21: Protocols in molecular parasitology. Totowa, New Jersey: Humana Press; pp. 67-89.

IV:D. Production of *Plasmodium falciparum* oocysts and sporozoites

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The production of different sexual stages of *P. falciparum* and their isolation from infected mosquitoes facilitates a variety of cellular, molecular, immunochemical, and transmission-blocking studies that examine the parasite-vector relationship. The procedures of mosquito rearing, parasite cultivation, and infection and dissection of mosquitoes are highly specialized skills requiring considerable experience to perform with competence. Additionally, the timing of the two life cycles, that of the parasite and that of the mosquito, requires precise planning so that the mosquitoes are the correct age to infect during the brief period in which the parasites are viable for infection.

Mosquitoes capable of transmitting *P. falciparum* should be maintained within a limited access, dedicated, high security humidified insectary. It is recommended that all rooms be painted in a light color and all cages be constructed of white materials in order to make highly visible any mosquito that may temporarily escape. A nonresidual insecticide spray should also be available in the event of an emergency. Infected mosquitoes to be dissected are first stunned or anesthetized by chloroform and then rendered incapable of movement (dewinged and delegged). Any potentially infective mosquitoes that are not to be dissected are frozen by placing the complete container at -20°C prior to autoclaving and disposal.

Equipment required for blood feed of mosquitoes

(A) *Pooter*—A pooter is a device used to transfer mosquitoes by aspiration. We use a polycarbonate plastic-tubed pooter, but any toughened material is suitable provided that it is transparent. Ours are custom-made (contact details upon request) but an efficient pooter can equally well be modified from a 10-mL plastic pipette. This is prepared for use as follows:

- Insert a stainless steel (or nylon) fine-meshed disc of the internal dimensions of the pooter tube 2 cm from one end of a 20- to 25-cm tube. A ring may be etched in the internal surface of the tube in which the disc can sit securely.
- Attach a length of clear, transparent silicon rubber tubing (approximately 30 cm) over the meshed end of the polycarbonate tube.
- Use the pooter by placing the free end of the silicon rubber tubing in your mouth and aspirating by a 'suck-blow' technique. Transfer the mosquitoes by sucking them into the open end of the polycarbonate tube and then blowing them out into the container.

(B) *Mosquito container*—The ideal container should minimize the risk of mosquito escape or injury or of the handler being bitten. The container used successfully by ourselves and several other research groups is customized from a 500-mL cylindrical ice-cream container made of waxed cardboard (of the type used by Ben & Jerry's and Häagen-Dazs). This is prepared for use as follows:

- Cut out a circular piece of filter paper (Whatman no. 1, or similar) to the internal dimensions of the container and tape it to the floor of the container to soak up any spillage that may occur (from nutrient feed, diuresis drops, etc.) when in use.
- Cut a 3-cm square in the side and cover the hole with 2 layers of dental latex (HCM rubber dental dam or similar, available from your local dental supplies

distributor) into which two 1-cm cuts are made, one of which is then placed perpendicular to the other. Hold both squares of latex firmly in place with electrician's insulating tape. This results in a cross-shaped entry port through which, with the aid of a pooter, mosquitoes can be safely introduced or removed.

- To complete the container, secure a double layer of 'bridal veil' netting over the mouth of the container with elastic bands and insulating tape.
- Other than during the actual blood feed, keep the containers inside a gauze-covered cage that acts as a secondary security barrier.

(C) *Blood feeder*—A glass membrane feeder is used to deliver the infectious blood meal to the mosquitoes. Blood feeders consist of a water-jacketed chamber into which the infectious blood feed can be introduced. Ours are custom-made by a professional glass blower (contact details upon request). This is prepared for feeding use as follows:

- Cover the blood feed chamber with a Baudrouch membrane derived from bovine intestine (Joseph Long Inc.). Wet the membrane with tap water to ensure that, when dry, it is taut. This will encourage the mosquitoes to feed. If washed and dried thoroughly after each use, a membrane can be used 5 or 6 times before it loses its integrity. In fact, in our experience, previously used membranes are preferred to new membranes because their use results in a greater proportion of mosquitoes taking a blood meal.
- Hold the membrane in place with an elastic band.
- Pass tap water at 38 °C through the water jacket via a recirculating pump (water inlet via the lower tube, outlet via the higher tube).

Rearing of *Anopheles stephensi* mosquitoes

- Keep each stock colony of *An. stephensi* in a gauze-covered, plastic-covered wire-framed cage. We use cages of frame dimensions 30 cm³, which are covered with mosquito netting or 'bridal veil' netting to incorporate a 15-cm front sleeve.
- Keep cages at 26.0 ± 0.5 °C and 80.0 ± 2.0% relative humidity. Maintain all colonies on a reverse light cycle with 12 h artificial lighting from 2000 to 0800 h. This enables all manipulations to be performed in keeping with the nocturnal activity of the mosquito during normal working hours.
- Feed adult female mosquitoes twice a week on uninfected rodent blood. We routinely feed directly by bite of naive outbred mice, but rats or guinea pigs are also suitable. Anesthetize the mouse by intraperitoneal injection of 0.1 mL per 10 g body weight of 10% Hypnorm (Janssen Pharmaceutica) and 20% Valium (Roche) in sterile distilled water. Place the mouse abdomen-down, with the four limbs stretched out, on top of the cage in which the mosquitoes to be fed are kept, and allow feeding for 20 min. The abdomen may be shaved of fur to facilitate access for feeding. We use mice under terminal anesthesia in accordance with project and personal licences issued by the UK Home Office and following institutional guidelines. Your local regulatory authority may permit rodents to be reused for the purposes of mosquito feeding, in which case they can be allowed to recover from the anesthetic.
- Eggs are laid by fed female mosquitoes 2 to 3 d after a blood feed. Place a small bowl lined with filter paper (Whatman no. 1, or similar) and filled with tap water on the floor of the stock cage overnight. The following morning, remove the bowl to another cage, and cover it with a square of transparent Perspex until the eggs begin to hatch (within 2 d).

- Rear the larvae in clean plastic washing-up bowls (preferably colored white) containing 3 inches of water. Carefully transfer the contents of the hatching bowl into a larval rearing bowl (approximately 300 larvae per bowl) and discard the filter paper. The water must not contain large amounts of chlorine so it may be necessary to allow your local tap water to stand for a minimum of 24 h before use to allow any chlorine to dissipate ('chlorine-free' water). We recommend the continuous maintenance of a reservoir of chlorine-free water ready for use. Alternatively, water can be boiled before use or bottled water can be used. Whatever the source and treatment of the water, it must be at room temperature before eggs are added.
- Anopheline mosquito larvae are primarily surface feeders and must be provided with a form of food that floats on water. We use koi carp pellets (Wardley Corporation premium koi staple, or a suitable alternative supplied by your local aquarist). Baby weaning formulation milk powder ('Farex' or similar) may also be used but should be finely sieved before sprinkling on the water.
- Feed the larvae morning and evening with fresh pellets (a 'little but often' regime). Remove and discard any surplus 'old' food. In this way, the larvae receive sufficient food, but not so much that the bowl becomes contaminated with uneaten food.
- After 2 to 3 d, reduce the mosquito numbers. By keeping the larvae numbers between 200 and 250 per bowl, large well-nourished mosquitoes are produced.
- Larvae should pupate 6 to 8 d post emergence from eggs if kept at 26 °C and 80% relative humidity. Pupation can be delayed by 2 or 3 d by reducing the ambient temperature by 1 or 2 °C. Kill surplus larvae by immersion in hot water. Do not discard them live down the sink.
- Collect pupae and place them in a bowl of chlorine-free water. Cut an inch off of the point of a filter-paper cone and place the cone over the bowl. This system allows emergence of adult mosquitoes (2 to 3 d postpupation), but also deters emergent mosquitoes from flying back into the bowl and drowning and keeps fed females from laying eggs in the pupae bowl. Place the pupae bowl in the stock cage.
- Provide a solution of 5% glucose (Sigma) and 0.05% *p*-aminobenzoic acid (PABA; Sigma) in chlorine-free water for all adult mosquitoes. Filter-sterilize this nutrient solution through a 0.22- μ m filter (Sterilin or similar) before soaking a cotton wool pad and placing it in the cage. The pad should be moist but not dripping as this leads rapidly to fungal contamination, and should be changed daily.

On the day before the feed

- Prepare the mosquito container(s) and place into each, with the aid of a pooter, 4 to 6-d-old virgin female mosquitoes (i.e., they should be 5 to 7 d postemergence from pupae on the day of the feed). Add a suitable number of mosquitoes for the volume of the container to be used; for a 500-mL cylindrical container, we add between 100 and 250 mosquitoes. Up to 20 mosquitoes can be accommodated comfortably within a pooter at any one time to enable counting during the transfer procedure. It is important to handle the mosquitoes carefully so as not to damage them.
- Test mosquitoes for their readiness for a blood feed by evaluating their attempt to feed on a warmed, water-filled, tissue culture flask placed on top of the gauze-

- covered stock cage in which they are housed. If the pooter is used for transfer to the feed container while the culture flask is in place, those mosquitoes that are attracted by the warm object can be positively selected. This procedure acts not only to select older individuals more eager for blood among a population of slightly mixed ages (due to variation in the timing of emergence from pupation) but also to segregate female from male mosquitoes. Only females are attracted by the warmed flask, as its temperature mimics that of a human (from which they require blood in order to lay eggs); male mosquitoes do not blood feed and are ambivalent to the presence of the flask.
- Feed mosquitoes on a cotton wool pad soaked in 5% glucose/0.05% PABA which is placed on top of the mosquito container. Remove the cotton wool pad 12 h before the blood feed. Starving the mosquitoes for 12 h prompts the vast majority to feed to engorgement on blood.

On the day of the feed

- Wash fresh group O, Rhesus group positive whole blood and warm it to 37 °C.
- Warm group O, Rhesus group positive serum to 37 °C.

For details of preparation of human RBC and pooled serum, refer to the Appendix of MOSQUITOES AND PARASITES, section II:B.

- Place a Baudruch membrane on the blood feeder, wet it, and allow it to dry. Once the membrane is dry (approximately 20 min), switch on the water pump and allow the water jacket to warm up to 38 °C (approximately 60 min).
- Take *P. falciparum* to be used for the feed from 14- to 17-d-old cultures which contain mature, stage V gametocytes. As male gametocytes mature more rapidly than do females, in practice it is best to use either: a) exclusively cultures that are 17-d post-subculture, which should contain a sufficient number of gametocytes of both sexes; or b) a preparation of blood of different ages (such as a 1:1 mix of 14- and 16-d-old cultures), which will show a sex ratio bias in favor of male or female parasites, respectively. For method of cultivation, refer to “Growth of blood cultures for gametocyte production” of MOSQUITOES AND PARASITES, section II:B.

If at all possible, perform the following procedures entirely at 37 °C and as rapidly as safe handling and good microbiological practice permits in order to minimize the possibility of gametocytes committing to activation/exflagellation before the blood meal is taken up by the mosquito.

- Transfer infective cultures to centrifuge tube(s) prewarmed to 37 °C.
- Centrifuge the cultures at 800 × g for 2 min in a centrifuge prewarmed to 37 °C.
- Remove and discard the supernatant.
- Measure the volume of the remaining pellet, containing packed PRBC, and add an equal volume of serum.
- Make the volume of infective blood/serum from step 8 up to that of the blood feeder to be used (1 to 2 mL) by diluting the suspension up to 3× volume with a mixture of 1:1 washed human RBC/serum. Mix gently but thoroughly. We routinely dilute blood in this manner because: a) better infection rates are achieved using blood that contains a high but submaximal gametocytemia; b) addition of fresh blood/serum to the 17-d-old cultured PRBC promotes mosquito

- feeding, in terms of both extending the feeding time of individual mosquitoes (qualitative) and increasing the number that feed (quantitative).
- Rapidly transfer the diluted infective blood from step 9 into the blood feeder using a prewarmed syringe with a blunt-ended needle, ensuring that there are no air bubbles present.
 - Remove a mosquito container from its outer cage. Place the container underneath the blood feeder, ensuring that the blood feeder and the netting are in intimate contact.
 - Turn off any artificial lighting and allow the mosquitoes to feed undisturbed for 12 to 15 min. We recommend that the high security insectary in which the mosquitoes are held be without windows; this enables feeding to take place in the dark, mimicking feeding behavior in the wild.
 - Remove the mosquito container from underneath the blood feeder, thus terminating feeding, and return it to a gauze-covered cage. Most mosquitoes that have been starved of nutrients for the previous 12 h will feed to engorgement within a few minutes. These will have a very distended, red abdomen and can be easily distinguished from mosquitoes that have not fed. If the mosquitoes have fed well, the floor of the container will be covered with red splashes of fluid passed by diuresis. Depending on the ease with which the pooter can be manipulated within the container, it may be possible at this stage to remove individual unfed mosquitoes.
 - During the time the blood feed is taking place, examine any infective blood/serum mix that was not added to the membrane feeder for gametocyte activation and/or exflagellation. Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. The cytoplasm of the male gametocyte becomes agitated, followed rapidly by the sudden and vigorous protrusion and detachment of up to 8 flagellated gametes (spermatozoa) from the surface of the parasite cell body. This phenomenon also occurs spontaneously *in vitro* and thus may be observed in cultures of fresh infected blood under the light microscope (1000× magnification under oil immersion). It is controlled *in vitro* solely by the change from 37 °C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO₂ tension as the blood equilibrates with the atmosphere. Viewing of gametogenesis (typically between 10 and 25 min after removal of a blood culture from the 37 °C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, in our experience, observable exflagellation is not a prerequisite for successful mosquito infectivity.

On days after the feed

- Subsequent to a blood feed, feed mosquitoes daily on a 5% glucose/0.05% PABA diet, as previously described. Delaying the start of feeding by 24 h post blood feed enriches for blood-fed mosquitoes as the vast majority of 'die off' mosquitoes under these conditions are those that did not blood feed and therefore were starved of nutrients for at least 36 h.
- Mosquitoes may be dissected for the presence of oocysts at 10 to 12 d postblood feed. Dissection for sporozoites may take place from 14 d postblood feed. The number of live mosquitoes in each container at the time of dissection must be verified and recorded by 2 people independently (usually by observing carefully the pootering process). The total number of mosquitoes either dissected or

undissected at the end of the experiment should tally with the records made. The protocol for dissections is described below.

Dissection of blood-fed mosquitoes

Dissection of mosquito midgut to determine the presence of oocysts

- For the detection of *P. falciparum* oocysts, dissect mosquitoes 10 to 12 d postinfective blood feed.
- Remove mosquitoes from the container via a pooter, the end of which is immediately plugged with cotton wool and sealed with masking tape to prevent escape. Mosquitoes may be collected one at a time, but up to 5 mosquitoes can be held in the cavity of the pooter without compromising handler safety.
- Strike the pooter against the palm of the hand firmly several times to knock the mosquitoes out for a sufficient period of time to allow their safe removal onto a dissection board. In order to stop each mosquito from flying, and also as an aid to dissection of the body, remove its legs and one wing with a scalpel blade.
- Place dewinged and delegged mosquitoes in a watch glass containing dissection medium (PBS + 0.0001% FCS). Using a pair of fine forceps, pick up each mosquito by its one remaining wing and dip it into 70% ethanol to ensure both sterility and killing of the mosquito.
- Remove individual mosquitoes, mount them on glass microscope slides onto which a drop of dissection medium had been previously placed, and remove the remaining wing.
- Dissect the mosquito under a binocular light microscope using a 40× objective (total magnification 400×).
- Separate the abdomen from the rest of the mosquito. Remove the gut by holding the anterior of the abdomen with one dissecting needle while at the same time making a small cut with a second needle in the tegument on each side of the seventh abdominal segment. Using the second needle, gently pull on the apex of the abdomen until the gut and Malpighian tubules are exposed.
- Sever the alimentary canal sufficiently far forward to bring away (and discard) all of the foregut except for the section immediately proximal to the midgut.
- Anchor the remainder of the gut by placing the point of one needle on the posterior section of foregut. Using the other needle, cut through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the Malpighian tubules. Discard the hindgut and Malpighian tubules, leaving only the midgut on the slide.
- When an especially rapid dissection is required (with least chance of disruption to the oocysts), once the gut has emerged completely from the abdomen, cut off and remove the hindgut only, leaving the midgut exposed but with the foregut and esophagus still attached.
- Using a needle, pick up the dissected midgut and transfer it to a fresh slide onto which has been placed a drop of dissecting medium. Lower a coverslip gently onto the moistened midgut, which then can be viewed for the presence of oocysts. The volume of medium bathing the preparation is important as too little will cause the midgut to rupture (so releasing oocysts and thereby preventing their examination *in situ*) and too much will prevent adequate flattening of the

- midgut for optimal viewing. Draw off excess medium by holding a filter paper to the edge of the slide.
- View guts by under 400× magnification with reduced light when oocysts, if present, should be easily identified as circular refractive bodies on the gut wall of the dissected mosquito.
 - In our experience, the number of oocysts recovered per infected mosquito varies between 1 and >200, average 30. Mosquitoes that are not infected, as determined by a lack of oocysts, are usually those that did not blood feed and will not contain eggs. Occasionally, an infected mosquito (with oocysts) that has no eggs may also be seen.

Dissection of mosquito salivary glands to determine the presence of sporozoites

- Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.
- Sporozoites may first be observed in the salivary glands 14 to 17 d postinfective blood feed. The junction of the mosquito head and thorax contains a pair of glands, each of which is tri-lobed (or very occasionally four-lobed).
- Repeat steps 1 through 6 as described above for oocyst dissection.
- Place a dissecting needle gently on the thorax of the mosquito, just below the region where the salivary glands lie.
- Place a second needle on the 'neck' of the mosquito (apex of head and thorax) without cutting it, then with a gentle pulling action detach the head.
- The salivary glands should now be exposed and can be detached from the head. If the salivary glands are not apparent, then press with a needle again on the thorax, when the salivary glands should emerge from the thorax and can be detached.
- Transfer the dissected glands and a small volume of dissection medium into a 0.5-mL microcentrifuge tube using a 100- to 200- μ L Gilson pipette. Keep the microcentrifuge tube on ice.

Liberation of sporozoites from salivary glands

- Cover the microcentrifuge tube containing the salivary glands with Parafilm to reduce the chance of leakage. Release the sporozoites by gently rupturing the glands using a laboratory benchtop whirlimixer set to maximum for 3 min or by vigorous pipetting using a 200- μ L Gilson pipette.
- Once liberated from the salivary glands, the sporozoite yield can be estimated by counting using an hemocytometer. Place 10 μ L of the sporozoite suspension into the hemocytometer chamber and then using 200 to 400× magnification phase-contrast microscopy to visualize the sporozoites, count the number present in a 4 \times 4 grid: this number relates to the number of sporozoites per $\text{mm}^2 \times 10^4$.
- If a qualitative examination for sporozoites is all that is required, transfer salivary glands directly to a glass microscope slide. Lower a coverslip carefully onto the moistened glands and tap it gently to disrupt the glands and release any sporozoites present. These should be easily identified as comma-shaped, motile bodies.
- In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.

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IV:E. Cultivation of *Plasmodium berghei* ookinetes with insect cells for production of young oocysts

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In the method described, *Plasmodium berghei* ookinetes invade co-cultured *Aedes aegypti* Mos 20 cells and form intracellular oocysts, expressing CSP after 6-7 days; development does not appear to continue further. Other insect cell lines tested also sustain oocyst development, such as *Drosophila melanogaster* S2 cells and *Anopheles gambiae* Sua5B cells. When using other cell lines the method should be adjusted for the optimal growth of the cells used.

Equipment

haemocytometer

LabTek 8 well chamber slides (Nunc) or multiwell plates

incubator at 19 °C.

epifluorescence or confocal microscope

Materials and reagents

In vitro cultured ookinetes enriched by the magnetic bead method (this book) or using any of the methods described in references 1-3.

Insect cell line *Aedes aegypti* Mos20 (ATCC # CCL-125) (see Appendix)

M199 insect cell culture medium (see Appendix)

PBS

fixative for example: acetone/methanol 1:1 or 4% paraformaldehyde, 0.2 % triton in PBS

normal goat serum (NGS) or bovine serum albumin (BSA)

suitable primary antibodies

secondary antibodies conjugated to a suitable dye for detection; for example Alexa-conjugated secondary antibodies (available from Invitrogen)

Procedure

Setting up the co-cultures

- Determine the concentration of the purified ookinetes by counting in a haemocytometer. Note that the ookinetes are often seen in aggregates after purification, and one has to count the ookinete in the aggregates as well. Diluting the ookinetes to lower cell density ($<10^6$ /mL) in PBS and leaving them for 15 minutes at room temperature results in looser aggregates, which are easier to count.
- Determine the concentration of the Mos20 cells in a haemocytometer.
- Mix 1×10^4 ookinetes in PBS (should not correspond to more than 10 μ L) and 2×10^5 cells in a total of 0.4 mL of medium M199. Add to the culture dish. Incubate at 19 °C for the requested number of days.
- The culture medium should be changed twice a week during prolonged incubations.

Monitoring the co-cultures

The co-cultures can be monitored in a number of ways.

- If the parasite is expressing GFP (green fluorescent protein) the culture can be monitored directly under a fluorescence microscope.
- Oocyst development can be detected by staining the culture with antibodies. Young oocysts will express the P28 surface antigen, and at day 6 -7 the CSP (circumsporozoite protein) antigen.
- Nuclear division can be detected using a suitable DNA binding dye, e.g. DAPI, Propidium iodide or Hoechst 33 342 .

Antibody staining of co-cultures (from LabTek chamber slides)

- Carefully remove the medium from the well. The Mos20 cells and ookinetes/oocysts stick to the plastic.
- Leave the dish at room temperature to dry. Warming the slide on a slide warmer at ~50 °C for about 10 min. improves morphology of the sample.
- All the following steps are carried out at room temperature.
- Fix the sample in suitable fixative. We have had good results using the following two protocols but each antibody should be tested for the best fixation method. Aceton/methanol 1:1, 2 min or 4% paraformaldehyde, 0.2 % triton in PBS for 10 min
- After fixation rinse twice with PBS.
- Add the primary antibody diluted in PBS with 5 % NGS or 0.1 % BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Add the secondary antibody diluted in PBS with 5% NGS or 0.1 % BSA. Incubate 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- In addition, the sample can be incubated with a suitable DNA stain diluted accordingly.
- Incubate for 5-15 min.
- Wash twice with PBS.
- Mount the sample and analyze in an epifluorescence or confocal microscope.

Appendix. Growth of *Aedes aegypti* Mos20 cells.

The cells are grown in tissue culture flasks in M199 medium (Gibco) supplemented with 10% foetal calf serum and penicillin/streptomycin 50ug/mL/50µg/mL. The flasks are incubated in a stationary incubator at 25 °C. No gassing is necessary. Medium should be changed twice a week and the flasks should be split once a week.

References

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IV:F. Enrichment of *Plasmodium berghei* ookinetes from *in vitro* cultures using Dynabeads

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The method, using magnetic beads coated with a monoclonal antibody against the Pbs21 surface antigen, provides a rapid protocol for purification of small scale *in vitro* cultures of *P. berghei* ookinetes (originating from 1 to ~5 mice). Because the antigen is also present on female gametes and zygotes the purified material will contain also these stages.

Materials and reagents

in vitro cultures of *Plasmodium berghei* ookinetes, prepared as described in references 1-3.

Dynabeads® M-450 Goat anti-Mouse IgG (Invitrogen; cat. no. 110.05)

13.1 monoclonal antibody (can be obtained from Prof. R. E. Sinden, Division of Cell and Molecular Biology, Imperial College London, Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, United Kingdom)

sterile PBS (per litre)

NaCl 8.0 g

KCl 0.2 g

Na₂HPO₄ 1.15 g

KH₂PO₄ 0.2 g/litre

pH=7.2

Equipment

Magnetic Particle Concentrator, MPC, (for example Dynal MPC™-S Magnetic Particle Concentrator from Invitrogen, cat. no. 120.20D)

table top centrifuge

tubes suitable for the MPC

centrifuge tubes

microscope

haemocytometer

rotating wheel

Procedure

Preparation of magnetic beads coated with 13.1 antibody

- Re-suspend the magnetic beads in their vial by mixing with the use of a vortex.
- Remove 200 µL to a suitable microfuge tube. Put the tube on the MPC for 2 min.
- Remove the supernatant while keeping the tube on the MPC.
- Washing: Add 200 µL PBS, re-suspend the beads by using the vortex, then put the tube on the MPC. Remove the PBS.
- The washing step is repeated a total of four times.

- After the last wash, remove the PBS and add 100 μ L of the 13.1 antibody. Gently re-suspend the beads in the antibody solution, and then incubate for 30 min. at 4 °C, on a rotating wheel.
- Separate the now coated beads from the antibody solution by putting the microfuge tube on the magnetic rack for 2 min.
- Remove the antibody to a fresh tube; the antibody can be re-used for coating several times if it is kept at 4 °C under sterile conditions.
- Wash the coated beads with PBS four times.
- Re-suspend the beads in 200 μ l PBS. Keep at 4 °C until use. The beads can be kept for > 1 month.

Purification of ookinetes using magnetic beads coated with the 13.1 antibody

- Set up an *in vitro* culture of ookinetes, starting from the blood of 1-5 infected mice as described in ref. 1-3. Incubate at 19 °C for 20-24 hours.
- Check the culture under a phase contrast microscope for the presence of ookinetes.
- Spin down the cells using a table-top centrifuge at 500xg for 5 min.
- Re-suspend the pellet in 0.5 mL PBS (per 1 mL of blood added to the *in vitro* culture) and transfer to a suitable tube for the MPC.
- Add 2.5 μ L of the 13.1 antibody coated beads.
- Invert the tube for 5 min, by hand or on a rotating wheel at room temperature.
- Put the tube on the MPC for 2 min. Carefully remove the supernatant. The ookinetes attached to the beads should form a thick “pellet” close to the magnet.
- Wash the ookinetes by adding 200 μ L PBS while the tube is still on the MPC. Remove the PBS carefully.
- Resuspend the ookinetes in 100 μ L to 1 mL of PBS.
- Dilute a small sample (1 to 5 μ l) in PBS and count the ookinetes in a haemocytometer. We usually obtain 5×10^6 to $>10^7$ per 1 mL of mouse blood added to the *in vitro* culture. Note that the ookinetes are often seen in aggregates after purification, and one has to count the ookinete in the aggregates as well. Diluting the ookinetes to lower cell density in PBS ($<10^6$ /mL) and leaving them 15 minutes at room temperature results in looser aggregates, which are easier to count.
- The ookinetes can be kept in solution for several hours at room temperature without loss of viability.

References

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Rodriguez MC, Margos G, Compton H, Ku M, Lanz H, Rodriguez MH, Sinden RE. 2002. *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. Exp Parasitol 101(1):73-6.

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IV:G. Motility assays of *Plasmodium berghei* ookinetesby **Inga Siden-Kiamos** and **Christos Louis***Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology
– Hellas, GR 711 10 Heraklion, Greece**e-mail: inga@imbb.forth.gr, louis@imbb.forth.gr*Equipment

haemocytometer

LabTek 8 well chamber slides (Nunc) or multiwell plates.

incubator at 19 °C.

microscope equipped with a camera for time-lapse video microscopy (for Method 1) or fluorescence microscope (for Method 2)

slides and coverslips

Cytochalasin D

Materials and reagents*In vitro* cultured ookinetes enriched by the magnetic bead method (this book) or using any of the methods described in reference 1-3.insect cell line *Aedes aegypti* Mos 20 (ATCC # CCL-125) (see Appendix)

M199 insect cell culture medium (see Appendix)

PBS

fixative acetone/methanol 1:1

normal goat serum (NGS) or bovine serum albumin (BSA)

anti-Pb70 antibody against an ookinete cytoskeletal protein (can be obtained from Prof. R.E. Sinden Division of Cell and Molecular Biology, Imperial College London, Sir Alexander Fleming Building, Imperial College Road, London SW7 2AZ, United Kingdom)

secondary antibodies conjugated to a suitable dye for detection; for example

Alexa conjugated secondary antibodies available from Invitrogen

Vaseline

Method 1. Direct observation of motile ookinetes.

- Resuspend the purified ookinetes to a final concentration of $\sim 5 \times 10^6 - 10^7$ ookinetes/mL.
- Mix 1 μ L the purified ookinetes with 2×10^5 freshly harvested *Aedes aegypti* Mos20 cells in 10 μ L M199 medium.
- Add the cell mixture to a glass slide and cap the slide with a Vaseline rimmed cover slip.
- The slides should be observed immediately as motility is rapidly initiated. Motility of the ookinetes is monitored at room temperature, under a microscope using transmission optics. For time-lapse photography pictures are taken every 30 seconds. Alternatively, when using GFP expressing parasites a fluorescence microscope can be used. The processing of the time-lapse videos is easier using the fluorescent parasites as the insect cells will not be visible under these conditions.

Method 2. A quantitative assay of motility.Setting up the co-cultures

- Mix 1×10^4 ookinetes in PBS (should not correspond to more than 10 μ L) and 2×10^5 *Aedes aegypti* Mos20 cells in a total of 0.4 mL of medium M199.

- Add the cell mixture to the culture dish (8 well Lab Tek chamber slides or 24 well multiwell plate). In each experiment a control with Cytochalasin D at a final concentration of 10 μ M should be included.
- Incubate at 19 °C over night.

Antibody staining of co-cultures (from LabTek chamber slides)

- Carefully remove the medium from the well.
- Leave the dish at room temperature to dry. Warming the slide on a slide warmer at ~50 °C for about 10 min. improves morphology of the sample.
- Fix with acetone/methanol 1:1, for 2 min at room temperature.
- After fixation rinse twice with PBS.
- Add the anti-Pb70 antibody diluted in PBS with 5 % normal goat serum or 0.1% BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Add the secondary antibody diluted in PBS with 5% NGS or 0.1 % BSA. Incubate for 30 min. at room temperature.
- Wash twice with PBS for five min. each.
- Mount the sample.

Scoring the samples

- Score the stained samples under the fluorescence microscope:
 - In the control sample with wild type ookinetes most parasites should be seen as clearly demarcated, individual ookinetes.
 - In the control sample with Cytochalasin D most ookinetes should be seen in aggregates. Cytochalasin D inhibits motility of the ookinete and therefore the numbers from these samples represent the aggregated non-motile ookinetes, while the single ookinetes results from scattering of the ookinetes when the cultures are seeded.
- To achieve a quantitative estimate of motility count individual and aggregated ookinetes in random microscope fields.

Appendix. Growth of *Aedes aegypti* Mos20 cells.

- The cells are grown in tissue culture flasks in M199 medium (Gibco) supplemented with 10% foetal calf serum and penicillin/streptomycin 50ug/mL/50 μ g/mL.
- The flasks are incubated in a 25 °C stationary incubator.
- No gassing is necessary.
- Medium should be changed twice a week and the flasks split once a week.

References

Sinden RE. 1997. Infection of mosquitoes with rodent malaria. In: Crampton JM, C.B. Beard, C. Louis, editor. The molecular biology of insect disease vectors A methods manual. London: Chapman and Hall. p 67-91.

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III. Transmission blocking and sporozoite invasion assay

III:A. Transmission blocking assay (TBA) – membrane feeding

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Equipment

membrane feeder (glass), Lillie Glassblower, USA (Tel: + 1-404-436-8959)
silicon tubing to connect the feeders
aspirator tube
pint containers (Naptune Brand Round containers, Kahn Paper Co.)
dental dam, hygienic
nylon net, bridal net (any fabric store).
Parafilm
water pump, constant temperature circulator (Polystat, Cole-Parmer, or any other equivalent)
light microscope (e.g., Olympus)
dissecting microscope (e.g., Olympus)
dissecting forceps
dissecting glass slide
glass slides and coverslips
Eppendorf tubes
sterile pipettes
insectaries approved for handling *Plasmodium falciparum*-infected mosquitoes

Materials and reagents

parasite culture:

Maintain *Plasmodium falciparum* gametocyte culture for 14 to 18 days making blood smears on alternate days. From *Day 12* onward, make blood smears on a daily basis and look for mature C-shaped gametocytes as well as in vitro exflagellation of male gametocytes.

mosquitoes:

Maintain and breed *Anopheles stephensi* according to the standard procedure (see MOSQUITOES AND PARASITES, section II:C). Use 3- to 5-d-old mosquitoes for the experiment.

human RBC

heat-inactivated normal human serum (NHS)

heat-inactivated antiserum

incomplete medium used for *P. falciparum* cultures

mercurochrome solution

Methods

Important notes before starting

- *Plasmodium falciparum* cultures of at least 0.5% mature gametocytes and at least 1 to 2 exflagellation centers per field should be used for mosquito feeding.
- Before preparing blood meal, have mosquitoes and feeders ready.
- Take care to maintain the parasite and the blood always at 37 °C.

Exflagellation assay

- Using a sterile pipette, take out 1 mL of parasite culture and transfer it to an Eppendorf tube.
- Spin down the tube for 15 s at high speed and discard the supernatant.
- Add an equal volume of normal human serum to resuspend the pellet and allow it to stand for 10 min at room temperature.
- Place 10 μ L of this suspension on a glass slide and cover it with a coverslip.
- Determine the average number of exflagellation centers per field using a light microscope at 400 \times magnification. A polarizer filter may help in locating the parasitized RBC as in this configuration the malaria pigments look shiny. Scan at least 10 to 20 fields to get a proper estimate of exflagellation centers.

Making dilution of antibodies in 50% RBC

Premix the antibodies to be tested for transmission blocking activities with 50% red blood cells in heat-inactivated normal human serum at various dilutions (1:5 to 1:20).

Keep these diluted antibodies at 37 °C until the final feeding.

- Place 100 μ L of 50% RBC (in incomplete medium) in a microfuge tube, harvest the cells at 3,000 rpm for 1 minute, and wash them with heat-inactivated NHS.
- Resuspend the cells at 50% hematocrit in NHS and antiserum to obtain desired serum dilution.
- Incubate the tubes at 37 °C.

Preparation of mosquito pint

- Make a hole (2-cm diameter) in the paper can. Cover the hole with two squares (2 in \times 2 in) of dental rubber with perpendicular cuts on top of each other using adhesive tapes.
- Cover the top of the paper can with nylon net of appropriate size and securely tape the net to the paper can.

Collecting female mosquitoes in pint

Distribute 30 to 40 female mosquitoes per pint. The easiest way to collect female mosquitoes from a mixed pool of male and female is to place a warm water bottle (39 to 40 °C) against the wall of the mosquito container. The female mosquitoes are attracted to the warmer side of the cage, whereas the male mosquitoes will be all over. Starve the mosquitoes for 5 to 6 h.

Preparation of feeders

- Connect the glass feeders to each other and to the circulation water pump with silicon tubing to generate a continuous flow of 39 °C hot water through the water jackets of the membrane feeders.
- Stretch Parafilm membrane (approximately 2 in \times 2 in) in both directions and place on the glass feeder. Maintain the temperature of the membrane feeder by a glass water jacket (39 °C) and a circulating water bath.
- Place the pint with female mosquitoes under the warmed membrane feeder.

Membrane feeding

This step is the most crucial and must be performed as quickly as possible. During the whole procedure, take care not to allow the temperature of the *Plasmodium falciparum*

gametocyte culture to drop below 37 °C. A drop in temperature promotes exflagellation. The success of the experiment depends on the maximization of exflagellation as well as gametogenesis within the mosquito, not outside. Allow the mosquitoes to engorge for 15 min.

Experimental steps

- Place 10 mL of matured *Plasmodium falciparum* gametocyte culture in a 50-mL conical tube.
- Harvest at 1,000 rpm for 2 min and then allow the rotor to stop naturally (without the break).
- Remove the supernatant and resuspend the pellet at 50% hematocrit with prewarmed NHS.
- Keep the tube warm at 37 °C.
- Lower the membrane feeder over the nylon net covering the pint. Make sure that the mosquitoes are attracted to the membrane.
- Add 50 µL of gametocyte suspension kept warm at 37 °C to the diluted antibody and immediately transfer the mixture to the feeders.
- After 15 min, separate the unfed mosquitoes from the fed ones. It is very easy to separate the fed mosquitoes from unfed mosquitoes as the fed mosquitoes have swollen red abdomens.
- Feed sugar and water to the mosquitoes and maintain them for 7 to 9 more days in a room maintained at 26 °C and 60 to 80% relative humidity.

Scoring and statistical analysis

Perform any TBA experiment with at least 2 or 3 different concentrations of the test serum. The controls may include preimmune sera, irrelevant antisera (raised against asexual stage specific antigens), no antiserum added, and a serum known to possess transmission blocking activity.

- After 7 to 9 days, dissect the midgut of each and every individual mosquito from different groups under the dissection microscope and look for the presence of oocysts. Mosquitoes can be knocked down by dropping them in 70% ethanol and then transferred to a container with 1× PBS. Transfer the mosquitoes to glass slides and carefully pull the posterior part of mosquito with dissecting forceps, holding the anterior part of mosquito at the same time with another forceps. Treat the mosquito midguts thus separated with drops of 0.1% Mercurochrome solution and allow them to stain for a few minutes under coverslips.
- The oocysts will stain red on a light pink background of the midgut. Count the number of oocysts per midgut.
- Calculate the infection rates (number of mosquitoes infected per total number dissected) and number of oocysts (geometric means) in mosquitoes.
- Compare the infection rates and the number of oocysts in mosquitoes fed test serum to those obtained from feeds using appropriate control sera.
- Perform the Mann–Whitney test to assess the statistical significance.

Comments:

Apart from assessing the potency of different transmission blocking antibodies, the same experimental procedure can be used to address other biologically important questions. For example, effects of some chemotherapeutic drugs as transmission blocking agents

can easily be scored with the help of this assay. Also the involvement of different agents on parasite infectivity can be quantitated. A similar experimental design is also used to perform genetic crosses using different parasite strains.

Reference

Bhattacharyya MK, Kumar N. 2001. Effect of xanthurenic acid on infectivity of *Plasmodium falciparum* to *Anopheles stephani*. Int J Parasitol 31(10):1129-1133.

III:B. Sporozoite invasion assay

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The techniques used for assessing *Plasmodium berghei* and *P. falciparum* sporozoite invasion are identical, although the latter parasite obviously poses a substantial risk to any individual exposed to it, so due care should be taken in handling infectious mosquitoes and viable sporozoites. Sporozoites are layered over hepatoma (HepG2-A16) cells or primary human hepatocytes and recognized by monoclonal antibody or serum raised to circumsporozoite (CS) protein, which bind the cell surface of sporozoites and block sporozoite invasion into hepatoma cells in vitro.

The reagent that is recommended for sporozoite detection is an IgM monoclonal antibody (MAb 36) which reacts with the CS proteins of both *P. falciparum* and *P. berghei*, and which was isolated from *P. falciparum* sporozoite-immunized mice (Sina et al. 1992; available on request from MR Hollingdale). In assays of biological activity, MAb 36 induces the CS precipitation reaction with live sporozoites and blocks the invasion of hepatoma cells by sporozoites in vitro at concentrations much lower than those observed for other reported CS protein-specific MAb. However, suitable alternatives are available in the MR4 repository: rabbit antiserum against *P. falciparum* CS protein (MRA-24) and rat antiserum against recombinant *P. falciparum* CS protein region I to C-terminus (MRA-21).

Equipment

CO₂-incubator
200- μ L Gilson pipette
hemocytometer
phase-contrast microscope

Materials and reagents

immortalized human hepatocyte cell line HepG2 (available from MR4 as either a frozen ampoule or a growing culture)
entacin–collagen–laminin (ECL)
serum-free RPMI 1640 medium
mosquitoes (see MOSQUITOES AND PARASITES, section II:C)
PBS
PBS + 0.0001% FCS
PBS + 1% BSA
MEM; minimal essential medium (Sigma)
10% heat-inactivated FCS (referred to as 'binding medium')
100% methanol
MAb 36
goat anti-mouse peroxidase conjugate (DAKO)
distilled water
DAB tablet (Sigma 'Fast' DAB kit)
H₂O₂ (Sigma 'Fast' DAB kit)

DPX mountant (HD Supplies)
8-well Nunc slides

Producing a confluent HepG2 monolayer to study sporozoite invasion

The growth of HepG2 cells on 8-well Nunc coverslips is achieved using standard culture techniques, splitting confluent cultures between 1:3 and 1:6. However, the surface of slide wells should be precoated with an extracellular matrix (entacin–collagen–laminin; ECL) to promote cell attachment.

- Dilute a 40- μ L frozen aliquot of ECL (stock concentration at 1 mg/mL) in 4 mL of serum-free RPMI 1640 medium. Add 200 μ L of this solution to each well of the 8-well slides.
- Leave at 37 °C for a minimum of 2 h.
- Aspirate off the ECL solution and add a 250- μ L suspension of HepG2 cells to each well.
- Place slides in an incubator (37 °C, CO₂) for 2 to 3 d to allow the cells to attach and grow before using them in an invasion assay.

Invasion assay

Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.

- Dissect the mosquitoes for sporozoites between 14 and 17 d after an infectious feed, as described in detail previously (see MOSQUITOES AND PARASITES, section II:C).
- Dissect out both salivary glands from the apex of head and thorax of each mosquito and remove any unwanted body tissue. Collect salivary glands into ice-cold PBS + 0.0001% FCS. In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.
- Using a 200- μ L Gilson pipette, vigorously pipette the suspension containing mosquito salivary glands up and down to disrupt the glands in order to release free sporozoites.
- Determine the density of sporozoites in this suspension using a hemocytometer. Place 10 μ L of the sporozoite suspension into the hemocytometer chamber, use phase-contrast microscopy to visualize the sporozoites, and count the number present in a 4 \times 4 grid. This number relates to the number of sporozoites per mm² \times 10⁴.
- Dilute and divide the sporozoite suspension so that there are between 10,000 and 20,000 sporozoites per 300 μ L of minimal essential medium (MEM) + 10% heat-inactivated FCS (referred to as 'binding medium'). Ensure that this density is the same for all groups that are to be compared.
- Remove 8-well slides containing HepG2 cells from the 37 °C CO₂ incubator, wash each well once with 300 μ L of binding medium, and add 300 μ L of sporozoite suspension. Place slides in a loose-lid container and return them to the 37 °C CO₂ incubator for 3 to 4 h.
- Gently aspirate off the sporozoite suspension, add 300 μ L of warmed MEM (without FCS), and leave the slides for 5 min.
- Repeat the wash.

- Aspirate off the MEM and flood the wells with 100% methanol. After 5 min, remove the methanol, add PBS, and leave them at 4 °C overnight.

Visualization of sporozoites

Visualize the sporozoites using immunohistochemical techniques.

- Discard the PBS solution and remove the plastic well surrounds and the rubber gaskets from the 8-well slides.
- Block each monolayer with a 100- μ L solution of PBS+ 1% BSA for 10 min.
- Aspirate off the solution and add 100 μ L of PBS + 1% BSA + 5 μ g/mL of MA b 36 (shows immunological cross-reactivity between *P. falciparum* and *P. berghei* CS proteins).
- Incubate the slides in a humidified chamber at room temperature for 30 min.
- Aspirate off the primary antibody solution and wash the cell monolayers by placing the slides in a Coplin jar containing PBS for 2 min. Repeat this step twice more.
- Add 100 μ L of PBS+ 1% BSA containing a 1:200 dilution of goat anti-mouse peroxidase conjugate. Incubate the slides in a humidified chamber at room temperature for 30 min.
- Repeat the PBS washes.
- In 1 mL of distilled water, dissolve one DAB tablet and one H₂O₂ tablet from the Sigma 'Fast' DAB kit. Add 100 μ L of this solution to each cell monolayer. Allow the reaction to proceed for 2 min and then terminate it by washing off the DAB solution with tap water. Note: **DAB is very toxic**. Always wear gloves when handling it and use copious amounts of water to wash solutions containing DAB down a suitable laboratory sink.
- Allow slides to dry and then mount them using DPX mountant and glass coverslips. Visualize sporozoites under either 200 \times or 400 \times magnification using phase contrast microscopy.

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ANIMAL MODELS

I. Infection of monkeys with *Plasmodium* spp.

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Equipment

compound microscope
centrifuge
water bath (37 °C)
liquid N₂ freezer
Neubauer chamber
Monolet

Materials and reagents

syringes
16g, 19g, 21g, and 27g needles
25 × 75-mm microscope slides
cryovials (Nunc)
Glycerolyte 57 (Baxter Health Care - Fenwall Division)
Giemsa stain
heparinized vacutainer tubes
phosphate buffer (pH 7.0)
RPMI 1640
50-mL centrifuge tubes
12% NaCl
1.6% NaCl
0.9% NaCl + 2% dextrose solution

Preparations

Infection with fresh parasitized erythrocytes

- Collect blood from donor animal (using femoral vein) into a heparinized tube.
- Based on the parasite count, aseptically dilute blood in RPMI 1640 to give the required number of parasites in 1 mL or less.
- Restrain (New World) or anesthetize (larger Old World) monkeys.
- Inject blood slowly into femoral vein using a 25 g needle; maintain pressure on the needle exit hole until bleeding stops.

Infection with frozen parasitized erythrocytes

- Thaw vial(s) of frozen blood rapidly in 37 °C water bath.
- Rapidly transfer cells to a 50-mL tube by sterile pipette (note volume).
- Poke a hole in the cap of the 50-mL tube with a 16g needle.
- Draw 0.2× blood volume of 12% NaCl into a syringe and 21g needle.
- Add 12% NaCl dropwise through the hole while shaking the tube.
- Let the tube stand at room temp for 5 min without shaking.
- Take up 10× volume (of cells) of 1.6% NaCl.
- Add the NaCl solution dropwise through the hole while shaking the tube.

- Spin the tube at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 10× cell volume of 0.9% NaCl and 2% dextrose solution as above.
- Spin at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 1 mL of RPMI 1640.
- Inoculate recipient monkey via the femoral vein using a syringe with 25g needle.

Comment

Viable parasites have been stored frozen for >25 years; thawing and refreezing has markedly reduced their viability.

Infection via mosquito bite

- Cage infected mosquitoes in a small container covered with nylon netting.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps (the center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage).
- Allow mosquitoes to feed to engorgement (usually 10 to 15 min).
- After feeding, dissect mosquito salivary glands and examine them for presence of sporozoites to determine number of infected mosquitoes.
- Determine the number of sporozoites remaining in the glands:
 - 1+ = 1 to 10 sporozoites
 - 2+ = 11 to 100 sporozoites
 - 3+ = 101 to 1,000 sporozoites
 - 4+ = >1,000 sporozoites
- Record the total number of +'s for all mosquitoes fed on the animal to determine the exposure index.

Infection with sporozoites harvested by dissection

- Kill mosquitoes by exposure to chloroform.
- Remove legs and wings.
- Transfer bodies to a clean slide (wiped with alcohol just prior to use).
- Remove salivary glands from the thorax by gently pulling the base of the head from the body using either a fine needle or a 27g needle on a 1-mL syringe into a drop of 20% heat-inactivated monkey serum/saline (homologous species).
- Cut the salivary duct and remove the body and head. Ten or more sets of glands can be harvested into one drop of serum/saline.
- Add a coverslip and apply gentle pressure to rupture glands.
- Gently remove the coverslip and wash sporozoites from coverslip onto the slide with several drops of serum/saline.
- Aspirate the sporozoite suspension into a syringe with 25g needle.
- Transfer the suspension to a Nunc vial.
- Adjust the volume to approximately 1 mL.
- Repeatedly aspirate and express the suspension from the syringe to further dissociate sporozoites from salivary gland tissue.
- Transfer a small volume to a Neubauer cell counting chamber and allow it to set for 15 min.
- Calculate the number of sporozoites available.
- Dilute the suspension to the desired number of sporozoites in 1 mL serum/saline.

- Inject into the femoral vein using a 25g needle.

Comments

Infection of New World monkeys with *P. falciparum* and *P. vivax* via sporozoites has required the injection of large numbers of sporozoites, either via the bites of many heavily infected mosquitoes or via needle-injection of dissected sporozoites. Increasing the number of sporozoites injected has resulted in greater transmission rates and shorter prepatent periods.

Monitoring parasitemia in the infected monkey

- Remove the animal from the cage and immobilize it.
- Thoroughly clean either an area of the tail or the back of the leg.
- Prick with a "Monolet", carefully avoiding the vein.
- From the drop of blood expressed, make thick and thin blood films on a precleaned, prelabeled microscope slide. Label the slide with the animal number and date.
- After the thin film dries, fix it with methanol.
- Stain the slide with Giemsa stain for 35 to 45 min.
- Examine the slide for presence of parasites.
- Express the parasite counts as
 - 1) parasites per 10,000 RBC,
 - 2) parasites per 100 WBC, or
 - 3) parasites per μL .
- If the latter, the method of Earle and Perez is preferred. Using a 5- μL pipette, collect the blood and express it onto a 15-mm \times 5-mm area of a precleaned, prelabeled microscope slide (area is usually prescribed on the slide tray under the slide). Add a second drop of blood to the same slide to make the thin film.

Freezing parasitized erythrocytes

- Collect blood from the femoral vein into a heparinized tube.
- Spin the tube for 10 min at 1,200 rpm.
- Estimate the packed cell volume.
- Remove the plasma.
- Wash the cells 2 times with RPMI.
- Calculate $2 \times$ RBC volume (volume of Glycerolyte needed).
- Dropwise add one-fifth of the Glycerolyte volume to the tube at 1 or 2 drops per second with shaking, then let the tube stand for 5 min.
- Add the remaining Glycerolyte dropwise at same rate with shaking.
- Aliquot 0.5 mL to 1.0 mL per labeled Nunc vial.
- Transfer the vials to a -70°C freezer overnight; transfer them to a vapor phase liquid nitrogen freezer for storage.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. *J Lab Clin Med* 17:1146-1168.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. *J Parasitol* 87(6):1398-1403.

II. Infection of mosquitoes with *Plasmodium* spp. in monkeys

by **William E. Collins**

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Equipment

enamelware or plastic pans
incubator (25 °C)

Materials and reagents

deionized water
baker's yeast
mosquito larval food
monkey/rat chow
lactalbumen (Sigma L-7252)
brewer's yeast (Sigma YBD)
10% sugar solution
cotton balls
blood source (rabbit/Guinea pig)
Parafilm

Preparation

Rearing anopheline mosquitoes

- Grind monkey or rat chow in a mill and sieve through a 60-mesh screen.
- Combine lactalbumen, inactive brewer's yeast, and chow (1:1:1).
- Store the mixture in a refrigerator.
- Allow mosquito eggs to hatch in a pan lined with strips of paper toweling.
- Feed a "pinch" of active baker's yeast on *Day 1*.
- On succeeding days, feed increasing amounts of mosquito larval food.
- Maintain the water depth at 2 to 3 cm.
- Every 3 or 4 days, split pans until *Day 8* to give approximately 1 larva/cm² of water surface.
- When pupae appear, strain and suspend larvae and pupae in ice water.
- Pour floating pupae into an emergence cup.
- Return the larvae to the rearing pan.
- Place the pupae in a cage for emergence.
- After they emerge, feed them a 10% sugar solution daily on a cotton ball.
- Beginning 3 days after emergence, offer them a blood meal from an anesthetized rabbit or guinea pig every 3 or 4 days. (Shave hair from the feeding area.)
- Three days after each blood meal, put a small bowl with water (1 to 2 cm depth) into the cage overnight to collect eggs.

Comments

Most commonly reared anopheline mosquitoes such as *Anopheles stephensi*, *A. atroparvus*, *A. quadrimaculatus*, *A. gambiae*, and *A. albimanus* will mate in cages; others such as *A. dirus* require force mating. The most suitable vector depends on the species and strain of *Plasmodium* being investigated.

Infection of mosquitoes by feeding on monkey

- Transfer female mosquitoes (3 to 7 days after emergence) to infection cages.
- Concentrate female mosquitoes by placing a warm hand to the side of the holding cage.
- Aspirate and transfer the mosquitoes to the infection cage.
- Partially starve mosquitoes overnight with 5% sugar solution.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps. (The center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage.)
- Allow mosquitoes to feed to repletion (usually 10 to 15 min); remove and discard unfed mosquitoes.
- Store cartons of mosquitoes in a 25 °C incubator.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.
- Seven to 10 days after feeding (depending on the species of *Plasmodium*), dissect aliquots of mosquitoes to determine presence and numbers of oocysts.
- If oocysts are present, return undissected mosquitoes to the incubator.
- Dissect and examine salivary glands 10 to 18 days after feeding (depending on the temperature and species of *Plasmodium*).

Comments

Development of oocysts depends on the temperature and the species of *Plasmodium*. When incubated at 25 °C, the quotidian parasite *P. knowlesi* completes sporogonic development in 10 days; the tertian parasites *P. vivax*, *P. ovale*, *P. cynomolgi*, *P. gonderi*, *P. coatneyi*, *P. fragile*, *P. fieldi*, *P. simium*, and *P. simiovale* in 10 to 15 days; the quartan parasites *P. malariae*, *P. brasilianum*, and *P. inui* in 15 to 20 days.

Infection of mosquitoes by membrane feeding

- Transfer mosquitoes to infection cage (as above).
- Collect blood in heparin from the donor animal.
- Centrifuge blood at 1,500 rpm for 10 min.
- Remove plasma and replace it with serum or heparinized blood from an unexposed animal or human.
- Cover the feeding surface of a water-jacketed feeding bell with stretched Parafilm.
- Place the blood in the chamber.
- Commence circulation of water (37 °C).
- Lower the bell onto the netting of the feeding cage and allow mosquitoes to feed to repletion for 5 to 10 min.
- Remove and discard unfed mosquitoes.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.

Comments

Blood is collected in heparin, ACD, or is defibrinated with glass beads; EDTA inhibits infection. Transmission-blocking immunity often develops rapidly in monkeys. Removal of plasma and its replacement with serum or blood from an uninfected host allows continued mosquito infection if infectious gametocytes are present. Membrane feeding provides a means of assessing the transmission-blocking activity of immune sera by combining dilutions of sera from immunized

monkeys with infectious gametocytes. Reduction in oocyst densities measures transmission-blocking activity.

References

Earle WC, Perez M. 1932. Enumeration of parasites in the blood of malarial patients. J Lab Clin Med 17:1146-1168.

Rutledge LC, Ward RA, Gould DJ. 1964. Studies on feeding response to nutritive solutions in a new membrane feeder. Mosq News 24:407-409.

Sullivan JS, Strobert E, Yang C, Morris CL, Galland GG, Richardson BB, Bounngaseng A, Kendall J, McClure H, Collins WE. 2001. Adaptation of a strain of *Plasmodium vivax* from India to New World monkeys, chimpanzees, and anopheline mosquitoes. J Parasitol 87(6):1398-1403.

III. Experimental malaria: using bloodstage infections of rodent malaria

by **Helena Helmbly and Brian de Souza**

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Equipment

hemocytometer
light microscope
liquid N₂ freezer

Materials and reagents

syringes and needles for i.p and i.v injections
microscope slides
cryovials (e.g. Nunc)
Plasmodium freezing medium: 15% Glycerol in RPMI with 5% fetal calf serum and heparin (10units/mL)
Giemsa stain (Gurr's improved Giemsa, BDH Laboratory Supplies)
phosphate buffer (Na₂HPO₄ - 1g/L; KH₂PO₄ – 0.7g/L)
acridine orange (AO) (optional)
heparinized tubes
methanol
frozen stabilate of rodent malaria (e.g. *Plasmodium chabaudi*, *P. berghei*, *P. yoelii* etc)

To make frozen stabilates

- Collect blood, from mice with ascending parasitemia, directly into parasite freezing medium at a ratio of 1mL blood to 4mLmedium.
- Working aseptically, mix the blood and aliquot (100 – 500 µL/tube) in cryotubes and freeze immediately in liquid nitrogen. (For short periods, the tubes can be frozen and stored at –70 °C.)

NB. Parasite virulence can change after a number of *in vivo* passages, therefore it is important to start from a fresh stock of stabilates regularly.
(Freeze down stocks of new stabilates regularly)

To start an infection

- Thaw a cryotube of parasites quickly at room temperature.
- Inject undiluted contents immediately i.p. into a naive recipient mouse. Dilute the stabilate 1:2 with PBS for i.v injections.
- Start monitoring parasites in peripheral blood after 3-4 days.

Counting parasitemia

- Make a thin blood film from tail blood, fix with methanol for 2 min, and stain with Giemsa (Dilute Giemsa stain1:10 in phosphate buffer).
- Count percent infected cells (100× oil immersion).
(Instead of Giemsa staining, acridine orange (AO) staining of thin blood films is possible. After methanol fixing, add a small drop of AO on a slide, add a

coverslip, and count parasitemia under a light and UV-light microscope (see PARASITES, sections III:A, C)).

Passage of infection into new mice

- Collect blood from the donor mouse during ascending parasitemia. Transfer 10 μ L of tail blood into 5 mL chilled PBS and mix.
- Count parasitemia as above and the number of erythrocytes per microliter in a hemocytometer.
- Dilute the blood in sterile PBS to 5×10^5 infected erythrocytes per milliliter.
- Inject 200 μ L i.p. or i.v. per mouse (10^5 infected erythrocytes per dose).
- Parasites normally appear in the blood stream after 2 to 3 days.

N.B.

If you inject i.v., only one-tenth of the i.p. dose is needed to obtain similar parasitemias.

Reference

Cox FEG. 1988. Major animal models in malaria research. *In*: Wernsdorfer WH, McGregor I, eds. Malaria: principles and practice of malariology. Vol. 2. Edinburgh: Churchill Livingstone; pp. 1503-1543

IV. *In vivo* imaging of pre-erythrocytic forms of murine *Plasmodium* parasites

by **Samantha Blazquez, Sabine Thiberge, Rogerio Amino and Robert Ménard**

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Real-time *in vivo* studies of *Plasmodium* sporozoites within host tissues, will lead to a better understanding of how the parasite invades and then develops in host cells. In this method, we describe the observation of pre-erythrocytic parasites in the dermis and the liver of mice. At early time-points, parasites can be tracked from the time they arrive in the tissue until they invade cells. At later time-points, parasite development in host cells can be analysed.

Equipment

stereomicroscope equipped with epi-fluorescence
fluorescence microscope, equipped with objectives that have a long working distance (for higher penetration into the tissue), and coupled to a camera microscope platform to hold animal

Materials and reagents

Plasmodium berghei and/or *P. yoelii* fluorescent sporozoites (GFP or RFP)
70% Ethanol
1x PBS
microscope glass slides
50 I.U. and 100 I.U. Insulin syringes (Terumo)
Eppendorf microtubes
1x PBS, 5% bovine serum albumin (BSA)
hemocytometer (CML)
SKH1 Hairless (dermis) or C57BL/6 (liver) mice, if possible Pentobarbital or a mix containing 2% Rompun (Xylazine, Bayer) and Imalgene 1000 (Ketamine, Merial)
24 x 60-mm cover slips
BSA coupled to a fluorochrome (Alexa 488, 555 or 647) (Invitrogen)
Specific to each tissue:
For skin: Double-sided Scotch-tape
Nanofil intradermic needles with 35G needles
3M Scotch-tape
For liver: Surgical equipment (tweezers, scissors, electro-cautery)
SuperGlue 3

Procedure

Selection of mosquitoes positive for salivary gland sporozoites (infection by natural bite)

- At least 24 hours before the bite session gently aspirate mosquitoes and then blow them into a tube on ice. Transfer the mosquitoes to a Petri dish on ice.
- Under a fluorescence stereomicroscope, determine which mosquitoes contain fluorescent parasites in the salivary glands and place the mosquitoes in a recipient covered with netting.
- Keep the mosquitoes at 21°C and 70% humidity without nourishment (sugar solution) until the bite session.

Dissection of mosquitoes to obtain salivary gland (SG) sporozoites

- Anaesthetise mosquitoes by placing on ice. Place 10 seconds in 70% ethanol then transfer to 1x PBS. Place 10-12 mosquitoes on a microscope slide.
- Under the stereomicroscope, determine which mosquitoes are positive for SG sporozoites.
- Place one needle on the thorax of the mosquito and one needle at the base of the head. Gently pull the head away from the body, removing the SG from the thorax.
- Cut the salivary duct freeing the SG from the head and place the SG in an Eppendorf microtube containing 20 μ L of PBS 1x.
- Repeat this for around 50 mosquitoes.
- To determine the number of sporozoites, crush the SG, liberating the sporozoites. Take 1 μ L of sporozoite suspension and add to 9 μ L of 1x PBS, 5% BSA. Place the 10 μ L in a hemocytometer and count the number of sporozoites
- It is preferable to also determine the percentage of sporozoites that are gliding (moving in circles) as this has been shown to be related with a good infectivity.

Anaesthesia of mice

- Inject i.p 100 μ L of an appropriately diluted solution of Pentobarbital (50 mg/ 20 g mouse bodyweight). Anaesthesia with Pentobarbital reduces the chances of hyperventilation by the animal; however, the dose needed, is harder to control as the product is stocked in fat and then diffuses.
- A solution containing 2 volumes of Imalgene 1000, 1 volume of 2% Rompun and 5 volumes of 1x PBS can also be used but may lead to hyperventilation and the anaesthesia is generally considered to be of shorter duration. Inject i.p. 100 μ L of this solution for a mouse weighing 20 g.

Infection of mice

- For the dermis, the infection is generally done in the ear, but another part of the body such as the abdomen can be used:
 - By natural bite, once the animal has been anaesthetised, hold it gently over the cage containing the positive mosquitoes until one comes to feed. Allow the mosquito to bite for 1 minute (if left longer, move the mouse regularly). Note the time the bite started and the location, for imaging afterwards. The mouse can be observed immediately in order to follow the sporozoites or at later time points for parasite development.
 - By intradermal injection, after anaesthetising the animal, tape the ear (as flat as possible), internal face down, onto the double-sided tape and inject i.d., on the external face of the ear, 1 μ L of the sporozoite suspension, in 3 to 5 different places. The animal is observed immediately or after different times after injection depending on what developmental stage of the parasite is to be observed.
- For the liver, the infection is different depending on whether an early time-point or a later time-point will be observed
 - For early time-points, once the animal has been placed on the microscope platform, the sporozoites are injected in the retro-orbital sinus, at the same time as the fluorescent BSA, and observed immediately.
 - For later time-points, the sporozoites are injected i.v. in the mouse tail vein. The mouse is observed from 45 hours after infection. The fluorescent-BSA is injected in the retro-orbital sinus just before imaging.

Exposure of the ear for imaging

- The animal is anaesthetised.
- A cover glass is Scotch-taped to the microscope platform.
- The mouse is placed on the platform so that the infected ear can be Scotch-taped to the cover glass. It is important that the ear is placed as flat as possible on the cover glass.

Exposure of the liver for imaging

- Anaesthetise the animal.
- Disinfect the abdomen with 70% ethanol. The animal is not pinned to a dissection board.
- Incise the skin 0.5 cm below the xiphoid process, and enlarge the opening to about 1 cm. Hairs that may have entered the incision must be removed with 1x PBS.
- Make a horizontal cut through the muscle layers. Do this carefully, with round-edged scissors, to avoid damaging the liver. This may lead to bleeding if capillaries are sectioned, the haemorrhage must be stopped immediately with the cautery and any blood removed, as this will perturb the observation.
- Gently push on the abdomen so that the lower tip of the left lobe appears. The incision must be big enough to allow the liver to extrude but small enough that it remains exposed and does not fall back into place. Place a drop of 1x PBS on the lobe so that it does not dehydrate during observation. Place several drops, about 5, of SuperGlue 3 on the skin around the exposed lobe and place the cover glass horizontally over the lobe. Push gently to stick the cover glass into position.
- Note: If it is difficult to extrude the liver lobe, but a sufficient amount is visible then the cover glass can be placed without emerging the lobe.

General procedures for imaging parasites in murine tissues

- Place the platform holding the animal on the microscope.
- Using the autofluorescence of the tissues (dermis of the ear or capsule of the liver), set an arbitrary $z=0$ at this focal point. For imaging the liver sinusoids, the retro-orbital injection should be done at this point.
- Then using the focus wheel, penetrate into the tissue until a zone with parasites is observed. Delimit the zone to be observed, and choose the z -step to be used (the smaller the z -step the higher the resolution but the slower the acquisition and the larger the files will be).
- The zone to be observed can be acquired in a single stack (per fluorochrome to be observed) over time. Depending on what will be observed, the space between stacks must be set up accordingly. For instance, sporozoites that are highly motile should be filmed continuously. In this case, this should be done for short periods (5-10 minutes) to avoid cell damage due to phototoxicity.

Analysis of acquired images

- Once the images have been acquired they can be analysed using the corresponding software or can be analysed using the open source software ImageJ. For the second option, it is best if images are in .tif format.
- Films can be made to visualise the parasite during the time-lapse; this allows the visualisation of sporozoite motility.

- 3D-reconstructions can also be processed to visualise the position of parasites in comparison with tissue structures. For instance, to determine whether a hepatic sporozoite localised in a liver sinusoid or in the hepatic parenchyma.

Different types of analysis possible

- Tracking of sporozoites within the different tissues.
- Localisation of sporozoites within different zones of the tissue.
- Analysis of interactions between sporozoites and host cells, for instance immune cells.
- Evaluation of merozoite volume and release of merozoites.

References

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IMMUNOCHEMISTRY

I. Studies of the *Plasmodium falciparum*-infected erythrocyte surface

I:A. Surface iodination of PRBC (Lactoperoxidase method)

by **Victor Fernandez**

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Materials and reagents

parasite culture at 5% hematocrit and >5% parasitemia

¹²⁵I-Na 1 mCi/10 µL (Amersham IMS.30)

PBS (pH 7.2) (stored at 4 °C)

lactoperoxidase, 2 mg/mL (Sigma L8257) (stored at -20 °C)

30% H₂O₂ (stored at 4 °C)

Labeling buffer:

100 mL PBS (pH 7.2)

0.087 g K₂HPO₄

0.1 mL 1 mM KI

washing buffer:

100 mL PBS (pH 7.2)

0.83 g KI

Procedure (On ice at all times)

- Use cells from a culture of 10 to 20% parasitemia (not lower than 5% at any case) with a majority of trophozoite stages.
- Wash the cells 3 times with PBS, then transfer 200 µL of the washed packed PRBC into a fresh centrifuge tube and add 800 µL of Labeling buffer.
- Add 0.5 to 1 mCi of ¹²⁵I-Na to the cell suspension.
- Add 100 µL of lactoperoxidase stock solution (2 mg/mL).
- Dilute the stock solution of H₂O₂ 1,000-fold in PBS just before use.
- Add 25 µL of the diluted H₂O₂ to the cells; mix gently.
- Wait 1 min and repeat the addition 3 times (4 times altogether).
- At 1 min after the last addition of diluted H₂O₂, wash the cells 4 times in ice-cold washing buffer.
- The cells are now ready for separation/enrichment of mature stages in Percoll/sorbitol gradients as described in PARASITES, section IV:D.

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

I:B. Solubilization/extraction of surface-radiolabelled proteins**by Victor Fernandez**

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Equipment

microfuge, refrigerated

Materials and reagents

Solution containing:

1% Triton X-100

1 mM PMSF

1 mM EDTA

1 μ M leupeptin2 μ M pepstatin1% ϵ -amino-*n*-caproic acid in PBS

SDS-PAGE sample buffer:

2% SDS

5% 2-mercaptoethanol

Procedure

- Use the pellet of labelled PRBC enriched for mature stages on Percoll/sorbitol gradient as described above (see PARASITES, section IV:D).
- Resuspend the pellet in 0.25 to 0.5 mL of a solution containing 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1 μ M leupeptin, 2 μ M pepstatin, and 1% ϵ -amino-*n*-caproic acid in PBS. Incubate the pellet on ice for 1 h with periodic vortexing.
- Centrifuge the samples in a microfuge at 4 °C, at maximum speed for 15 min. Separate and save the supernatant (the fraction soluble in Triton X-100).
- Resuspend/extract the pellet in SDS-PAGE sample buffer, mix well by pipetting, boil for 4 min, mix again, and centrifuge it for 3 min at maximum speed in a microfuge.
- Separate the supernatant (the fraction soluble in SDS).
- Run aliquots of Triton X-100 and SDS-soluble fractions on SDS-PAGE gels (6% homogeneous or 5 to 8.5% gradients for proteins above Band 3; i.e., M.W. ~100,000, 7.5 to 17.5% gradients for proteins below Band 3).

Reference

Fernandez V, Treutiger CJ, Nash GB, Wahlgren M. 1998. Multiple adhesive phenotypes linked to rosetting binding of erythrocytes in *Plasmodium falciparum* malaria. Infect Immun 66(6):2969-2975.

I:C. Alternative solubilization protocolby **Denise Mattei and Artur Scherf**

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e-mail: dmm@pasteur.fr, ascherf@pasteur.fr**Equipment**SDS-PAGE gel apparatus
microfuge**Materials and reagents**

2% SDS

PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors

PBS containing 1% Triton X-100, 1 mM PMSF and 1% ϵ -amino-*n*-caproic acid
protein A-Sepharose (Amersham Biosciences 17-0780-01 or Sigma)**Preparation**

- Solubilize ~200 μ L of erythrocytes (the pellet from one flask) in 4 to 8 mL of 2% SDS (dissolved in PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors).
- Incubate the sample at room temperature for 30 to 90 min. Any possible background radiation from the protein A-Sepharose can be adsorbed by mixing washed protein A-Sepharose with the solubilized sample and letting it incubate for 1 h prior to the immunoprecipitation.

Solubilization

- Extract surface-radioiodinated proteins by using 0.2 mL of pellet plus 1.8 mL of PBS containing 1% Triton X-100, 1 mM PMSF, and 1% ϵ -amino-*n*-caproic acid and incubating the tube at 4 °C on ice for 1 h.
- Centrifuge the extract in a microfuge at maximum speed for 15 min. Remove the Triton X-100 soluble supernatant, then freeze the supernatant at -70 °C.
- Extract the insoluble pellet in SDS-PAGE sample preparation buffer.
- Separate the proteins on a 7.5 to 17.5% linear gradient SDS-PAGE gel.

Reference

Mattei D, Scherf A. 1992. The Pf332 gene of *Plasmodium falciparum* codes for a giant protein that is translocated from the parasite to the membrane of infected erythrocytes. *Gene* 110(1):71-79.

I:D. Immunoprecipitation of ¹²⁵I-labelled PfEMP1

by **Victor Fernandez**

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Equipment

microcentrifuge
PhosphorImager

Materials and reagents

¹²⁵I-labelled SDS extract, preferably use 100,000 or more cpm per sample
¹²⁵I-labelled Triton X-100, if desired
2% Triton X-100 in PBS
hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4),
0.5% Triton X-100, 5 mM EDTA, and protease inhibitors
Protein A-sepharose (Amersham Biosciences 17-0780-01)

NETT buffer:

150 mM NaCl, MW 58.44 (8.77 g/liter)
5 mM EDTA, MW 380.2 (1.9 g/liter)
50 mM Tris-base, MW 121.1 (6.06 g/liter)
0.02% sodium azide
0.5% Triton X-100
Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT with final 0.3 M NaCl

NETT with 10 mg/mL Ig-free BSA containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 2 mM pepstatin, and 1% ε-amino-*n*-caproic acid in PBS)

NETT with 10 mg/mL BSA Fraction V

secondary antibodies as appropriate

protein A–Sepharose equilibrated with NETT with Ig-free BSA without protease inhibitors, make up as a 1:1 suspension (i.e., 50 μL of Sepharose gel : 50 μL of suspension)

5% SDS Sample Buffer (= 2× concentration)

ProcedureDay 1

- Reconstitute SDS extract by adding an equal volume of 2% Triton X-100 in PBS and 20 volumes of NETT plus Ig-free BSA containing protease inhibitors.
- Aliquot the reconstituted SDS extract in microfuge tubes allowing about 100,000 cpm per tube (or more if it is available).
- Add primary antibody:
For sera, use 10 μL/sample for monoclonal antibodies (you can go up to 20 μL with human or *Aotus* sera if desired), but rabbit sera generate a high MW aggregate which messes up this region on the gel. Use 2 to 5 μL of hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors.
- Incubate the sample overnight at 4 °C with slow rotation/mixing.

Day 2

- Add secondary antibodies if using primary antibodies not binding protein A. Leave the tubes for 1 h at room temperature.
- Add 100 μ L of the above equilibrated protein A–Sepharose.
- Leave it at room temperature for 1 h with intermittent mixing.
- Take off the supernatant and put in 125 I liquid waste. Wash the gel–slurry with the following solutions and quick, \sim 30-s spins in a microcentrifuge on the high setting:
 - 1 mL NETT–BSA
 - 1 mL NETT
 - 1 mL NETT with 0.3 M NaCl
- Leave the slurry for 20 min to allow NaCl to equilibrate into the beads. Then wash again:
 - 1 mL NETT with 0.3 M NaCl
 - 1 mL NETT
- Take off all supernatant.
- Add 50 μ L of 2 \times Sample Buffer to the pellet. Mix and freeze at -70 $^{\circ}$ C until use in SDS–PAGE gel electrophoresis.

Notes on the capacity of protein A–Sepharose

According to the manufacturer's specification, the capacity is 20 mg of human-IgG per milliliter of gel or 3 to 10 mg of mouse-IgG per milliliter of gel. Taking 10 mg/mL as above, then 50 μ L of gel used for immunoprecipitation has a maximum capacity of 500 μ g of IgG. Based on the principle that it is best to use less than 20% of capacity to get complete binding, one should aim to use maximally 100 μ g of IgG.

e.g., 10 μ L of rat serum = 100 μ g of IgG. Add 50 to 100 μ g of secondary antibody IgG.

Autoradiography for the measurement of γ rays

Stain, destain, and dry the SDS–PAGE gel. Expose a PhosphorImager screen (or an X-ray film using an intensifying screen) at room temperature.

Reference

van Schravendijk MR, Rock EP, Marsh K, Ito Y, Aikawa M, Neequaye J, Ofori-Adjei D, Rodriguez R, Patarroyo ME, Howard RJ. 1991. Characterization and localization of *Plasmodium falciparum* surface antigens on infected erythrocytes from West African patients. *Blood* 78(1):226-236.

I:E. Surface biotinylation of infected erythrocytesby **Julius Nyalwidhe, Stefan Baumeister, and Klaus Lingelbach**address: Philipps-Universität, FB Biologie, Zoologie/Parasitologie, Karl-von-Frisch-Str. 8
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Equipmentcentrifuge (10.000 × g), refrigerated
appropriate reaction tubes for centrifuge**Materials and reagents**

sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin; Pierce Chemicals). Prepare fresh each time.

inhibitors of “*New permeation pathways*”:

Furosemide (Sigma) or 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; Sigma). Prepare stock solution (10 mM in DMSO)

washing buffer (PBS 2+): PBS containing 0.6mM CaCl₂, 1mM MgCl₂, pH 7.6 and PIC (1:500)

biotinylation buffer: 1 mg/mL sulfo-NHS-LC-biotin in PBS 2+

blocking buffer: PBS 2+ containing 100 mM glycine

buffers contain:

protease Inhibitor Cocktail Set III (Calbiochem), dilution 1:500

Procedure

- prior to biotinylation wash 10⁸ infected erythrocytes three times in PBS 2+
- incubate cells in PBS 2+ containing 1 mg/mL sulfo-NHS-LC-biotin and 100 μM of furosemide or NPPB for 30 min on ice.
- sediment cells by centrifugation at 10,000 x g for 15 s at 4 °C. (analyze supernatant photometrically at 570 nm for the release of haemoglobin)
- to block and remove unbound biotin, wash cells three times in blocking buffer
- cells are now ready for further analyses.

Comments

Late schizonts tend to disintegrate during the process leading to lysis and internal biotinylation.

ReferencesNyalwidhe, J., Baumeister, S., Hibbs, A.R., Tawill, S., Papakrivov, J., Volker, U., and Lingelbach, K. (2002) A nonpermeant biotin derivative gains access to the parasitophorous vacuole in *Plasmodium falciparum* infected erythrocytes permeabilized with streptolysin O. J Biol Chem 277: 40005–40011.Baumeister, S., Endermann, T., Charpian, S., Nyalwidhe, J., Duranton, C., Huber, S., Lang, F., Kirk, K and Lingelbach, K. (2003) A biotin derivative blocks parasite induced novel permeation pathways in *Plasmodium falciparum*-infected erythrocytes. Mol Biochem Parasitol 132: 35–45.

II. Studies of the nuclear components of *Plasmodium falciparum*

II.A. Chromatin Immunoprecipitation (ChIP) Assay

by **Jose-Juan Lopez-Rubio, Ana-Paola Rojas-Meza, and Artur Scherf**

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Equipment

centrifuge 4 °C
microcentrifuge (4°C and room temperature)
Douncer homogenizer
light Microscope
Bioruptor UCD-200 (Diagenode)

Materials and reagents

malaria culture with 6-8 % parasitemia
PBS
saponin
3M sodium acetate pH 5.2
glycogen
protease inhibitors
absolute Ethanol
70% Ethanol
Proteinase K
phenol:Chloroform:isoamyl alcohol
SDS 10%
NaHCO₃ 1M
Salmon Sperm DNA/protein A agarose slurry 50%
formaldehyde 37%
1.25M glycine
Nonidet-P40 10%

Buffers

Lysis Buffer:
10 mM Hepes pH 7.9
10 mM KCl
0.1 mM EDTA pH 8.0
0.1 mM EGTA pH 8.0
1 mM DTT (add just before using)
The Lysis Buffer is supplemented with protease inhibitors

SDS Lysis Buffer
1% SDS
10 mM EDTA
50 mM Tris-HCl pH 8.1

ChIP Dilution Buffer
0.01% SDS
1.1% Triton X-100

1.2 mM EDTA
16.7 mM Tris-HCl pH 8.1
150 mM NaCl

Low Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8.1
150 mM NaCl

High Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8.1
500 mM NaCl

LiCl Immune Complex Wash Buffer
0.25 M LiCl
1% NP-40
1% Deoxycolate
1mM EDTA
10 mM Tris-HCl pH 8.1

TE Buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0

Elution Buffer
1% SDS
0.1 M NaHCO₃
To be prepared before using

SAMPLE
6 x 10⁹ rings (~8 immunoprecipitations)
2 x 10⁹ trophozoites (~8 immunoprecipitations)
1 x 10⁹ schizonts (~12 immunoprecipitations)
(~15 µg of chromatin per immunoprecipitation)

Procedure

Preparation of cross-linked chromatin

- Add 37% formaldehyde to the cultures to get a final concentration of 1%. Mix immediately and incubate at 37 °C with agitation for 5 min.
- Note: Cross-linking time influences the efficiency of chromatin shearing and the efficiency of precipitating a specific antigen. For histone modifications 5 min is sufficient. For transcription factors longer cross-linking times may be necessary.

- To stop crosslinking add the amount of 1.25 M Glycine needed to achieve a final concentration of 0.125 M. Place the flask in ice and agitate for 5 min.
- Wash sample 3 times with cold PBS (centrifugations should be carried out at 4°C) Note: The formaldehyde may cause some red blood cell lysis.
- Add saponine so that the final concentration is 0.06%. Incubate for 5-10 min (until complete RBC lysis). If RBC lysis is not complete, add more saponine until the final concentration is 0.15%.
- Spin the sample (4000 rpm 4 °C 10 min) and wash the pellet with cold PBS until the supernatant becomes clear.
- Parasite pellets can be stored at -80 °C.
- Prepare nuclei by resuspending the crosslinked parasites in Cold Lysis Buffer:
 - for $\sim 6 \times 10^9$ rings: 2 mL of Cold Lysis Buffer
 - for $\sim 2 \times 10^9$ trophozoites: 2 mL of Cold Lysis Buffer
 - for $\sim 1 \times 10^9$ schizonts: 3 mL of Cold Lysis Buffer.
- Transfer to a pre-chilled douncer homogenizer and set on ice for 30 min. Add 10% Nonidet-P40 to reach a final concentration of 0.25%. Lyse the parasite with:
 - 200 strokes for $\sim 6 \times 10^9$ rings/2 mL of Cold Lysis Buffer
 - 100 strokes for $\sim 2 \times 10^9$ trophozoites/2 mL of Cold Lysis Buffer
 - 100 strokes for $\sim 1 \times 10^9$ schizonts/3 mL of Cold Lysis Buffer
- Check for parasite lysis with the help of a light microscope.
- Centrifuge the lysate for 10 min at 14000 rpm 4 °C.
- Resuspend the pellet in:
 - 400 μ L of SDS Lysis Buffer for $\sim 6 \times 10^9$ rings
 - 400 μ L of SDS Lysis Buffer for $\sim 2 \times 10^9$ trophozoites
 - 600 μ L of SDS Lysis Buffer for $\sim 1 \times 10^9$ schizonts.
- Note: 1% SDS improves the efficiency of sonication (next step) but could negatively affect the recovery for some antibodies. Performing the sonication in a 0.1% SDS-containing buffer compromises shearing efficiency; therefore, sonication conditions should be carefully controlled. Another option is to use SDS Lysis Buffer containing 1% SDS, followed by dialysis against the same buffer with a lower SDS concentration.

Chromatin sonication

- A "pre-cooling" of the Bioruptor's tank with crushed ice 30 min before starting is recommended to avoid water heating too quickly.
- Bioruptor settings: Power: High, Cycling parameter: 30 sec ON 30 sec OFF.
- Sonicate for 8 min (200 μ L is the maximum volume per 1.5 mL tube).
- Replace the water with cold water and crushed ice.
- Sonicate for 8 min.
- Remove debris by centrifuging for 10 min at 12500 g 4°C 10 min.
- Dilute supernatant fraction 10 fold in ChIP Dilution Buffer. Add protease inhibitors. Keep a portion of this chromatin solution (20 μ L) as DNA input and another portion (80 μ L) to check chromatin shearing. These two portions will be processed with the other immunoprecipitations during DNA purification.

Chromatin immunoprecipitation

- To reduce non-specific background, pre-clear the chromatin solution with 50 $\mu\text{L}/\text{mL}$ of salmon sperm DNA/protein A agarose slurry for 2 hours at 4 $^{\circ}\text{C}$ with agitation. Pellet agarose by brief centrifugation and transfer the supernatant to a new tube.
- Make aliquots of the chromatin solution (500 $\mu\text{L}/\text{aliquot}$).
- Add the antibody (the concentration of the antibody should be empirically determined) and incubate overnight at 4 $^{\circ}\text{C}$ with rotation.
- To collect immune complexes, incubate the sample with 50 μL per sample of Salmon sperm DNA/protein A agarose slurry for 2 hours at 4 $^{\circ}\text{C}$ with agitation.
- Pellet beads by gentle centrifugation (1000 g x 1 min). Carefully remove the supernatant that contains unbound chromatin.
- Wash the protein A agarose/antibody/chromatin complex for 5 min on a rotating platform with 1 mL of each of the buffers listed below. Discard the wash buffer between steps:
 - Low Salt Immune Complex Wash Buffer (at 4 $^{\circ}\text{C}$)
 - High Salt Immune Complex Wash Buffer (at 4 $^{\circ}\text{C}$)
 - LiCl Immune Complex Wash Buffer (at 4 $^{\circ}\text{C}$)
 - TE buffer, two washes at room temperature
- Elute immune complexes by adding 100 μL of fresh Elution Buffer. Vortex briefly to mix, and incubate at room temperature for 15 min with rotation. Spin down beads, carefully transfer the supernatant fraction (eluate) to another tube and repeat elution with 150 μL of fresh Elution Buffer. Combine eluates.

DNA purification

- To reverse cross-linking, incubate the immunoprecipitations and the input at 65 $^{\circ}\text{C}$ for 6 hours.
- Proteinase K treatment. Add 2.5 μL of 20 mg/mL Proteinase K and incubate for 2 hours at 45 $^{\circ}\text{C}$.
- Phenol/chloroform/isoamyl alcohol extraction.
- Ethanol precipitation.
- Resuspend the pellet in 300 μL of distilled water.
- Quantify immunoprecipitated DNA by dot blot or semiquantitative or quantitative PCR. In this last case, use 5 μL of sample in a 20 μL PCR reaction.

- II.B. Immunolocalization of Nuclear Antigens in *Plasmodium falciparum*
by **Liliana Mancio-Silva, Lucio Freitas-Junior and Artur Scherf**
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Equipment

Waterbath at 37 °C
Microscope

Materials and reagents

Saponin (Sigma)
RPMI 1640 (Gibco)
Microscope slides (Cell-Line) and Coverslips
10% Paraformaldehyde (Electron Microscopy Sciences)
Bovine serum albumin (BSA) (Sigma)
VECTASHIELD mounting medium with DAPI (Vector Laboratories)
Nail polish

Procedure

Parasite fixation

- Treat the parasites (~2,5% parasitemia) with Saponin (20min for ring stages and 5min for mature stages), then centrifuge 4000rpm for 5min
- Wash twice in RPMI (centrifuge 6000rpm 1min)
- Resuspend the parasites in Paraformaldehyde 4% (in PBS) for 10-15min, on ice
- Wash once with cold PBS (centrifuge 6000rpm 1min)
- Resuspend in 100µL cold PBS (fixed parasites can be stored for at least a week at 4°C prior to IF analysis)

Antibodies:

- Dilute the primary antibody in 100µL PBS-BSA 1%
- Centrifuge the fixed parasites, resuspend the pellet in the diluted primary antibody and incubate for 30-45 min at 37°C
- Wash twice in PBS (centrifuge 6000rpm)
- Resuspend the pellet in the diluted secondary antibody conjugated with fluorochrome (in PBS-BSA 1%) and incubate for 30 min at 37°C (protected from light)
- Wash twice in PBS (centrifuge 6000rpm)
- Resuspend the pellet in PBS and deposit on microscope slides
- Let air dry the slide and mount using Vectashield with DAPI
- Analyze the slide by fluorescence microscopy
- If z-stack analysis will be performed the coverslips must be previously coated with poly-lysine or poly-ethylenimine 1%

Comments

All the steps are performed in suspension, so the nuclear architecture is better preserved.

The saponin lysis does not allow analysis of erythrocyte-surface antigens, but produces preparations with very reduce background.

II.C. Extraction and purification of *P.falciparum* Histones

by **Neha Issar** and **Artur Scherf**

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Equipment

microcentrifuge (4°C)
vortex

Materials and reagents

malaria culture of 6-8% parasitemia, enriched for mature stages (preferably tophozoites, 30-40 hours post invasion).
PBS 1x (Ice cold)
saponin
hemoglobin Removal Buffer
 25 mM Tris-HCl (pH 7.8)
 1 mM EDTA
 0.2% (v/v Nonidet P40)
Distilled H₂O
0.8 M NaCl
0.25 M HCl
20% TCA (Tri-chloro acetic acid, stored at 4 °C)
acetone (stored at -20°C)
SDS PAGE Sample Buffer

Procedure

- Pellet 2-3 mL of healthy malaria culture with at least 6-8% parasitemia and lyse with saponin
- Resuspend pellet with 1.5 vol. PBS and add saponin to obtain a final concentration of 0.06%.
- Mix gently and incubate on ice for 5-10 min.
- Add 5 vol. of PBS and centrifuge at 3000 rpm for 5 min.
- Discard the supernatant and transfer the pellet to a 1.5 mL microcentrifuge tube
- Wash the resulting pellet at least 3 times (6000g for 2 min each) with ice cold PBS till the supernatant is clear of visible haemoglobin.
- Resuspend the resulting pellet in 2 vol. of haemoglobin removal buffer to further remove contaminating membranes and haemoglobin.
- Centrifuge briefly at 12000g for 30 s.
- Wash the resulting pellet twice (till the supernatant is clear) in the same buffer 12000g for 30 s.
- Wash 1 time with ice cold distilled H₂O (12000g for 30 s).
- Wash the pellet 2 times in 0.8 M NaCl. (Care should be taken while removing the supernatant subsequent to each wash as the pellet is viscous and gooey and may quickly expand into the supernatant or sucked up with it. It may be advisable to draw up minimal amount of supernatant and the gooey pellet and transfer to a new eppendorf tube after each wash to avoid sucking up and disintegrating the pellet each time).

- Acid extract the resulting pellet by adding 8 vol of 0.25 M HCl followed by vigorous vortexing and subsequent incubation at 4 °C for 2 hours to overnight.
- Recover the acid soluble proteins in the supernatant by centrifuging at 12000g for 30 minutes. The pellet contains acid insoluble contaminants.
- To the supernatant mix equal volume of 20% TCA, mix by inverting and incubate on ice for 15 minutes.
- Centrifuge at 12000g for 15 minutes. (At the end of the spin, immediately place the samples on ice. As the samples are removed from the microfuge, a miniscule separate phase may appear at the bottom of the tube. This may be clear or cloudy, depending on which care should be taken in the next step while removing the supernatant).
- Carefully remove the supernatant. (If the pellet is not visible, place the pipett tip in the centre of the tube but not touching the bottom or the sides of the eppendorf while removing the supernatant).
- Add 500 μ L of acetone to each sample, flick the tube gently and incubate at 20 °C for at least 1 hour. (After adding acetone to each sample, care should be taken to keep everything in the lower half of the tube as the protein precipitates as a thin chip or aggregate and may easily be lost somewhere on the lid of the tube).
- Centrifuge at 12000g for 15 minutes.
- Carefully remove the supernatant and set tubes on their sides at RT for 5-10 minutes to dry the pellet and evaporate the acetone.
- Dissolve the protein precipitate in SDS PAGE Sample buffer and analyse on 15% SDS Polyacrylamide gels. Individual histones should separate out in 5 distinct bands as in example coomassie stained extract in Fig. 1.

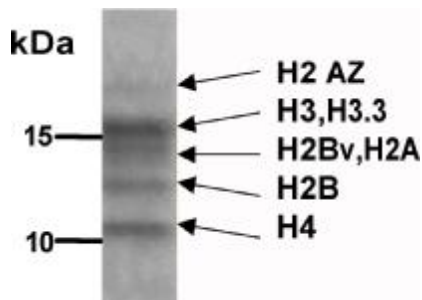


Fig. 1

II. Metabolic Labeling of *Plasmodium falciparum*

II:A. Metabolic Labeling of *Plasmodium falciparum*-infected erythrocytes
by **Victor Fernandez**
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Materials and reagents

RPMI custom medium, containing everything including serum, but without methionine
healthy parasites at ring-stage
0.5 mCi of ³⁵S-methionine (Amersham SJ1015)
RPMI 1640

Procedure

- Use ring-form parasites to label, then let them develop into late stages (20 to 30 h) for analysis.
- Use RPMI custom medium, containing everything including serum, but without methionine.
- Use as high a parasitemia as possible (i.e., healthy parasites at ring-stage).
- Use 0.5 mCi of ³⁵S-methionine per small culture flask (25 cm²).
- Remove the old medium, as usual, leaving a few drops of the old medium in the flask.
- Mix 0.5 mCi of ³⁵S-methionine with 5 mL of medium and add to the flask.
- Incubate the flask as usual at 37 °C for approximately 30 h, until late parasite stages develop.
- Wash the parasites 3 times with RPMI 1640.
- Freeze the parasite pellet at –20 °C.

Comments on autoradiography for measuring β rays

- Stain and destain the gel. If it will be exposed on an X-ray film, use Amplify (Amersham) after the last destaining bath. Use enough Amplify for the gel to float freely and let it soak for 15 to 20 min. This step is not needed if a PhosphorImager screen will be used.
- Dry the gel. Expose the X-ray film to the gel at –70 °C using an intensifying screen, or simply use a PhosphorImager screen at room temperature.

Reference

Helmby H, Cavalier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

II:B. Immunoprecipitation using metabolically labelled parasite extracts*by Victor Fernandez**Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden**e-mail: victor.fernandez@smi.ki.se*Equipment

immufuge

Materials and reagents

NETT-buffer 1 (NETT-1):

150 mM NaCl, MW 58.44 (8.77 g/L)

5 mM EDTA, MW 380.2 (1.9 g/L)

50 mM Tris-base, MW 121.1 (6.06 g/L)

0.02% sodium azide

0.5% Triton X-100

Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT-buffer 2 (NETT-2):

same as above except use 500 mM NaCl (29.22 g/liter).

Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

10% Triton X-100

1% BSA

50% protein A–Sepharose (washed with NETT-buffer 1)

SDS–PAGE sample buffer:

100 mM Tris-HCL, pH 6.8

10% Glycerol

5% SDS

2% 2-mercaptoethanol

bromophenol blue

0.25% Coomassie blue R-250

Amplify (Amersham)

Procedure

- Mix in a conical centrifuge tube:
 - 300 μ L NETT-1
 - 100 μ L 10% Triton X-100
 - 25 to 100 μ L antibodies
 - 100 μ L solubilized sample
 - 1% BSA
- Incubate the mix for 90 min at room temperature or 4 °C, slowly rotating/mixing.
- Add 75 μ L of 50% protein A–Sepharose. Incubate the mix for 30 to 60 min at room temperature. Shake the tube a little now and then.
- Wash the Sepharose mixture and centrifuge it in a blood centrifuge (Immufuge) for 15 s on low setting:
 - twice with NETT-1
 - once with NETT-2
 - twice with NETT-1
- Add 50 μ L of sample prep. buffer and boil the solution for 2 min. Run the sample on SDS–PAGE.

- Stain and destain the gel, use Amplify for no more than 20 min (see IMMUNOCHEMISTRY, section II:A above), then dry the gel at 60 °C.
- Put the dried gel on film, without intensifying screen, in a cassette at –70 °C for at least two weeks or simply use a PhosphorImager screen at room temperature.

Reference

Helmbj H, Cavelier L, Pettersson U, Wahlgren M. 1993. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 61(1):284-288.

III. Resolution of giant proteins (200 kDa to 1 MDa) of *Plasmodium falciparum* on polyacrylamide–agarose composite gels

by **Denise Mattei** and **Artur Scherf**

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Equipment

SDS–PAGE gel apparatus

Materials and reagents

polyacrylamide–agarose composite gel:

375 mM Tris–HCl (pH 8.8)

0.5% agarose

3% acrylamide (29:1, acrylamide:bisacrylamide)

0.1% SDS

ammonium persulfate

N,N,N',N'-tetramethyl-ethylenediamine (TEMED)

sample buffer:

10% SDS

250 mM Tris–HCl (pH 8.8)

20% glycerol

0.1% bromphenol blue

running buffer:

25 mM Tris–HCl (pH 8.3)

250 mM glycine

0.1% SDS

transfer buffer:

48 mM Tris

39 mM glycine

0.0375% SDS

3% 2-mercaptoethanol (optional)

Preparation

- Prepare the polyacrylamide–agarose composite gel. Dissolve the agarose by boiling it in the Tris buffer, cool it to 50 °C, and then add the acrylamide and SDS.
- Initiate the polymerization by adding ammonium persulfate and TEMED to a final concentration of 0.1% and 1 µL/mL of gel, respectively. Cast the gel in a standard horizontal chamber without a stacking gel layer.
- Boil the protein extracts for 5 min in sample buffer and load them on the gel. The addition of 3% 2-mercaptoethanol to the sample buffer is optional. In the presence of 2-mercaptoethanol, samples are denatured by heating to 50 °C for 15 min.
- Size-fractionate the proteins at 15 V/cm in running buffer for 1 to 2 h.
- Transfer the size-fractionated proteins to a nylon membrane using a semidry transfer device at 3.5 mA/cm² in transfer buffer for 2 h.

Reference

Wiesner J, Mattei D, Scherf A, Lanzer M. 1998. Biology of giant proteins of *Plasmodium*: resolution on polyacrylamide–agarose composite gels. *Parasitol Today* 14(1):38-40.

IV. Recovery of *Plasmodium falciparum* native proteins and active enzymes

IV:A. Solubilization in the presence of non-detergent sulphobetaines, mild solubilization agents for protein purification

by **Catherine Braun-Breton**

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Equipment

centrifuge

Materials and reagents

RPMI 1640

10% Triton X-100 (stock solution)

mixed ion-exchange beads AG 501-X8 (Bio-Rad)

3 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka) dissolved in warm water and repurified on mixed ion-exchange beads; kept at $-20\text{ }^{\circ}\text{C}$

2% Triton X-100, 2 M NDSB 201 (extraction solution; kept at $-20\text{ }^{\circ}\text{C}$)

leupeptin and pepstatin (10 $\mu\text{g}/\text{mL}$) (optional) (stock solutions)

double-distilled water

sulphobetaine SB3-14 (optional)

Preparation

- Wash pelleted PRBC twice in RPMI 1640.
- Resuspend the pellet in 1 volume of double-distilled water.
- Freeze at $-20\text{ }^{\circ}\text{C}$ and thaw at room temperature.
- Add 2 volumes of:
 - 2% Triton X-100 (or detergent sulphobetaine SB3-14)
 - 2 M NDSB 201
 - leupeptin and pepstatin (10 $\mu\text{g}/\text{mL}$), if needed
 - no** Tris buffer (usually 10 mM [pH 7.5]) if followed by isoelectric focusing
- Incubate the pellet for 30 min at room temperature.
- Centrifuge the pellet for 30 min at $15,000 \times g$ at $4\text{ }^{\circ}\text{C}$; keep the supernatant (extract) frozen at $-20\text{ }^{\circ}\text{C}$.

Reference

Vuillard L, Braun-Breton C, Rabilloud T. 1995. Non-detergent sulphobetaines: a new class of mild solubilization agents for protein purification. *Biochem J* 305(1):337-343.

IV:B. Isoelectric focusing of proteins in a Rotofor cell: a first step in protein purificationby **Catherine Braun-Breton***Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724**Paris, France**e-mail: cbb@pasteur.fr*

The Rotofor cell is a preparative scale, free solution, isoelectric focusing apparatus useful in a protein purification scheme. The cell can achieve 25-fold purification of a protein in a single 4-h run. This technique is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins are in solution in their native conformation.

Equipment

Rotofor cell (Bio-Rad)

pH meter

Materials and reagents

0.1 M NaOH

0.1 M H₃PO₄

1% Triton X-100

1 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka)

2% ampholytes (Pharmalytes of the appropriate pH range, usually between pH 3 and pH 10, 40% stock solution, Pharmacia)

Preparation

- Equilibrate the ion exchange membranes by overnight incubation in the appropriate electrolyte solution: 0.1 M NaOH for the anion exchange membrane and 0.1 M H₃PO₄ for the cation exchange membrane.
- Cool the Rotofor cell by connecting the cooling finger to a source of circulating coolant.
- Assemble the focusing chamber according to the instruction manual.
- Fill the electrolyte chambers with electrolytes immediately after assembly to prevent the membranes from drying: 25 mL of 0.1 M NaOH for the cathode chamber (–) containing the anion exchange membrane and 25 mL of 0.1 M H₃PO₄ for the anode chamber (+) containing the cation exchange membrane.
- Fill the focusing chamber with 45 mL of focusing solution, using a 50-mL syringe with a needle:
 - 1% Triton X-100 (or SB3-14; not necessary for soluble proteins)
 - 1 M NDSB 201
 - 2% ampholytes
- Prerun the chamber for 1 h at 4 °C, 11 W, 34 mA, and 995 V to preform the pH gradient.
- Load 1 to 5 mL of the sample (protein solution or extract) in 1 M NDSB 201, 2% ampholytes.
- Add the sample in the central channels of the Rotofor focusing chamber to prevent exposure of the proteins to very low or high pH.
- Protein concentration should be adjusted for desired yield. Assuming each component will focus in 1 to 3 fractions; preparative fractionation of 1 to 2 g of proteins has been successfully performed!
- Run the sample for 3 h at 4 °C, 11 W, 34 mA, and 995 V.

- Collect the 20 fractions on ice.
- Measure the pH of each fraction. Take a 100- μ L aliquot, add 1 mL of double-distilled water, and keep it at room temperature for 10 min before measuring the pH.
- Store the fractions at $-20\text{ }^{\circ}\text{C}$.

Reference

Roggwiller E, Morales Bétouille MR, Blisnick T, Braun-Breton C. 1996. A role for erythrocyte band 3 degradation by the parasite gp76 serine protease in the formation of the parasitophorous vacuole during invasion of erythrocytes by *Plasmodium falciparum*. Mol Biochem Parasitol 82(1):13-24.

SEROLOGY

I. Antibody staining of *Plasmodium falciparum*-infected erythrocytes

I:A. Erythrocyte membrane immunofluorescence (EMIF)

by **Hedvig Perlmann**

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e-mail: hedvig.perlmann@imun.su.se

Equipment

centrifuge
humid chamber (wet filter paper in a plastic box)
microscope (UV and light)

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)

glutaraldehyde (GDA)

biotinylated anti-Ig of the appropriate specificity (Vector Labs)

FITC–Avidine D, F/P 4.9 (Vector Labs).

ethidium bromide (Sigma)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

Tris (Sigma)

Hanks' balanced salt solution (Gibco)

multitest slides (8 wells; ICN Biomedicals)

Pasteur pipettes

micropipettes

TH-buffer (0.15 M Tris-buffered Hanks', pH 7.2):

2.11 g Tris–HCl

0.2 g Tris-base

7.88 g of 0.9% NaCl

Dissolve the ingredients in distilled water and bring volume to 1 liter.

Mix 1 volume of Tris buffer with 1 volume of Hanks' BSS.

Add 0.02% NaN₃ (200 mg NaN₃/1 liter).

coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃

200 mg NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Monolayers

- Wash cultures (5 to 10% parasitemia) in TH and resuspend them to 1% hematocrit.
- Pretreat multitest slides, 8 wells with one drop (~20 µL) per well of coating buffer for 30 min.

- Add one drop per well of the 1% *P. falciparum* culture directly after aspiration of the coating buffer and leave the slides to settle for 30 min.
- Rinse off unbound erythrocytes by gently shaking the slides immersed upside down in TH.

Glutaraldehyde (GDA)-fixation of monolayers

- Quickly but gently cover the wet monolayers with 1 to 2 mL of 1% GDA (in PBS) on top of the slide.
- Decant the GDA after about 10 s and repeat the GDA fixation once.
- Wash the slides with distilled water and air-dry them. Slides can then be stored long-term in a freezer (>10 years).

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with one drop (~20 µL) of serial dilutions of sera containing malaria antibodies.
- Use 15 µg/mL of biotinylated anti-Ig of the appropriate specificity followed by 50 µg/mL of FITC–avidin D.
- Wash the slides between the incubations with TH or PBS.
- For counter-staining, use one drop of ethidium bromide solution (10 µg/mL) in each well and rinse the slides with distilled water after a few seconds.

Reference

Perlmann H, Berzins K, Wahlgren M, Carlsson J, Björkman A, Patarroyo ME, Perlmann P. 1984. Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum*. J Exp Med 159(6):1686-1704.

I:B. Erythrocyte membrane staining by enzyme linked antibodies (EMEAS)by **Rachanee Udomsangpetch***Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th*

Prepare glutaraldehyde-fixed monolayers as in the protocol for EMIF (see SEROLOGY, section I:A).

Equipment

light microscope with a 100× oil-immersion lens
centrifuge
humid chamber (wet filter paper in a plastic box or dish)

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)

multitest slides (8 wells; ICN Biomedicals)

Pasteur pipettes

micropipettes

biotinylated anti-Ig (Vector Labs)

streptavidin/ALP (Mabtech)

PBS

ALP-conjugated Substrate Kit (Bio-Rad 170-6432)

coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃,

200 mg NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Staining

All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with serial dilutions of immune sera.
- Use biotinylated anti-Ig of the appropriate specificity and streptavidin/ALP.
- Wash the slides between the incubations with PBS.
- Add freshly prepared substrate.
- Wash slides under distilled water after 20 min, then air-dry them.
- Examine the slides under a light microscope with a 100× oil-immersion lens.

Reference

Udomsangpetch R, Kulane A, Berzins K, Perlmann H, Perlmann P, Wåhlin Flyg B. 1993. Enzyme-linked staining method for light microscopic detection of antibodies to parasite antigens on the membrane of *Plasmodium falciparum* infected erythrocytes. *Acta Trop* 55(1-2):79-86.

I:C. Parasite immunofluorescence (PARIF)by **Rachanee Udomsangpetch***Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th*

This protocol allows for antibody staining of intracellular *Plasmodium falciparum* using air-dried monolayers of infected erythrocytes. For staining of the live, intact PRBC surface, please refer to PARASITES, section IX:B.

Equipment

fan

Materials and reagents

Plasmodium falciparum cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia of primarily late stages
PBS

coating buffer (pH 9.6):

1.59 g Na₂CO₃2.93 g NaHCO₃200 mg NaN₃

Add distilled water up to 1 liter.

Monolayers

- Use *Plasmodium falciparum* cultures in human type O⁺ erythrocytes, 5 to 10% parasitemia of primarily late stages.
- Wash cells in PBS and resuspend them to 1% parasitemia.
- Pretreat multitest slides with one drop (10 to 20 µL) per well of a coating buffer for 30 min.
- Aspirate the wells and immediately add one drop of the washed culture to each well.
- Leave it to settle for 30 min.
- Gently aspirate the excess of red cells from each well and air-dry the slides under a fan.
- The slides can be stored in a freezer; dry them well before staining.
- For staining, see the protocol for EMIF in SEROLOGY, section I:A above.

Reference

Udomsangpetch R, Carlsson J, Wåhlin B, Holmquist G, Ozaki LS, Scherf A, Mattei D, Mercereau-Puijalon O, Uni S, Aikawa M, Berzins K, Perlmann P. 1989. Reactivity of the human monoclonal antibody 33G2 with repeated sequences of three distinct *Plasmodium falciparum* antigens. J Immunol 142(10):3620-3626.

I:D. Formaldehyde fixation for immunofluorescence analysis (IFA) of *P. falciparum*

by **Michael J. Blackman**

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See PARASITES, section IX:C, page 73

II. Antibody selection on immobilized antigen or peptide

by *Denise Mattei*

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Equipment

PD10 column equilibrated in PBS

Materials and reagents

0.45- μ m nitrocellulose membrane

PBS

PBS plus 5% powdered skim milk

3 M MgCl₂, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol

Preparation

- Prepare 5 mL of a 5 to 10 μ g/mL solution of peptide or protein in PBS.
- Soak a 0.45- μ m nitrocellulose-membrane filter in this solution for 30 min at room temperature with gentle agitation, and then soak a second filter in this solution.
- Incubate the filters for saturation in PBS plus 5% powdered skim milk for 90 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Incubate each filter in 5 mL of a 1:20 dilution of appropriate serum in PBS plus 5% powdered skim milk for 60 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Wash the filters in PBS plus 5% powdered skim milk for 30 min at room temperature with gentle agitation.
- Wash the filters twice in PBS.
- Soak the filters in 5 mL of a solution of 3 M MgCl₂, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol.
- Incubate the filters for 20 min at room temperature with gentle agitation.
- Recover the solution and load it on a PD10 column equilibrated in PBS for collection of 500- μ L fractions.
- Check for the presence of IgG in the fractions by dot blot analysis (5 μ L of each fraction).

Reference

Tsang VCW, Wilkins PP. 1991. Optimum dissociating condition for immunoaffinity and preferential isolation of antibodies with high specific activity. *J Immunol Methods* 138(2): 291-299.

III. ELISA

III:A. Equipment, materials, and reagents

by **Hedvig Perlmann**

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Equipment

micropipette, multichannel pipette (e.g., Thermo Electron)
microplate washer (e.g., Thermo Electron, Skatron Instruments)
microplate reader (e.g., V max kinetic microplate reader with computer program
SOFTmax Cat. No. 79-200 105, 79-200 100)
centrifuge, swing out rotor

Materials and reagents

With many proteins or peptides, PBS can be used as a coating solution at least with the above plates; test your system first.

flat-bottomed microtiter plates (Maxisorp from Nunc A/S or High Binding from
Costar, Cat. No. 3590)
disposable pipette tips
tubes for preparations of dilutions (Bio-Rad, Cat. No. 223-9391)
cell culture flasks
RPMI-HEPES (see PARASITES, section I:A)
Hanks' balanced salt solution (Gibco)

PBS 10× stock solution:

40 g Na₂HPO₄ · 12 H₂O

5 g KH₂PO₄

81 g NaCl

1.0 mL 20% NaN₃

Dissolve in distilled water and bring volume to 1 liter.

Coating buffer (pH 9.6):

1.59 g Na₂CO₃

2.93 g NaHCO₃

1.0 mL 20% NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Blocking buffer:

0.5% BSA in coating buffer

Tween buffer for dilutions and incubations:

100 mL PBS stock (10×, see above)

5 g BSA

0.5 mL Tween

1.0 mL 20% NaN₃

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Tween wash:

45 g NaCl

2.5 mL Tween

Dissolve the ingredients in distilled water and bring the volume to 5 liters.

Enzyme substrate buffer:

97 mL diethanolamine

~700 mL distilled water

1 mL 20% NaN₃Adjust the pH to 9.8 with 1 M HCl (~100 mL), then add 101 mg of MgCl₂·H₂O. Bring buffer volume to 1 liter.

Substrate:

NPP (kept in freezer)

Dissolve 1 tablet of NPP in 5 mL of enzyme substrate buffer; keep the solution in the dark. Incubate plates with the substrate at room temperature and keep them in the dark until you are ready for a reading at OD₄₀₅ (usually in 20 to 40 min).

TH (0.15 M Tris-buffered Hanks', pH 7.2):

2.11 g Tris-HCl

0.2 g Tris-base

7.88 g NaCl

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Add 1.0 mL of 20% NaN₃. Mix 1 volume of Tris buffer with 1 volume of Hanks' balanced salt solution.

Sera:

For antibody determinations, sera may be kept in the refrigerator diluted 1:10 in Tris-buffered Hanks' with 0.02% NaN₃ for many months.

Conjugate:

ALP-conjugated or biotinylated anti-Ig of appropriate specificity at recommended concentration

Streptavidine-ALP (Mabtech) when biotinylated antibody has been used.

60% Percoll solution:

60 mL Percoll

7 mL 10× PBS

33 mL PBS

0.3 M NH₄OH

III:B. Antigens for coating

by **Hedvig Perlmann**

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Crude parasite antigen

Late stage-infected erythrocytes enriched by gradient centrifugation. For 40 tubes, 80-mL of culture:

- Prepare fresh 60% Percoll solutions before each use. Keep all solutions on ice throughout the preparation.
- Wash a late stage *Plasmodium falciparum* culture once in RPMI-HEPES and resuspend it to 10% hematocrit in cold RPMI-HEPES.
- Distribute in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge the tubes in a swing-out rotor at $1,500 \times g$ and 4°C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw the cells at the interphases with care, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Freeze-thaw or sonicate the isolated interphases, determine protein after centrifugation, and coat the plate at $10\ \mu\text{g}/\text{mL}$ in coating buffer.
- Contaminating HGG in this antigen is avoided by using Albumax to replace serum in the medium for the parasite cultures (see PARASITES, section I:A).

Comment: 12 medium size cultivation flasks ($75\ \text{cm}^2$, 20 mL of culture) with late stages of 10% parasitemia give about 1.5 mg of protein in the extract.

Air-dried monolayers of human erythrocytes (of the same type as used for parasite cultures)

- Pretreat the ELISA plate with 50 μL of coating buffer for 30 min.
- Add 50 μL of freshly washed human erythrocytes (in TH or PBS) at 1% hematocrit.
- After 30 min gently and carefully rinse off any unbound erythrocytes and leave the plates to air-dry.
- Proceed as usual (see SEROLOGY, section III:C, ELISA procedure).

Peptide/BSA conjugation

- Dissolve 1 mg of peptide in 0.1 mL of distilled water.
- Add 0.3 M NH_4OH , if necessary, until the peptide is dissolved, then add PBS to a total volume of 0.5 mL.
- Dissolve 0.5 mg of BSA in the peptide solution.
- Add 0.5 mL of 0.25% glutaraldehyde in PBS dropwise while stirring.
- Mix the solution gently on a roller drum in the cold, overnight.
- Dialyse against PBS, then store at -20°C . We use $10\ \mu\text{g}/\text{mL}$ for coating.

Comment: Several malaria peptides are commercially available from Bachem. Bigger peptides as MAPs can be used for coating without BSA conjugation.

III:C. ELISA procedureby **Hedvig Perlmann**

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e-mail: hedvig.perlmann@imun.su.seProcedure

- Coat the plate with 50 μ L of peptide solution or crude parasite antigen at 10 μ g/mL in coating buffer.
- Keep the plate at 4 °C overnight. With many proteins or peptides, PBS can be used as a coating solution. Test your system first.
- Block with 100 μ L of 0.5% BSA in coating buffer for 3 to 4 h at 37 °C.
- Wash 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μ L of serum samples diluted 1:1000; leave them for 1 h at 37 °C.
- Wash them 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 μ L of ALP-conjugated or biotinylated anti-Ig of appropriate specificity at the recommended concentration in Tween-buffer; leave for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- If biotinylated antibody has been used, add 50 μ L of streptavidin–ALP diluted 1:2000 in Tween-buffer; leave the sample for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- Develop the sample with 50 μ L of NPP (1 tablet/5 mL of substrate buffer) (see SEROLOGY, section III:A) under Materials and reagents and read at OD₄₀₅.

References

Paulie S, Perlmann P, Perlmann H. 2003. Enzyme-linked immunosorbent assay. *In*: Celis JE, ed. Cell biology: a laboratory handbook. 3rd edition. San Diego: Elsevier Science.

Perlmann H, Perlmann P, Berzins K, Wåhlin B, Troye-Blomberg M, Hagstedt M, Andersson I, Högh B, Petersen E, Björkman A. 1989. Dissection of the human antibody response to the malaria antigen Pf155/RESA into epitope specific components. *Immunol Rev* 112:115-132.

Rougon G, Ceard B, Van Rietschoten J, Jordan B, Barbet J. 1984. Induction with a synthetic peptide of antibodies to HLA class I C-terminal intracytoplasmic region. *Mol Immunol* 21(6):461-468.

Wahlgren M, Berzins K, Perlmann P, Björkman A. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria. I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. *Clin Exp Immunol* 54(1): 127-134.

CELLULAR IMMUNOLOGY

I. Preparation of human peripheral blood mononuclear cells (PBMC)

by *Marita Troye-Blomberg* and *Jacob Minang*

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Equipment

centrifuge (swing out rotor)
Burker chamber
light microscope

Materials and reagents

heparinized tubes (CPT sodium heparin blood collection tubes, BD Vacutainer Systems)

Pasteur pipettes

conical centrifuge tubes

Ficoll-Paque (Pharmacia)

RPMI 1640 with 20 mM HEPES (Gibco)

L-glutamine (Gibco)

penicillin–streptomycin combination (PEST) (Gibco)

fetal bovine serum (FBS) (Gibco)

tissue culture medium (TCM):

RPMI 1640–HEPES

2 mM L-glutamine

penicillin–streptomycin combination (final concentration of 100 units/mL of penicillin G sodium and 100 µg/mL of streptomycin sulfate)

10% heat-inactivated FBS

Turk gentian violet staining solution:

40 mg gentian violet

12.5 mL acetic acid

Make up to 200 mL with distilled water.

Procedure

- Collect venous blood into heparinized 10-mL tubes.
- Save and freeze a small fraction of the plasma in Eppendorf tubes (1 to 2 mL for each tube) for antibody determinations.
- Mix blood in a 1:1 ratio with RPMI 1640 containing 20 mM HEPES solution and carefully layer 7 mL of the resultant suspension on top of 3 mL of Ficoll-Paque in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 2,800 rpm for 20 min. Collect the interphase with a Pasteur pipette.
- Pool and dilute (\approx 10 times) the cells in RPMI 1640 containing 20 mM HEPES.
- Wash the cells 2 times, first at 1,800 rpm for 10 min and then at 1,200 rpm for 7 min and resuspend the cells in each 10-mL tube in 3 to 5 mL of TCM depending on the density/size of the pellet.

- Pipette 10 μ L of the resultant cell suspension in TCM into 40 μ L of TURK for counting of the lymphocytes in a Burker-chamber.

Alternative preparation of whole blood cells for cultures

- Collect 10 mL of venous blood as above.
- Pipette 10 μ L of cell suspension into 30 μ L of Turk for counting of the lymphocytes.
- For the cultures, dilute the blood 2, 5, or 10 times in TCM.

Reference

Smedman L, Joki A, da Silva APJ, Troye-Blomberg M, Aronsson B, Perlmann P. 1994. Immunosuppression after measles vaccination. *Acta Paediatr* 83(2):164-168.

II. Antigen preparations and peptides for cellular work

II:A. Crude parasite antigen (Percoll-band)

by **Marita Troye-Blomberg**

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For working with parasites and Percoll, please refer also to PARASITES and for crude antigen, please refer to SEROLOGY, section III, ELISA.

Equipment

centrifuge (swing out rotor)
sonicator
freezer at $-70\text{ }^{\circ}\text{C}$

Materials and reagents

RPMI 1640-HEPES
PBS
conical centrifuge tubes
Pasteur pipettes
0.45- μm filters (Millipore)

60% Percoll:

60 mL Percoll
7 mL 10 \times PBS
33 mL 1 \times PBS

Procedure

- Prepare parasite extracts from late stage cultures. Wash them once in RPMI 1640-HEPES and suspend them to 10% hematocrit in cold RPMI 1640-HEPES.
- Layer 2 mL of extract gently on top of 2.5 mL of cold 60% Percoll in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at $1,500 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$. Collect the interphase with a Pasteur pipette, pool and wash the cells 3 times with cold PBS ($1,500 \times g$ for 7 to 8 min), and resuspend them in a small volume of PBS. The interphase contains trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Sonicate (25w) the cells with short intervals for 2 min, determine protein content, and dilute to about 2 mg/mL.
- Centrifuge the sample at $1,500 \times g$ for 10 min, filter-sterilize it, make a final protein determination, then store it in aliquots of 1 mg/mL in a $-70\text{ }^{\circ}\text{C}$ freezer.

Reference

Troye-Blomberg M, Perlmann H, Patarroyo ME, Perlmann P. 1983. Regulation of the immune response in *Plasmodium falciparum* malaria II. Antigen specific proliferative responses in vitro. Clin Exp Immunol 53(2):345-353.

II:B. Desalting of synthetic peptidesby **Marita Troye-Blomberg**

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Equipment

speed-vac

Materials and reagentsSep-Pak C₁₈ cartridge (Waters)

5-mL syringe

distilled water

methanol

Procedure

- Use a Sep-Pak C₁₈ cartridge connected with a 5-mL syringe to desalt peptides. Prewet the cartridge 3 times with 1.5 mL of methanol and wash it 2 times with 5 mL of water before use.
- Dissolve 10 mg of peptide in 10 mL of distilled water (or 10% methanol in distilled water) and pass the solution 3 times through the cartridge.
- Wash out the salts 2 times with 5 mL of distilled water, elute the peptide 2 times with 1.5 mL of 60% methanol and 2 times with 1.5 mL of 100% methanol.
- Pool the eluates, evaporate them in a speed-vac, weigh them, dissolve them in distilled water at 1 mg/mL, freeze them, and store them frozen.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson A, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM, Perlmann P. 1989. T and B cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. *J Immunol* 143(9):3043-3048.

III. T-cell proliferation

by **Marita Troye-Blomberg**

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Equipment

incubator (37 °C, 5% CO₂ in a humid atmosphere)
scintillation counter
cell harvester (TomTec Cell Harvester)

Materials and reagents

round-bottomed 96-well microplates (Costar 3799, Corning)
(6-³H)-thymidine (specific activity 2 Ci/mmol; Amersham)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
peripheral blood mononuclear cells (PBMC)

Procedure

- Dilute PBMC in TCM to 1 × 10⁶ cells/mL and seed 1 × 10⁵ cells/well in 96-well round-bottomed microplates in triplicates per test.
- Add antigens (parasite extracts or recombinant proteins) or peptides at 2 to 10 µg/mL representing epitopes of interest at the initiation of the culture.
- Incubate the plates for 5 days at 37 °C in an atmosphere of 5% CO₂.
- Harvest 100 µL for cytokine determination at the time of pulsing, and then pulse with 1 µCi [6-³H]-thymidine in 100 µL of TCM for 18 h.
- Harvest the cells and measure the thymidine incorporation by liquid scintillation.
- Results are expressed as stimulation index (SI), defined as mean cpm of test triplicates divided by mean of cpm of control triplicates. An SI ≥ 2.5 is considered positive.

Reference

Troye-Blomberg M, Riley EM, Perlmann H, Andersson G, Larsson Å, Snow RW, Allen SJ, Houghten RA, Olerup O, Greenwood BM, Perlmann P. 1989. T- and B-cell responses of *Plasmodium falciparum* malaria-immune individuals to synthetic peptides corresponding to sequences in different regions of the *P. falciparum* antigen Pf155/RESA. J Immunol 143(9):3043-3048.

IV. Interleukin ELISPOT

by **Gehad El Ghazali**

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The following description should be considered as a guideline on how to stimulate and analyse cells for IFN- γ production by the ELISPOT technique. Conditions for cell preparation, stimulation, and the ELISPOT may be changed to fit with the test situation.

Equipment

incubator (37 °C, 5% CO₂ in a humid atmosphere)
multi-channel pipette
dissection microscope (40 \times)

Materials and reagents

nitrocellulose plates (Millipore Corp.)
mAb anti-human IFN- γ , 7-B6-1-Biotin (Mabtech)
biotinylated mAb anti-human IFN- γ , 7-B6-1-Biotin (Mabtech)
streptavidin–alkaline phosphatase (Mabtech)
BCIP/NBT substrate (Bio-Rad Laboratories)
TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
5-mL round-bottomed tissue culture tubes (Falcon 2058, BD Labware)
PHA (phytohemagglutinin)
PBS (filter-sterilized phosphate buffer saline) (pH 7.2)
FCS (fetal calf serum)

Procedure

Stimulation of cells can be carried out directly in the ELISPOT plate, but better results may be obtained if cells are first stimulated for a shorter period in culture tubes. In the following description cells are incubated for 2 to 4 h in tubes and thereafter transferred to ELISPOT plates for further incubation.

- Prepare sterile PBMCs.
- Count cells and suspend them in TCM to a concentration of 2×10^6 cells/mL.
- Transfer 0.5 to 1 mL of cell suspension to 5-mL round-bottomed tissue culture tubes.
- Add antigens (parasite extracts or recombinant proteins) or peptides representing epitopes of interest at a concentration of 2 μ g/mL at the initiation of the culture. As a positive control, add phytohemagglutinin (PHA) at a concentration of 5 μ g/mL.
- Incubate cells at 37 °C for 2 to 4 h.

Coating of ELISPOT plates

- Dilute the coating antibody (mAb anti-human IFN- γ , 7-B6-1-Biotin) to 15 μ g/mL in filter-sterilized phosphate buffer saline (PBS).
- Add 100 μ L/well to the nitrocellulose plates and incubate them overnight at 4 °C.

Transfer of cells to the nitrocellulose plate (sterile)

Before transferring stimulated cells to the coated ELISPOT plates, wash them 4 times with sterile PBS (200 μ L) to remove unbound coating antibody.

- Add stimulated cells to the plates. For IFN- γ , a cell concentration of 20,000 to 50,000 cells/well is usually suitable. If the number of cells exceeds 250,000 cells/well, this may result in blurry spots due to multiple cell layers (especially in the wells with cells stimulated with PHA).
- Incubate cells for 10 to 40 h at 37 °C in a humid atmosphere.

Addition of the secondary antibody (nonsterile)

- Remove cells by washing them 6 times (200 μ L/well) using PBS (multi-channel pipette).
- Dilute biotinylated mAb (anti-human IFN- γ 7-B6-1-Biotin) to 1 μ g/mL in PBS with 0.5% fetal calf serum (PBS-0.5% FCS) and add 100 μ L/well.
- Incubate the plates for 2 to 4 h at room temperature.
- Wash them in PBS 6 times (200 μ L/well).
- Add 100 μ L of streptavidin–alkaline phosphatase (diluted 1:1000 in filtered PBS-0.5% FCS) and incubate the plates for 1 to 2 h at room temperature.
- After washing them in PBS 6 times (200 μ L/well), add 100 μ L of substrate and incubate them until spots emerge (may be up to 1 h).
- Stop color development by washing the plates under running tap water (3 times at 200 μ L/well).
- Leave the plate to dry; inspect and count the spots with a dissection microscope (40 \times).

One-Step ELISpot kits:

Very sensitive and easy to use kits are available at Mabtech AB.

Reference

Elghazali G, Esposito F, Troye-Blomberg M. 1995. Comparison of the number of IL-4 and IFN- γ secreting cells in response to the malaria vaccine candidate antigen Pf155/RESA in two groups of naturally primed individuals living in a malaria endemic area in Burkina Faso. *Scand J Immunol* 42(1):39-45.

V. Reverse transcription PCR for detection of cytokines

by **Ankie Söderlund**

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Equipment

- 70 °C freezer
- incubator (37 °C, 5% CO₂ in a humid atmosphere)
- RT-PCR kit with recombinant *Ampli Taq* DNA polymerase (Applied Biosystems)
- PCR using cytokine specific primers

Materials and reagents

- Ficoll-Paque (Amersham Biosciences)
- round-bottomed tubes
- cryotubes
- liquid nitrogen
- PBS
- acid guanidinium thiocyanate–phenol–chloroform
- DEPC-treated distilled water

Procedure

- Isolate PBMC from venous blood by Ficoll-Paque centrifugation as described in CELLULAR IMMUNOLOGY, section I.
- To detect any spontaneous cytokine production in the controls, wash unstimulated PBMC 4 times in PBS. Before the last wash, transfer cells to a cryotube, centrifuge them at 300 × *g*, and carefully remove all remaining PBS. Immediately freeze the cells in liquid nitrogen.
- To detect cytokines after antigen stimulation, place cells in round-bottomed tubes, one (or two) for each time point, and add the desired antigen. Place the tubes at 37 °C, 5% CO₂, for 4, 7, and 22 h.
- Wash the cells 4 times in PBS as described above, remove the supernatant, and freeze the cells in liquid nitrogen.
- For total RNA extraction, either use commercial kits available or the acid guanidinium thiocyanate–phenol–chloroform method by Chomczynski and Sacchi, 1987 (see also MOLECULAR BIOLOGY, section II).
- Dilute the RNA in 50 µL of DEPC-treated distilled water and thereafter keep it stored at -70 °C.
- Use an RT-PCR kit with recombinant *Ampli Taq* DNA polymerase for reverse transcription and subsequent PCR using cytokine specific primers.

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Söderlund A, Gabrielsson S, Paulie S, Hammarström ML, Rak S, Troye-Blomberg M. 1997. Allergen induced cytokine profiles in type I allergic individuals before and after immunotherapy. *Immunol Lett* 57(1-3):177-181.

VI. Detection of cytokine-gene polymorphism

VI:A. Extraction of human DNA from peripheral blood or buffy coat by **Ben Gyan**

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Equipment

incubator
centrifuge
gel electrophoresis apparatus
speed-vac
gel drier or oven
spectrophotometer
camera
automated thermal cycler
microscope with UV light

Materials and reagents

lysing buffer:
1.6 M sucrose
50 mM Tris-HCl (pH 7.5)
25 mM MgCl₂
5% Triton X-100

DNA buffer:
10 mL 1 M Tris-HCl (pH 7.5)
2.5 mL 0.5 M NaCl
2.5 mL 0.5 M EDTA
1.25 mL 20% SDS
Adjust volume to 50 mL using double distilled H₂O.

sterile vacutainers with EDTA (ethylamine tetra-acetic acid)
Falcon tubes, 50-mL
proteinase K solution (Invitrogen 25530 049)
20% SDS
sterile double-distilled H₂O
Pasteur pipettes
Eppendorf tubes
phenol
chloroform
3 M sodium acetate
absolute ethanol
70% ethanol

Procedure

- Draw 1 to 8 mL of venous blood into sterile vacutainers containing EDTA and keep it at room temperature (or frozen).
- Transfer blood into a 50-mL Falcon tube and add 10 to 40 mL of lysing buffer.
- Turn the tube gently upside down for 2 to 3 min.

- Centrifuge the tube at 2,000 rpm for 10 min (with brake off) and discard the supernatant.
- Add 40 μL of proteinase K solution, 40 μL of 20% SDS, 180 μL of DNA buffer, and 340 μL of sterile double-distilled H_2O .
- Mix the sample well by gently pipetting it up and down until it becomes gelatine-like.
- Digest the blood by incubating it overnight at 37 to 42 $^{\circ}\text{C}$.
- Transfer the sample into Eppendorf tube and add 600 μL of phenol.
- Extract the DNA by revolving/mixing the tubes until the solution becomes milky.
- Centrifuge the tube at 13,000 rpm for 10 min at room temperature.
- Transfer the upper aqueous phase (DNA) into a new Eppendorf tube.
- Add 800 μL of chloroform.
- Extract the DNA and centrifuge the tube at 6,000 rpm for 10 min.
- Transfer the upper phase into a new Eppendorf tube.
- Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at -20°C . The DNA can be stored this way as well.
- Centrifuge the precipitate for 30 min at 4 $^{\circ}\text{C}$, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 μL of distilled water, depending on its size.
- Determine the DNA concentration at OD_{260} ; 2 mL of culture may yield $\sim 2 \mu\text{g}$ of genomic DNA. Visualize $\sim 0.5 \mu\text{g}$ on a 0.8% agarose gel to see that the DNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

VI:B. PCR for amplification of nucleotide repeat (VNTR) polymorphisms of cytokine genes

by **Ben Gyan**

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Equipment

automated thermal cycler
UV-transilluminator
camera

Materials and reagents

dNTPs
primers
Taq polymerase
PCR buffer with MgCl₂
sterile H₂O
Taq gold (Applied Biosystems)
agarose gel
0.1% ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

TE buffer (pH 8.0):
10 mM Tris Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure

- Prepare the mix at the following final concentration (total volume 25 μ L):
 - 100-250 ng DNA
 - 200 nM dNTPs
 - 0.1 μ M of each primer
 - 1.5 U *Taq* polymerase
 - PCR buffer (with MgCl₂), 8% (v/v)
 - sterile H₂O, 46.4% (v/v)
- Program the automated thermal cycler according to manufacturers' instructions. Use the following guidelines to set annealing temperature and extension time according to primer and product considerations.
- Hot start at 95 °C for 10 min when using *Taq* Gold.
- Denature the DNA for 1 min at 94 °C. If the GC content is \leq 50%, anneal it at 55 °C; if it's >50%, anneal at 65 °C. Extend for 5 min at 72 °C. Run the program for 30 cycles.
- Prepare 2% agarose gel in TE buffer. Run the gel at 80 to 100 V and stain it with 0.1% ethidium bromide. Visualize the gel under UV light. Take a photograph.

VI:C. PCR for amplification of single nucleotide polymorphisms or RFLP of cytokine genes

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Procedure

- Use the same procedure as for VNTR polymorphisms, CELLULAR IMMUNOLOGY, section VI:B. The PCR product will give the same band for all samples. To detect polymorphisms, further digest the PCR product with digestive enzymes according to the manufacturers' instructions.
- Run the digested PCR product on 3% agarose gel (use NuSieve GTG) electrophoresis at 80 V and stain the gel with 0.1% ethidium bromide. Visualize the gell under UV light and take a photograph

VI:D. Amplification Refractory Mutation System (ARMS) PCR for detection of previously characterised mutations in cytokine genes

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Equipment

automated thermal cycler
agarose gel electrophoresis
UV-transilluminator

Materials and reagents

Tris-HCl (pH 8.8)

MgCl₂

Taq polymerase

dNTP

Tween 20 (ABgene)

primers

ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

PCR master mix:

25 mM Tris-HCl (pH 8.8)

1.5 to 4.0 mM MgCl₂

0.25 *Taq* polymerase

200 μM dNTP

0.01% (v/v) Tween 20 (ABgene)

Primer mix:

specific primer mix (10 μM of generic primer and 10 μM of specific primer)

5 μM of positive internal control (10 μM of internal control forward and 10 μM of reverse primers).

Procedure

- Put 100 to 250 ng of each DNA into two tubes or wells.
- Add other PCR reagents as indicated in the master mix above.
- Program the automated thermal cycler according to manufacturers' instructions.
- Use guidelines in CELLULAR IMMUNOLOGY, section VI:B for VNTR polymorphisms to set annealing temperature and extension time according to primer and product considerations. Run the program for 30 cycles.
- Analyze the PCR products by agarose gel electrophoresis followed by ethidium bromide staining and visualisation under UV light.
Amplification should occur in only one tube if the DNA template contains wild-type sequence or mutation sequence. Heterozygous individuals show amplification in both tubes. A positive control sequence is required to measure that absence of product is not due to PCR failure.

Reference

Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. 1999. ARMS-PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta 1 gene polymorphisms. *Transpl Immunol* 7(2):127-128.

VII. Haptoglobin phenotyping using PAGE

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Equipment

gel electrophoresis apparatus
gel drier or oven

Materials and reagents

polyacrylamide gel
Tris-HCl
Tris-base
glycine
distilled water
10% ammonium persulphate
TEMED (tetramethyl-ethylenediamine)
haptoglobin
erythrocyte haemolysate
benzidine stain
filter paper

Buffers:

1.5 M Tris-HCl (pH 8.8)
54.5 g Tris-base
150 mL distilled H₂O

0.5 M Tris-HCl (pH 6.8)
6 g Tris-base
60 mL distilled H₂O

10× running buffer
15.5 g Tris-base
72.1 g glycine
500 mL distilled H₂O

Procedures and preparations

- Cast a polyacrylamide gel with separation gel of concentration 4.7% and stacking gel of 2.5%.

	<u>separating gel (4.7%)</u>	<u>stacking gel (2.5%)</u>
30% bis acrylamide	7 mL	1.3 mL
Tris-HCl	11.25 mL	2.5 mL
distilled water	26.7 mL	6.0 mL
10% ammonium persulphate	150 µL	50 µL
TEMED	37.5 µL	18 µL

- Add 3 µL of erythrocyte haemolysate to 10 µL of serum or plasma and control haptoglobin.

- Mix the solution well and incubate it for 5 min to enable haptoglobin in serum to bind to hemoglobin.
- Add 10 μ L of loading buffer. Load the sample onto the gel using a template.
- Run electrophoresis at a constant voltage of 80 to 120V using 1 \times running buffer.
- Stain the gel for 5 to 15 min with benzidine stain.
- Wash the gel in distilled water.
- Transfer the gel onto filter paper and dry it using a gel dryer or oven.

References

Davis I, Orstein J. 1968. Disc electrophoresis, acrylamide gel columns: *In*: Williams, CA, Chase MW, eds. Methods in Immunology and Immunochemistry. Vol. 2. New York: Academic Press; pp. 34-47.

Coligan JE, et al., eds. 2003. Current protocols in immunology. Vol. 2. Electrophoretic separation of proteins. Chapter 8, section III. New York: Wiley-Interscience.

MOLECULAR BIOLOGY

I. DNA isolation from *Plasmodium falciparum*

I.A: Small-scale genomic DNA isolation from *Plasmodium falciparum* by **Martha Schlichtherle** and **Mats Wahlgren**

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Equipment

centrifuge (4 °C)
speed-vac
agarose electrophoresis unit
spectrophotometer

Materials and reagents

infected erythrocytes (a few mL with about 10% parasitemia)
phosphate-buffered saline (PBS) (pH 7.2)
5% saponin solution
lysis buffer:
40 mM Tris-HCl (pH 8.0)
80 mM EDTA (pH 8.0)
2% SDS, 0.1 mg/mL
proteinase K (Add the proteinase K just before using the buffer.)
phenol equilibrated with 0.1 M Tris-HCl (pH 7.0)
chloroform
RNase (Stratagene, RNase-It Ribonuclease Cocktail)
3 M sodium acetate (pH 5.0)
absolute ethanol
70% ethanol
TE-buffer:
10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure

- Centrifuge the infected erythrocytes at $3,000 \times g$ for 2 min. Wash the cells once in cold PBS.
- Resuspend the cells from one microfuge tube (1.7 mL) in 1 mL of PBS.
- Add and gently mix 10 μ L of 5% saponin (for a final concentration of 0.05%).
- Immediately centrifuge the tube at $6,000 \times g$ for 5 min after lysis is observed.
- Remove the supernatant.
- Add 25 μ L of lysis buffer and 75 μ L of distilled water to the pellet.
- Incubate the tube at 37 °C for ~3 h with intermittent stirring by hand.
- Add 100 μ L of distilled water, then 200 μ L of phenol. Mix well and centrifuge at $2,000 \times g$ for 8 min.
- Extract likewise with 200 μ L of chloroform.
- Add 2 μ L of RNase-It cocktail for 30 min at 37 °C.
- Extract with phenol and chloroform as above.

- Precipitate the gDNA by adding one-tenth volume of sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at $-20\text{ }^{\circ}\text{C}$. The DNA can be stored this way as well.
- Centrifuge the precipitate at $2,000 \times g$ for 30 min at $4\text{ }^{\circ}\text{C}$, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 μL of distilled water, depending on its size.
- Determine the DNA concentration at OD_{260} ; 2 mL of culture may yield $\sim 2\text{ }\mu\text{g}$ of gDNA. Visualize $\sim 0.5\text{ }\mu\text{g}$ on a 0.8% agarose gel to see that the gDNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.

I.B: Preparation of *P.falciparum* genomic DNA.

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see: Transfection, IV.A, page 289

II. RNA extraction from *Plasmodium falciparum*

II:A. RNA extraction from *Plasmodium falciparum* using guanidine thiocyanate and acidic phenol

by **Ulf Ribacke**

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Equipment

centrifuge (4 °C)
JA-20 Beckman rotor
small-blade Polytron homogenizer (Brinkmann)

Materials and reagents (see also Solutions below)

PRBC (Check PRBC to determine the parasitemia and rosetting rate. Usually eight 75-cm² bottles with a parasitemia of 7 to 10% will yield ~200 to 300 µg of RNA.)
cold sterile PBS (pH 7.2)
complete solution D (with 72 µL 2-mercaptoethanol (2-ME)/10 mL of Solution D or 288 µL of 2-ME/40 mL of Solution D). Remove 1 mL into a microcentrifuge tube; store the rest at 4 °C for Day 2. Solution D with 2-ME added is stable for 2 weeks at 4 °C.)
2 M sterile sodium acetate (pH 4)
acidic phenol:chloroform:isoamylalcohol (125:24:1) (Ambion 9722)
ice cold 2-propanol
distilled water
50-mL Falcon tubes (BD Labware)
tubes (see under MOLECULAR BIOLOGY, sections II:C)

Solutions

Solution D for RNA isolation:

100 g guanidine thiocyanate
117.2 mL sterile distilled water
7 mL 0.75 M sodium citrate (pH 7.0) (autoclaved)
10.6 mL 10% N-lauroyl-sarcosine (Sigma L9150; filtered; store stock at room temperature)
Check the pH, which should be ~7.0.
The solution can be stored for 3 months at room temperature.

Working solution:

720 µL 2-mercaptoethanol/100 mL of Solution D; keeps for 2 weeks if stored at 4 °C.

2 M sodium acetate, 50 mL:

Add 8.2 g of anhydrous sodium acetate to about half the final volume of distilled water; as the salt is dissolving, adjust the pH to 4.0 with ~16 mL of concentrated acetic acid; bring to volume and autoclave.

Tris–EDTA buffer (TE) (see also Sambrook et al. 1989):

10 mL 1 M Tris–HCl
2 mL 0.5 M EDTA (pH 8.0)
988 mL water

Combine Tris and EDTA in water; adjust pH to 7.4; autoclave. Store the solution at 4 °C; it is stable for 6 months.

10× electrophoresis buffer (0.2 M MOPS and 10 mM EDTA):

40 mL 1 M MOPS (pH 7.4) (autoclaved; 209.3 g/mol)
4 mL 0.5 M EDTA (pH 8.0) (autoclaved)
156 mL distilled water (autoclaved)

RNA loading buffer, deionized formamide:

25 g mixed-bead ion exchange resin (Bio-Rad, 142-6424)
250 mL of formamide (Sigma F7503)

Wrap an Erlenmeyer flask in foil. Add resin beads and formamide. Stir the mixture for 60 min in the hood. Filter through Whatman No. 1 filter paper. Store aliquots in dark bottles at –20 °C (stable for 1 year).

RNA loading buffer:

3.37 mL deionized formamide
1.08 mL formaldehyde (37%)
0.5 mL 10× electrophoresis buffer
20 µL 0.5 M EDTA (pH 8.0)
285 µL glycerol (sterile)
50 µL 10% SDS
50 µL bromphenol blue, 0.5% (or 5 mg)
50 µL xylene cyanole, 0.5% (or 5 mg)
Aliquot, then store at –20 °C.

Agarose gel (small 40 mL):

0.4 g agarose (Sigma A9539)
29 mL distilled water

Add agarose to sterile water, heat to boiling to dissolve the agarose, then cool to 65 °C.

Add:

4 mL 10× electrophoresis buffer
50 µL 0.5 M EDTA

In the hood, quickly add 7 mL of formaldehyde (or less if using Sigma's buffers). Pour the gel solution into a clean, taped gel tray containing a comb. Remove bubbles and allow the gel to cool.

20× SSC stock (5 L):

876.5 g sodium chloride
441.0 g sodium citrate

Add salts to distilled water and mix to dissolve. Add distilled water to total of 5 liters, adjust the pH to 7.0 with HCl, and autoclave.

Prehybridization/hybridization fluid:

0.5 M Na₂HPO₄ (pH 7)
1 mM EDTA
7% SDS

Probe removal solution

0.1% SDS

Place a nylon filter in the boiling solution and remove from heat. After 15 min remove and check the filter. Either repeat the treatment or simply expose the filter to check that the probe is really removed. Keep the filter moist at all times by placing it in a sealed hybridization bag. It may be stored at -20 °C.

Isolation of RNA, Day 1

- Cool the centrifuge for 50-mL Falcon tubes.
- Pool cells (PRBC) into two 50-mL tubes on ice.
- Centrifuge the cells at 1,800 rpm for 5 min at 4 °C.
- Remove the supernatant. Wash the cells with ~30 mL of cold PBS. Gently resuspend the cells. Centrifuge them again.
- Remove the supernatant. Resuspend the cells in cold PBS to ~20 mL/tube and transfer into centrifuge tubes that have lids (i.e., not Corex tubes) and that withstand 15,000 × g and organic solvents. Centrifuge them as above.
- Remove the supernatant and set the cells on ice.
- Rinse and clean a small-blade Polytron homogenizer by running distilled water in it, followed by some solution D.
- Add 10 mL of solution D per centrifuge tube and suck up and down to disrupt cells.
- Homogenize the cells on “medium” setting for 15 s. Avoid frothing. Keep the cells on ice.
- Clean the homogenizer by running solution D in it, then running distilled water, with changes of water until no trace of homogenized material appears on the blades.
- To each tube add:
 - 1.0 mL 2 M sodium acetate (pH 4) (× 0.1 volume)
 - 10 mL acidic phenol (× 1 volume)
 - 2.0 mL chloroform:isoamyl alcohol (× 0.2 volume)
- Cap the tubes with lids and vortex well for ~1 min. Set them on ice for 15 to 30 min. Meanwhile cool the centrifuge and rotor (JA-20 Beckman rotor) to 4 °C.
- Invert and vortex to mix the samples, then immediately centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- Remove and save the supernatant in two new centrifuge tubes (Nalgene 3110). Use lids. Avoid material from the interphase.
- Add an equal volume of 2-propanol (at -20 °C), mix, and set the sample at -20 °C overnight or at least for a few hours.

Further purification of RNA, Day 2

- Cool the JA-20 Beckman rotor. Mark the side of each tube where the “invisible” RNA pellet is expected to be. Centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- In a ventilated hood, carefully remove the supernatant with a 10-mL pipette, being careful to go down the opposite side of the tube from where the pellet is expected. There should be an almost transparent, gel-like pellet. Mark the tube where the pellet is.
- Dry the tubes some in the hood, but do not over-dry them.
- Resuspend both tubes’ RNA contents in a total of 0.5 mL of Solution D.
- Transfer the contents into a microcentrifuge tube. Precipitate it with an equal amount of 2-propanol overnight at –20 °C (or at least for a few hours).

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:B. Measurement of RNA yield and purity, and visualization on agarose–formaldehyde gel

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Equipment

microfuge

Materials and reagents

premixed RNA electrophoresis buffer (Sigma M5755)

RNA gel loading buffer (Sigma R4268)

10× electrophoresis buffer:

23.4 mL distilled water

600 µL 0.5 M EDTA (pH 8)

6 mL 1 M MOPS (pH 7.4)

This makes 30 mL total, approximately the amount needed for a regular small gel system.

formaldehyde

agarose

1 M EDTA (pH 8.0)

distilled water (or DEPC-treated water)

3 M sodium acetate (pH 5.2)

ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

bromphenol dye

Procedure

- Extract and purify the RNA as instructed in MOLECULAR BIOLOGY, section II:A, *Days 1 and 2.*

Day 3:

- Microfuge the RNA for 30 min at 12,000 to 14,000 rpm and 4 °C.
- Meanwhile prepare buffers and gel. Use EITHER:
 - premixed RNA electrophoresis buffer (Sigma M5755) and RNA gel loading buffer (Sigma R4268). The gel should then contain a lower amount of formaldehyde of 2.4%.
 - OR:
 - 10× electrophoresis buffer
- Have formaldehyde ready in the hood.
- Prepare a 1% agarose gel:
 - 0.4 g agarose
 - 29 mL of distilled waterMix, heat, and cook briefly. Cool gel liquid some. Then add:
 - 4 mL 10× electrophoresis buffer
 - 25 µL 1 M EDTA (pH 8.0)

- Bring the gel liquid to the hood, add 7 mL of formaldehyde, mix, then quickly pour the gel. Use a comb which is set aside for RNA-work so that no RNases from fingers are present on it.
- Allow the gel to solidify in the hood, but do not over-dry.
- Continue with the RNA sample. Wash precipitated RNA 2 times with 900 μ L of 70% ethanol at $-20\text{ }^{\circ}\text{C}$. Allow it to dry some in the hood.
- Set a small water bath to 60 to 65 $^{\circ}\text{C}$.
- Dissolve the RNA in $\sim 100\text{ }\mu\text{L}$ of autoclaved distilled water (or DEPC-treated water) depending on pellet size. If the RNA does not dissolve well with pipetting, heat it briefly at 65 $^{\circ}\text{C}$. Pipette to dissolve, then keep the RNA on ice.
- Use $\sim 1:100$ dilution of RNA for spectrophotometric measurement, preferably in a microcuvette to minimize the amount of RNA which is lost to absorbance measurements.
- Use the program for fixed wavelength at 260 and 280 nm.
- Take absorbance readings after blanking with distilled water.
- Calculate:
 - RNA concentration of stock: $(\text{Abs}) (\text{dil.}) (40\text{ }\mu\text{g}/\text{mL})/1000 = \text{RNA }\mu\text{g}/\mu\text{L}$
 - total yield: $(\text{above } \#) (\mu\text{L stock RNA}) = \mu\text{g RNA}$
 - purity: $\text{Abs}_{260}/\text{Abs}_{280} = \text{should be } \sim 1.8 \text{ to } 2.0$ if not too much protein contamination is present.
- Take out 5 μg or less of RNA to run in a lane on the gel.
- The RNA left to precipitate is: total yield – spec. reading – gel loading.
- Precipitate the RNA left by adding one-tenth volume of 3 M sodium acetate (pH 5.2), mix, then add 3 times volume of $-20\text{ }^{\circ}\text{C}$ absolute ethanol. Store RNA at $-70\text{ }^{\circ}\text{C}$.
- If RNA volume taken out for gel is $>10\text{ }\mu\text{L}$, speed-vac it down to dryness. Otherwise add RNA loading buffer to 19 μL (or less if using Sigma's premade solution, see manufacturer's suggestions). Mix and heat for 5 to 10 min at 60 to 65 $^{\circ}\text{C}$. Set on ice
- Add 1 μL of ethidium bromide (at $\sim 250\text{ ng}/\text{mL}$); mix. Pour $1\times$ electrophoresis running buffer over the gel and load.
- Run the gel at $\sim 50\text{ V}$ for more than 1 h with the bromphenol dye running about half way. Use commercial RNA marker(s) if needed (i.e., Gibco). For best results the gel should be run at 4 $^{\circ}\text{C}$. View and photograph the gel under UV-light. Two RNA bands should be quite visible corresponding to rRNAs. A third low rRNA band may at times also be visible; it is usually fuzzy. After a distilled-water rinse, the gel may be transferred to a nylon filter and be Northern probed using manufacturer's protocols.

References

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162(1):156-159.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

II:C. Reliable RNA preparation for *Plasmodium falciparum*

by **Sue Kyes**

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Equipment

benchtop centrifuge, refrigerated
 microfuge, ambient or refrigerated
 water baths/heating blocks (37 °C and 60 °C)
 spectrophotometer/cuvettes for reading absorbance at 260 nm and 280 nm
 (optional)

Materials and reagents

parasite-infected red blood cells (iRBC):

0.5 mL packed cell volume at 10% parasitemia, expected yields:

rings	50–150 µg total RNA
trophozoites, schizonts, gametocytes	250–750 µg total RNA

gloves

TRIzol Reagent: (Invitrogen) 15596-026 (Follow manufacturer's suggestions for safety and storage.)

chloroform (AnalAR from BDH, or Sigma C5312, or similar)

2-propanol (AnalAR from BDH, or similar)

formamide (molecular biology grade, Sigma F9037, or similar. It is not necessary to deionize before use. Open bottle in chemical fume hood.)

RNAse-free, chloroform/phenol-resistant plastics

screw-top test tubes*

1.5-mL microfuge tubes*

pipette tips*

**Straight from manufacturer is usually ok; autoclave if not sterile, taking care to wear gloves in handling.*

optional:

RNAse-free water (Sigma W4502 or Milli-Q purified or similar)

RNAse-free 3M sodium acetate solution (pH 5.2) (Sigma S7899 or similar)

Either buy RNAse-free water and sodium acetate solution or treat distilled water and sodium acetate solution with 0.1% diethyl pyrocarbonate (DEPC; Sigma, D5758) as described in Sambrook et al. 1989; p. 7.3; this requires autoclaving.

RNAse-free 75% ethanol/25% RNAse-free water

RNAse-free absolute ethanol (AnalAR from BDH, or Sigma E7023, or similar)

Protocol

Key RNA advice: Wear gloves for everything, and use RNAse-free plastics.

Preserve RNA in TRIzol

- Spin cells gently at 1,800 rpm (600 × g) for 4 min at room temperature. (example: 0.5 mL of packed infected RBC, about 10% parasitemia)^{1,2}
- Remove supernatant (s/n)³. Tap the tube to loosen the cell pellet⁴.

- Add TRIzol, prewarmed to 37 °C⁵.
For RINGS: add 10 pellet volumes of TRIzol.
(*example: 5 mL of TRIzol to 0.5 mL cells*)
For all other stages: add 20 pellet volumes of TRIzol.
(*example: 10 mL of TRIzol to 0.5 mL cells*)
- Shake the sample to dissolve any clumps.
- Incubate the sample at 37 °C for 5 min.
- Either store the sample at -70 to -80 °C⁶ or continue.

Continue extraction and precipitate RNA

- (Thaw samples at 37 °C, if necessary.)
- Add 0.2 TRIzol volumes of chloroform.
(*example: sample in 5 mL TRIzol, add 1 mL of chloroform*)
(*example: sample in 10 mL TRIzol, add 2 mL of chloroform*)
- Vigorously shake the sample; let it stand at room temperature for 2 to 3 min.
- Spin the tube at 4 °C for 30 min (microfuge = maximum 9,000 × g; or for 15-mL tubes in benchtop centrifuge, 1,000 to 1,400 × g is fine)⁷.
- Remove the aqueous (top, clear, RNA-containing) layer to a new tube⁸. Expect to recover up to 0.6 TRIzol volumes, but avoid the interface!
(*example: from sample in 5 mL of TRIzol, expect 3 mL of aqueous layer*)
- Add 0.5 TRIzol volumes of 2-propanol to the RNA; invert the tube several times to mix.
(*example: to 3 mL of aqueous layer, add 2.5 mL 2-propanol*)
- Transfer/split large samples to 1.5-mL snap-cap microfuge tubes.
- Precipitate the samples for AT LEAST 2 h (up to several days) at 4 °C.

EXAMPLE

Beginning with
0.5 mL of iRBC,

to the rings (*trophs*)

↓

add TRIzol
5 mL (*10 mL*)

↓

and chloroform
1 mL (*2 mL*).

Sample size is now:
3 mL (*6 mL*).

Add 2-propanol
2.5 mL (*5 mL*).

Finish preparation, check quantity/ quality of RNA

- Spin the sample at 12,000 × g (13,000 to 14,000 rpm, microfuge) for 30 min at 4 °C or room temperature⁹.
- Remove as much as possible of the supernatant¹⁰ (*Optional 75% ethanol wash¹¹).
- Air dry the sample, inverted, at room temperature, for no more than 5 min.
- Add formamide (usually a total of 100 µL for a starting pellet of 0.5 mL of infected RBC)¹².
- Heat the sample at 60 °C for 10 min; place it on ice.
- Resuspend the pellet by pipetting¹³.
- Check the absorbance at 260 nm¹⁴.
- Store the pellet at -70 to -80 °C.

Notes to the above procedure:

- 1: Especially for rings, cells pellet much more efficiently in 15-mL tubes than in 50-mL tubes. Subsequent steps are also easiest in 15-mL tubes, so harvest large volumes of cells directly in 15-mL tubes.
- 2: Use phenol/chloroform-resistant tubes.

- 3: After removing tubes, adjust centrifuge temperature down to 4 °C for the next spin.
- 4: Do not wash cells or lyse them with saponin. It is important to lyse the infected red blood cells directly in TRIzol as soon as possible after removing them from culture.
- 5: TRIzol seems to work best if it is prewarmed to 37 °C. Make sure lids are tightly sealed before shaking the tubes. Do the 37 °C incubation *before* freezing the samples to ensure complete complexing of nucleoproteins. The TRIzol can cope with extracting the RNA from hemoglobin, of which there is obviously plenty. TRIzol does not cope with separating RNA from DNA very well. Trophozoites/late stage parasites synthesize lots of DNA, and therefore require excess TRIzol.
- 6: Because the RNA is very stable at this point (if stored at –70 to –80 °C), collecting lots of samples is easy. Store them until you can process them all at once.
- 7: Do NOT spin at room temperature. If using a microfuge, use screw-cap tubes. Snap-cap tubes tend to leak and make a horrible mess of the inside of the centrifuge.
- 8: Avoid the interface to avoid DNA contamination. It is easier to recover more of the aqueous layer from 15-mL tubes than from 50-mL tubes. The DNA seems to collect right *above* the interface, so do not try to collect every last microliter of aqueous layer. The manufacturer's protocol for retrieving DNA from this does not seem to work due to massive amounts of hemoglobin. If anyone manages, please let me know!
- 9: Give tubes a quick vortex before spinning. Place tubes in the microfuge with hinges pointing outwards (for future identification of RNA pellet). Room temperature spin is fine, but 4 °C is ideal.
- 10: Remove supernatant in two stages:
 - a. Keeping pipette tip away from the putative pellet (directly beneath hinge, at bottom of tube), remove nearly all of the supernatant. Save the supernatant to a new tube if you are worried about losing your sample.
 - b. Give the tube a quick 'flick spin', again with hinges pointing outwards. This gets all those dribbles on the side of the tube down to the bottom again. Remove last bits of supernatant, as much as possible, taking care to avoid touching the very tiny, glassy pellet. If there is a large pellet, there is probably DNA or protein in it.
- 11: At this point, it is optional to add a 75% ethanol wash step: Add 0.5 mL of 75% ethanol: 25% DEPC–water, ice-cold; spin for ~5 min in a microfuge, and remove all supernatant. This gets rid of all traces of phenol and is particularly useful for cDNA preparations (see next note).
- 12: Add the formamide to the tube, avoiding touching the 'pellet'. Split the total formamide volume among the microfuge tubes if it was a large sample split to several tubes. RNA is very stable for a long time in formamide. However, the formamide affects downstream enzymatic applications. If you plan to make cDNA, it is worth considering resuspending the RNA pellet in DEPC–water, if you have it. If you have RNA samples in DEPC–water, store them in aliquots at –80 °C, but beware that the RNA is not very stable in water, and does not survive many freeze-thaw cycles. One way around this is to precipitate the RNA (add one-tenth volume of RNase-free 3M sodium acetate [pH 5.2], and 2.5 volumes of ethanol), then store at –80 °C. This is very stable, and will last 'forever'. When

- you need some RNA, just thaw the tube, vortex it, and remove the volume necessary to give the desired amount; spin it down in the microfuge for ~30 min.
- 13: By pipetting up and down, mix each sample thoroughly, making sure that nothing is stuck to the bottom of the tube. The pellet should have completely dissolved. Pool tubes of the same sample-type back together at this point. Set aside small volumes for measuring absorbance or running on gel.
- 14: Spectrophotometric measurement of quantity:
Check absorbance at 260 nm: Try 1 μ L in 500 μ L of water, remembering to blank against 1 μ L of formamide in 0.5 mL of water.
Conversion: OD 1 = 40 μ g RNA
Compare to OD 280. The ratio OD260/OD280 should be close to 2.0 for pure RNA. If there is any contaminating phenol, this number will be lower and the quantification will not be accurate.
If this of great concern, at the point marked *above, add the 75% ethanol wash step. It does not seem to make much difference, but gives some people peace of mind that the RNA is really clean.

Agarose gel measure of quality:

Using the procedure outlined for Northern blots below (see MOLECULAR BIOLOGY, section II:D), run a small gel, with small lanes. Run 1 to 2 μ L of each sample.

There should be two main ribosomal bands and a few minor bands of unknown provenance. Good RNA preparations have roughly equal amounts of both main ribosomal bands. If the RNA is degraded, there will be a 'comet tail' and no discrete bands. If there is plenty of DNA, there will also be a faint smudge close to the wells. Sometimes this does not matter, but sometimes it is really annoying. There are methods for getting rid of the DNA, but they are expensive and not fun.

Finally, if your sample had human white blood cells in it, you'll see more than two ribosomal bands!

References

Kyes S, Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. Mol Biochem Parasitol 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 7.3-7.4.

TRIzol is based on the following:

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162(1):156-159.

II:D. Simple Northernsby **Sue Kyes***Molecular Parasitology Group, Weatherall Institute of Molecular Medicine, Headington,
Oxford OX3 9DS, UK**e-mail: skyes@molbiol.ox.ac.uk***NO DEPC-treatment anywhere! Just wear gloves for everything.**Equipment

agarose gel tank, combs, and power pack
deep plastic trays for soaking gels (rinsed with 1× distilled water)
UV transilluminator and gel photography equipment
heating block/water bath (60 to 65 °C)
ice to chill samples

Materials and reagents

gloves
hydrogen peroxide, 30% (Sigma H1009)
deionized water (or 1× or 2× distilled water), but not autoclaved or DEPC-treated,
for all gel buffers and solutions, except as noted*.
No solutions are autoclaved

1× TBE (as for DNA gels):

0.089 M Tris
0.089 M boric acid
2 mM EDTA

5× TBE:

54 g Tris-base
27.5 g boric acid
20 mL 0.5 M EDTA
Combine ingredients; makes 1 liter.

agarose (normal grade as for DNA gels)
1 M guanidine thiocyanate (Sigma G6639; MW=118.2); i.e., 118 mg/mL of
sterile* water (non-DEPC, as used for DNA work is fine)
Make only as much as you need on day of use (e.g., ~0.5 to 0.6 mL).
TOXIC; be sure to dispose of waste properly.

DNA loading dye containing bromophenol blue
formamide (Sigma F9037, molecular biology grade).
Not necessary to deionize. Open in chemical fume hood.

fresh, dilute ethidium bromide solution (~0.1 to 0.5 µg/mL, ~200 mL)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and
use only in a chemical fume hood.

fresh 7.5 mM NaOH, ~1 to 1.5 liter (fresh, made on same day)
for blotting (see Sambrook et al.)
Hybond N+ (Amersham)

Whatman 3M paper
 paper towels
 2 glass plates, one big enough to fit over a shallow plastic tray
 shallow plastic tray
 2× SSC:
 0.3 M NaCl
 0.03 M sodium citrate
 (1 liter of 20× SSC = 175.3 g NaCl, 88.2 g sodium citrate, to pH 7 with HCl; dilute 1:10 for 2× SSC)

Protocol

Electrophoresis of RNA and capillary transfer to nylon membrane

- Clean gel apparatus by soaking everything including combs in 3% H₂O₂ for 10 min (1:10 dilution of 30% stock in 1× distilled H₂O)
or in 0.5 M NaOH for 2 h,
or in 1% SDS for 2 h
 then rinsing in 1× distilled H₂O (which is not necessarily sterile or RNase-free!).
- For a 100-mL, 1% agarose gel, using a relatively clean bottle, combine 100 mL of 1× TBE + 1 g agarose, and heat, with caution, in a microwave oven .
 The best resolution depends on the expected transcript size. Use the following guide for agarose concentration:
 0.8% agarose for large transcripts (greater than 7 kb)
 2% agarose for short transcripts (1.5 to 3 kb)
- Cool the gel to 55 to 60 °C. Check this with a relatively clean thermometer.
- Add 0.5 mL of freshly-made 1 M guanidine thiocyanate (final concentration = 5 mM G-SCN).
- Pour the gel and let it set for at least 30 min at room temperature.
- Prepare the samples while the gel sets.
 amount of RNA:
 For 0.8% agarose gel, use 6 to 10 µg of total RNA per lane.
 For 2% agarose gel, use 3 to 5 µg per lane.
 volume of RNA to load:
 total, approximately 5 to 20 µL per lane
 loading dye:
 Not necessary if samples are in formamide.
 If your samples are in water, add an equal volume of formamide (final 50% formamide or greater).
 NOTE: Don't put formaldehyde in your samples. It does not work on this type of gel!
 If you absolutely need loading dye, try formamide with final 1× TBE, 0.5% bromophenol blue. Add this AFTER denaturing your RNA in formamide.
- Denature ~2 µg of size-standard RNA, in ~10 µL of formamide for ~10 min; place it on ice.
- Denature samples at 60 to 65 °C for 2 min; place it on ice.
- Having rinsed the gel tank, place the set gel in it, pour in 1× TBE to cover it, then remove the comb and any end blocks.
- Load the gel. Load a blank lane with DNA loading dye between the size marker and the samples to track the running of the samples. This also keeps your

marker from spilling into a sample lane, which sometimes makes a mess of hybridizations.

- Electrophorese samples into the gel for approximately 10 to 15 min at 110 volts, then run at 70 to 80 volts (3.5 v/cm) until the bromophenol blue is approximately 8 to 10 cm from the well. The process takes about 3 to 4 h.
- Stain the samples in fresh, dilute, ethidium bromide solution for ~10 min.
- Destain the for a few minutes in 1× TBE using buffer left over from the gel box.
- Photograph them with a ruler next to the gel. Leave the gel on UV transilluminator for ~2 min. This nicks the RNA, helping with transfer of larger transcripts.
- Soak the gel in ~200 mL of 7.5 mM NaOH, twice, for 10 min each time.
- Set up the capillary transfer to Hybond N+ in 7.5 mM NaOH to run overnight (not less than 8 h, especially important for large transcripts). (See Sambrook et al. 1989.)

Disassemble blot and start hybridization

- Neutralize the filter in 2× SSC for 5 min.
- Air dry the filter. Put the filter on UV light box at this point and draw size marker positions directly on the filter with a pencil.
- UV-crosslink, if you have a cross-linker, but this is not necessary.
- Prehybridize and hybridize as usual (see Kyes et al. 2000 for solutions and conditions). To start, try 55 °C hybridization, 60 °C wash in 0.5× SSC/0.1% SDS.

References

Goda S, Minton N. 1995. A simple procedure for gel electrophoresis and northern blotting of RNA. *Nucleic Acids Res* 23(16):3357-3358.

Kyes S, Pinches R, Newbold C. 2000. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. *Mol Biochem Parasitol* 105(2):311-315.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; pp. 7.46-7.49.

III. *Plasmodium falciparum* cDNA library construction

by **Mats Wahlgren**

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Equipment

centrifuge (4 °C)
agarose electrophoresis unit
50-mL Falcon tubes (BD Labware)
Polytron homogenizer (Brinkmann)
centrifuge (Beckman)
speed-vac

Materials and reagents

Consider using kits from Stratagene for extraction of RNA and construction of cDNA libraries. They are generally more commonly employed than the Pharmacia kits used below. However, we did successfully make several cDNA libraries from two different strains using the Pharmacia kits.

Throughout the following protocols (MOLECULAR BIOLOGY III:A–E), there are references to the appropriate protocols from the companies from which library materials were bought. Further changes in these general “company-protocols” have been outlined below to facilitate *Plasmodium falciparum* library construction.

III:A. Cell preparations at desired cell stage(s)

Grow parasites in 75-cm² large flasks (12 mL per flask). You will need several sets of large flasks to be able to purify a workable amount of mRNA. We usually used 8 bottles/preparation where 8 bottles with ~10% parasitemia would yield 100 to 200 µg (sometimes even 300 µg) of total RNA. If rosetting parasites are desired, use parasites with ≥ 50% rosetting rate. See also protocols on cell culturing in PARASITES, section I, and synchronization of erythrocytic stages if that is desired (PARASITES, section IV).

III:B. RNA preparation (total RNA)

See separate protocol for RNA preparation in MOLECULAR BIOLOGY, section II above. Store RNA as a precipitate in 3× volume of ethanol with 0.3 M sodium acetate at –70 °C in microfuge tubes.

III:C. mRNA selection of pooled total RNA

See general protocol from 5Prime → 3Prime for mRNA isolation using oligo(dT) cellulose spin columns. (See also other companies for smaller amounts of mRNA isolation.)

Procedure

- Centrifuge microfuge tubes with RNA in microfuge at 4 °C for 30 min.
- Wash samples with 70% cold ethanol and centrifuge them again for ~20 min.
- Dry tubes upside down in a hood with good airflow or in a speed-vac.

- Add 50 to 100 μL of pure “kit- H_2O ” to each tube and resuspend the RNA. Pool the RNA into one tube.
- Heat the sample at 65 $^\circ\text{C}$ for a few minutes, then place it on ice.
- Load 1 to 5 mg of total RNA per column. See company protocol (5Prime \rightarrow 3Prime) for the rest.

Examples of mRNA yields:

One sample of ~ 2 mg total RNA gave:

~ 87 μg mRNA after the 1st selection ($\sim 3.5\%$)

~ 61 μg mRNA after the 2nd selection ($\sim 2.5\%$)

Another sample of ~ 1.05 mg total RNA gave:

~ 71 μg mRNA after the 1st selection ($\sim 6.8\%$)

~ 42 μg mRNA after the 2nd selection ($\sim 4\%$)

From gel analysis it was clear that a second mRNA selection is not necessary since no rRNA bands were shown after one round of selection.

mRNA was stored as a precipitate according to the protocol with muscle glycogen, sodium acetate, and ethanol.

Store pure mRNA in aliquots of 1 μg , 5 μg , and maybe more.

Run a few microgram-per-lane samples to visualize mRNA as a faint smear in the range of the two larger rRNA bands. The front of these two bands should however be sharp if unselected total RNA is also run in a separate lane along with mRNA to indicate lack of degradation. See MOLECULAR BIOLOGY, section II:C for RNA-gel protocol. If using pure mRNA for a gel, you may want to blot and fix it to nylon for possible later use in a Northern.

III:D. cDNA synthesis

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Pharmacia's kits: Timesaver cDNA Synthesis Kit was used with some components replaced by the Directional Cloning Toolbox.

Additional reagents needed are indicated in the protocol booklet. If one has good quality distilled water, there is no need to use DEPC which is very hazardous (and can be destructive to RNA if it remains after autoclaving DEPC-treated water).

One needs to have access to the following water bath temperatures:

12 °C overnight (next day at 16 °C)

room temperature

37 °C

65 °C

Optional: dry ice for dry ice/ethanol bath.

Procedure

- Cool the lyophilizer trap and have spin columns ready in autoclaved, sterile Corex tubes.
- Use 4 µg of very pure mRNA per cDNA synthesis (both for FCR3S1 and TM284 strains). One could use 5 µg to possibly increase the recombinant phage.
- Centrifuge the mRNA in an Eppendorf microfuge for 30 min at 4 °C, then wash it gently once with 70% ethanol to remove salts from the small white mRNA pellet. Centrifuge it again for 20 min, then dry it briefly.
- Follow Pharmacia's general protocol; use 1 mL of *NotI* dT₁₈ primer from the Directional Cloning Toolbox as the cDNA primer. (See the Pharmacia protocol.)
- Where phenol/chloroform extraction is indicated, use 50 µL of each, followed by an extra step of pure chloroform extraction.
- Ligate the adaptor for 2 to 2.5 h at 16 °C.
- Digest the adaptor with *NotI* for 1.5 h (not 1 h), then extract it again with chloroform/phenol and pure chloroform.
- Precipitate the cDNA in an ethanol/dry ice bath for 30 min.
- If one uses two purification steps with organics (i.e., phenol/chloroform, then chloroform), as opposed to one combined step of phenol/chloroform, one loses more cDNA material.
- It may be appropriate to use 20 µL of column effluent with 2 µg of vector to do the ligations, rather than the 15-10-5 µL scheme suggested in the protocol, since 15 µL of effluent gave more recombinant phage than 10 µL, which gave more than 5 µL. One strain did not yield any recombinant phage with 5 µL of column eluate.

III:E. cDNA insertion, propagation, and amplification in phage using Pharmacia's λ ExCell NotI/EcoRI /CIP kit

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See Pharmacia's Timesaver cDNA Synthesis Kit protocol along with comments for cDNA and phage ligations. Use the phage λ vector to set up and do ligation reactions with *Plasmodium falciparum* cDNA.

Use extra microfuge spin times (20 min, not 10 min), 70% ethanol washing, centrifugation, and drying of DNA. cDNA/phage may be heated for a few minutes at 50 °C to sufficiently resuspend the cDNA before ligations are done. Incubate ligation reactions for 30 to 40 min at 16 °C, then at 4 °C overnight.

Use Stratagene's Gigapack II packaging extracts to package ligated products into phage. (Now there is the Gigapack III kit, which is easier to use).

Use 3 to 4 μ L of the cDNA- λ ligation per packaging. See the Gigapack II protocols.

Control packagings are not done. It is imperative to work very quickly once the extracts are removed from -70 °C to thaw. Package for 1 h 45 min.

Procedure

- Use *Escherichia coli* strain NM522 as the host for phage grown on special minimal plates (see Pharmacia protocols). Inoculate a few colonies in 10 to 20 mL of Luria broth (LB) with 0.2% maltose and 10 mM MgSO₄.
- Shake the inoculum at 30 °C overnight.
- The next day, centrifuge the cell suspension at ~2,000 rpm for 8 min and remove the LB medium.
- Resuspend the cells in 10 mM MgSO₄ for an OD₆₀₀ of 0.5.
- Cool the cells at 4 °C until use; use within 5 days with phage.
- Restreak stock plates of NM522 about every 3 weeks. Freeze extra bacterial stock at -70 °C as described in the manual using ~20% glycerol in the medium.
- Titer these primary libraries, 3 primary libraries/strain since there were 3 ligation mixtures set up for each strain. Phage dilutions of 1:10 and 1:100 of the primary libraries are recommended to titer.
- For the 1:10 dilution titering, mix 1 μ L of phage with 9 μ L of SM-buffer (see Stratagene's picoBlue Immunoscreening kit protocol).
- Make another such serial 1:10 dilution to achieve 1:100 phage dilution.
- Of each dilution, mix 1 μ L with 200 μ L of NM522 bacteria in 10 mM of MgSO₄ (see above) and place at 37 °C for ~15 min.
- Add 3 mL of LB low-melt agarose (at 49 °C) to the mixture and pour it on a prewarmed LB plate.
- Incubate the plate at 37 °C overnight.
- About 100 to 250 phage may be visible on the lower dilution plates. Approximately 0.5×10^6 to 1×10^6 independent clones may be generated per "ligation-library".

Amplification of primary libraries

- One needs about 40 to 50 large petri dishes with LB agar (poured fresh 1 to 2 days previously and evenly on a FLAT surface) for a library amplification (~13-cm plates). Each plate can take 20,000 to 40,000 phage; maybe 25,000 is a good number. Follow the rest of the Gigapack protocol for phage amplification.
- Allow phage plaques to grow for 7 to 8 h until they are pinhead sized (not too large).
- Overlay the plates with 9 to 10 mL of SM buffer overnight. It is very important that the plates lie very flat so that there is liquid on the whole plate into which the phage can elute. If the plates don't lie flat, parts will dry up as the buffer soaks into the agar, thus causing some uneven amplifications of phage populations.
- Recover and pool about 7 mL of SM-buffer/plate into 1-liter sterile bottles.
- Remove the bacteria with chloroform as described in the protocol, and aliquot the phage suspension into sterile 50-mL Corex tubes for centrifugation. Freeze most of the amplified libraries as aliquots at $-70\text{ }^{\circ}\text{C}$ with some chloroform/DMSO as described.
- Test both primary and secondary libraries for blue/clear phage indicating ratios of phage with no cDNA insert to those with an insert. Those without an insert should have the *LacZ* gene intact and thus produce β -galactosidase that in the presence of inducer (IPTG) and substrate X-gal would form blue plaques. Phage with insert are clear.
- Mix 1 μL of unamplified phage or 5 to 8 μL of a 10^5 -dilution of amplified library with 200 μL of bacteria to get 200 to 300 phage plaques per plate.
- Incubate the phage and bacteria for ~20 min at $37\text{ }^{\circ}\text{C}$, quickly mix with 3.0 mL of $49\text{ }^{\circ}\text{C}$ top agarose, 7.5 μL of 1 M IPTG, and 50 μL of a solution of 250 mg of X-gal per mL of dimethylformamide (DMF), and pour on a prewarmed LB plate.
- Allow the top agarose to harden without a lid for 5 min, then incubate the plate upside down overnight at $37\text{ }^{\circ}\text{C}$.
- Remove the plates the following day and place them at $4\text{ }^{\circ}\text{C}$ for the day (or alternatively longer) for the blue color to fully develop. The primary libraries have 2 to 3% of phage that are blue (dark or light blue) indicating no insert. The rest should contain inserts. Amplified libraries are only 3 to 5% nonrecombinant.
- For initial titering of primary libraries, 1 μL of 10-fold dilutions is enough to use (see above).
- Dilute amplified libraries in steps of 1:100, where 10 μL of 10^{-6} , 10^{-7} , 10^{-8} (and possibly more) are plated and a range of about 800 down to 50 phage are seen on the increasingly diluted plates.

Examples of titres achieved:

FCR3S1 library: $\sim 3^9$ to 10^9 pfu/mL; TM 284 was at $\sim 9^9$ to 10^9 pfu/mL.

Libraries were aliquoted as 1-mL samples in microfuge tubes and stored with 50 μL of chloroform, along with some larger aliquots stored in screw-cap glass tubes with some chloroform at $4\text{ }^{\circ}\text{C}$ for library screening. The rest were kept for long-term storage as aliquots in 15-mL tubes with DMSO/chloroform at $-70\text{ }^{\circ}\text{C}$ (see also the Gigapack protocol).

Testing excision of inserts from libraries

To get an idea of insert sizes in the libraries, we used recombinant phage directly from the libraries to excise, or release, clones as phagemids. Best results were obtained with

pExCell release directly from the library as opposed to plaque-purified phage clones. For the in vivo excision, we used NP66 *E. coli* accompanying the kit as well as the specified medium and temperature shift from 32 to 39 °C (See Pharmacia λExCell protocol).

- Pick colonies the following day.
- Incubate them in LB-amp overnight.
- Perform plasmid mini-preps using the alkaline lysis method from “Current Protocols” with suggested modifications, such as phenol extraction, etc. (Do not use boiling mini-preps.)
- Use *PvuII* enzyme to cut around insert sites. Use about half of the digest on a gel. *PvuII*-cut sites are ~380 bp apart on the vector (i.e., without insert).
- The vector arms should appear as one band of ~2.5 kb. Insert sizes from a few random clones tested were seen from ~0.5 to 2.2 kb. Or simply test insert size by PCR using vector arm primers.

References

Protocols from the following companies' kits:

5Prime → 3Prime's Maxi oligo (dT) Cellulose spin column for mRNA purification
Pharmacia's Timesaver cDNA Synthesis
Pharmacia's Directional Cloning Toolbox
Pharmacia's λ ExCell NotI/EcoRI/CIP
Stratagene's Gigapack Packaging Extracts
Stratagene's picoBlue Immunoscreening

Ausubel FM, et al., eds. Current protocols in molecular biology. New York: Wiley-Interscience; 1988-1999.

IV. PCR of AT-rich genomes/regions

by **Mats Wahlgren**

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Noncoding regions of *Plasmodium falciparum* tend to be extremely rich in AT content (>90%). PCR amplification of these regions is significantly improved by lowering the *Taq* polymerase extension temperature from 72 to 60 °C. The other parameters do not need to be changed.

Note: The AT content of the entire genome is estimated to be ~82% while coding regions are thought to be 60 to 70% AT.

References

Bowman S, Lawson D, Basham D, Brown D, Chillingworth T, Churcher CM, Craig A, Davies RM, Devlin K, Feltwell T, et al. 1999. The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. *Nature* 400(6744):532-538.

Su XZ, Wu Y, Sifri CD, Wellems TE. 1996. Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. *Nucleic Acids Res* 24(8):1574-1575.

V. Single cell RT-PCR of *var* (DBL-1) mRNA from *Plasmodium falciparum*

by **Victor Fernandez**

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Equipment

PCR machine with hot lid

Materials and reagents

GeneAmp RNA PCR Kit (Applied Biosystems)

RNase-free DNase, 10 U/ μ L (Stratagene)

DNase

RNase inhibitor

Opti-Prime PCR buffer 3 and buffer 4, mixed 1:1 (Stratagene)

TaqStart antibody (Clontech)

Taq DNA polymerase, 5000 U/ μ L (Pharmacia)

- Prepare PCR mix for n samples:

deoxyadenosine triphosphate:	$2 \mu\text{L} \times n$
deoxycytidine triphosphate:	$2 \mu\text{L} \times n$
deoxyguanosine triphosphate:	$2 \mu\text{L} \times n$
deoxythymidine triphosphate:	$2 \mu\text{L} \times n$
GeneAmp 10 \times PCR Buffer II:	$2 \mu\text{L} \times n$
MgCl ₂	$4 \mu\text{L} \times n$
distilled water:	$1 \mu\text{L} \times n$
- Aliquot the PCR mix into n thin-walled PCR tubes (15 μ L/tube).
- Place a single infected erythrocyte into each tube and immediately freeze it on dry-ice (see PARASITES, section V:B).
- Release the DNA and RNA from the parasite by heating the tubes to 93 °C for 3 min in the PCR machine. Cool them on ice.
- Add 1 μ L of DNase and 1 μ L of RNase inhibitor to each tube. Degrade the DNA at 37 °C for 30 min in the PCR machine. Inactivate the DNase at 93 °C for 3 min.
- Add 1 μ L of RNase inhibitor, 1 μ L of random hexamers, and 1 μ L of reverse transcriptase to each tube. Perform the reverse transcription at 42 °C for 30 min and inactivate the reverse transcriptase at 93 °C for 3 min.
- Prepare the TaqStart mix for n reactions:

Taq polymerase:	$4.4 \mu\text{L} \times m$ (where $m = 4.8 \times n / 25.4$)
TaqStart antibody:	$4.4 \mu\text{L} \times m$
TaqStart buffer:	$17.6 \mu\text{L} \times m$

 Mix and incubate for at least 5 min at room temperature.
- Prepare the PCR master mix for n reactions:

Opti-Prime PCR buffer 3+4:	$8 \mu\text{L} \times n$
Specific primer DBL1-1 (DBL1-3):	$2 \mu\text{L} \times n$
Specific primer DBL1-2:	$2 \mu\text{L} \times n$
TaqStart mix:	$4.8 \mu\text{L} \times n$
distilled water:	$63.2 \mu\text{L} \times n$

- Add 80 μL of PCR master mix to each tube. The tubes already contain 20 μL from the previous steps; the final volume in each tube should thus be 100 μL .
- Amplify the *var* sequences using the following PCR conditions:
93° C for 20 s, 55 °C for 30 s, and 72 °C for 1 min.
Run 50 cycles followed by a last extension step at 72 °C for 7 min.
- Run 20 μL of the PCR products on a 1.2% agarose gel with TBE buffer.

Primer sequences

DBL1-1:5'-GGW GCW TGY GCW CCW TWY MG-3'

DBL1-2:5'-ARR TAY TGW GGW ACR TAR TC-3'

DBL1-3:5'-GCA CGA AGT TTY GCA GA-3'

Reference

Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature 394(6691):392-395.

VI. Fluorescent *in situ* hybridization (FISH)^{DNA-DNA} for *Plasmodium falciparum*

(Adapted from Ersfeld, K. & Gull, K, 1997)

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Reagents

thermal cycler with *in situ* adapter (Eppendorf)
waterbath at 37 °C
hybridization oven at 50°C
microscope

Materials and reagents

Saponin (Sigma)
RPMI 1640 (Gibco)
3 well microscope slides (Cell-Line) and coverslips
In situ Frame for 25 µL (AbGene)
Hybridization solution (HS):
 50% formamide (Roche Applied Science)
 10% dextran sulfate (Sigma)
 2× SSPE
 250 µg/mL Herring sperm DNA (Sigma)
Probes: To prepare the probes, use the “Fluorescein High-prime kit” (Roche Applied Science) for Fluorescein signals and “Biotin high-prime kit” (Roche Applied Science). For detection of the biotinylated probes use avidin-conjugate with rhodamine (Roche Applied Science).
M solution (pH 7.5):
 100 mM maleic acid (Sigma)
 150 mM NaCl (Sigma)
 1% blocking reagent (Roche Applied Science)
 4% bovine serum albumin (BSA) (Sigma)

TNT solution (pH 7.5):
 100 mM Tris-HCl
 150 mM NaCl (Sigma)
 0.5% Tween 20 (v/v) (Sigma)

20× SSC
10% paraformaldehyde (Electron Microscopy Sciences)
formamide (Roche Applied Science)
VECTASHIELD mounting medium with DAPI (Vector Laboratories)
nail polish

Procedure

Parasite fixation:

- Treat the parasites (10% parasitemia) with saponin to lyse the erythrocyte membrane (centrifuge 4000 rpm 5 min).
- Wash them twice in incomplete RPMI (centrifuge 6000 rpm 1 min).

- Resuspend the parasites in paraformaldehyde 4% (in PBS) for 10-15min, on ice.
- Wash once with cold PBS (centrifuge 6000 rpm 1 min).
- Resuspend in ~400 μ L cold PBS (fixed parasites can be stored at 4 °C in this step for at least a week).
- Deposit a monolayer of parasites on each well of the microscope slide.
- Air-dry the slides for 30 min at room temperature.

In situ hybridization:

- Fix the plastic frame around the wells containing the parasites.
- Wash the slides once in PBS for 5 min at room temperature.
- If necessary, permeabilize using 0,1% Triton X-100 for 5 min and wash twice with PBS)
- Apply the HS solution with the labeled probe (denatured at 95 °C for 5 min) on each well.
- Cover the well with the *In situ* Frame coverslip.
- Denature the slides in a thermal cycler with *in situ* adapter for 30 min at 80 °C followed by hybridization at 37 °C overnight.

Washing:

- Remove the *In situ* Frame coverslip and the HS solution.
- Wash the slides in 2 \times SSC/50% Formamide for 30 min at 37 °C, followed by 1 \times SSC for 10 min at 50 °C, 2 \times SSC for 10 min at 50 °C, and 4 \times SSC for 10 min at 50 °C.
- Equilibrate the cells in M solution for 5 min at room temperature in a humid chamber protected from light.
- Remove the M solution and replace it with M solution plus Avidin–Rhodamine (1:10.000) for detection using biotin probes. Incubate the slides for 30 min at room temperature.
- Wash the slides three times in TNT solution 10 min each at room temperature, with agitation.
- Let air dry the slide and mount using Vectashield with DAPI.
- Analyze the slide by fluorescence microscopy.

Comments

This protocol allows a dramatic decrease of the background generated by the erythrocyte membranes due to the saponin lysis, and better preservation of nuclear architecture since the fixation step is done in suspension.

VII. Separation and mapping of chromosomes of malaria parasites using pulsed-field gel electrophoresis (PFGE)

VII:A. PFGE Protocol 1

by **Rosaura Hernandez-Rivas**¹ and **Artur Scherf**²

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Equipment

Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) or CHEF-DR system (Bio-Rad)

centrifuge

water bath

15-mL Falcon tubes (BD Labware)

Materials and reagents

SeaKem GTG agarose (Cambrex) for agarose gels at a concentration of >0.6%

chromosomal grade agarose (Bio-Rad) for agarose gels ≤0.6%

low-melting point agarose (Cambrex) for embedding parasites in agarose blocks and preparative isolation of chromosomes

plastic gel molds (2 × 5 × 10 mm, 100-μL volume; Pharmacia)

nylon membrane: Hybond-N+ (Amersham) or Biotrans(+) (ICN Biomedicals)

These membranes allow the rapid alkaline transfer of DNA and their physical characteristics make them especially useful for multiple hybridization cycles (usually 5 to 10 cycles).

erythrocyte lysis buffer: 0.15% saponin (Sigma) in phosphate-buffered saline (PBS).

ethidium bromide in water: 10 mg/mL

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

alkaline transfer buffer: 0.4 M NaOH

20× SSC:

3 M sodium chloride

0.3 M sodium citrate

TE buffer:

10 mM Tris-HCl

1 mM EDTA (pH 8.0)

1 mM phenylmethylsulphonyl fluoride (PMSF) in TE

molecular weight size standards:

5 kb ladder (4.9 to 120 kb)

ladder (0.05 to 1 Mb)

Saccharomyces cerevisiae (0.2 to 2.2 Mb)

Hansenula wingei (1 to 3.1 Mb) (Bio-Rad)

cell lysis buffer (CLB):

0.5 M EDTA (pH 8.0)

10 mM Tris-HCl

1% sodium lauryl sarcosinate (Sigma)

Store at room temperature. Before cell lysis, add 2 mg/mL of proteinase K from a stock solution.

prehybridization and hybridization buffer:

0.5 M NaPO₄ (pH 7.2)

7% SDS

1% BSA

hybridization buffer:

7% SDS

0.5 M EDTA (pH 8.0)

1% BSA

saturated 2-butanol:

Mix 2-butanol with 1 M NaCl in TE (4:1 v/v). Add 9 volumes of saturated 2-butanol and 1 volume of the aqueous phase to the gel blocks. This is important to avoid gel shrinking!

10× TBE buffer:

108 g Tris-base

54 g boric acid

8.35 g disodium-EDTA per liter (pH ~8.5)

Preparation of DNA for PFGE

The ability to preserve the intact size of large DNA molecules is critical for the success of PFGE. To overcome the problem of DNA shearing, intact cells are embedded in low-melting agarose blocks. Cells are lysed and proteins are removed by proteinase K treatment. This procedure yields DNA that is both intact and susceptible to restriction enzyme digestion. The agarose block can be loaded directly into the well of a pulsed-field gel.

The host cells of the intracellular blood stage form of *Plasmodium falciparum* need to be lysed by saponin before the parasite cells are embedded into agarose blocks.

- Estimate the volume of the erythrocyte pellet and add 1.5 volumes of 0.15% saponin in PBS at room temperature.
- Resuspend the pellet and incubate it for 3 to 5 min. The liquid will clarify as the red blood cells lyse.
- Add 5 volumes of cold PBS and centrifuge the pellet at 5,000 rpm for 10 min.
- Resuspend the dark pellet which contains the parasite material in 2 mL of PBS.
- Pellet the cells by centrifugation at 3,000 rpm for 10 min.
- Resuspend the pellet at a concentration of approximately 5×10^8 parasites/mL of PBS.
- Equilibrate the cell suspension at 37 °C and add an equal volume of 1.6% melted low-melting point agarose (37 °C).
- Mix the cell suspension gently and dispense it immediately into plastic gel molds.
- Allow the gel to solidify at 4 °C for about 20 min.

- Place up to 20 solidified blocks in 10 mL of cell lysis buffer (CLB) and incubate them for 24 h at 42 °C.
- Replace the CLB with an equal volume of fresh CLB.
- Incubate the blocks for an additional 24 h.
- The blocks can be stored indefinitely in TE buffer at 4 °C.

Electrophoresis conditions

Optimal separation of the different chromosome size classes usually requires the use of a range of different PFGE conditions. Table 1 shows the running conditions of large DNA fragments of various sizes using the CHEF (clamped homogeneous electric field) system. These PFGE methods have been used in studies of *Plasmodium falciparum* and *Trypanosoma cruzi*, but also apply to other parasitic protozoa. The described electrophoresis conditions have been developed for the Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) based on the CHEF design. Similar results have been obtained with the CHEF-DR system (Bio-Rad).

Table 1. Electrophoresis conditions for separation of DNA fragments by PFGE (CHEF system)

Efficient separation	Pulse time (ramping)	Voltage	Run time	Agarose concentration	Buffer
5–100 kb	0.5 s – 1.0 s	400 V	7 h	1.3%	0.2× TBE
50–2000 kb	50 s – 90 s	200 V	22 h	1.0%	0.5× TBE
500–4000 kb	90 s – 300 s for 24 h followed by 300 s – 720 s for 24 h	95 V 85 V	48 h	0.7–0.8%	0.5× TBE
2000–6000 kb	120 s – 720 s	85 V	36 h	0.4–0.6% high gel strength agarose	0.5× TBE

- The agarose concentration and type of agarose used depends on the DNA size range to be separated. For example, pulsed field separations larger than 2 megabases are improved by using high gel-strength agarose (chromosomal grade agarose, Bio-Rad) allowing preparations of very low-percentage agarose gels (0.4 to 0.6%), which give significantly better separation of large chromosomes in the range of 2 to 5 megabases..
- For a 15 cm × 15 cm × 0.5 cm 1% agarose gel, dissolve 1.1 g of SeaKem GTG agarose in 110 mL of 0.5× TBE buffer by heating it in a microwave oven. Make sure that the volume has not changed. If it has, adjust again to 110 mL.
- Cool the agarose in a 50 °C water bath and pour it into the casting stand.
- After the gel is solidified, fill the wells with 0.5× TBE buffer and insert the blocks containing parasite DNA. About 2 to 5 mm of a block (1×10^7 to 2.5×10^7 parasites) generally contains enough material to visualize the chromosomes after ethidium bromide staining. (This number may also work for other parasite

species.) However, for best PFGE results it may be necessary to determine the optimal number of cells/block.

- Seal the blocks into the well with 1% low-melting point agarose.
- Fill the electrophoresis chamber with 2.2 liters of TBE buffer. Do not overfill; the buffer should just cover the top of the agarose gel. Keep the temperature of the running buffer (18 °C) constant during the run.

Staining of chromosomes

- After completing the PFGE run, the separated chromosomes can be visualized by staining the agarose gel for 15 min in ethidium bromide (1 µg/mL in water). Destain the gel by two washes in 0.5× TBE for 1 h with gentle agitation, and photograph it (do not forget the ruler) using a shortwave UV light (254 nm). **ATTENTION!** If chromosomes are intended for use in subsequent restriction mapping studies, longwave UV light (360 nm) must to be used to avoid nicking the DNA.

Southern hybridization

- Transfer the DNA from the agarose gel to a nylon membrane such as Hybond-N or Biotrans. In order to ensure efficient transfer of large DNA fragments, nick the chromosomal DNA by exposing the stained agarose gel for 5 min on a UV-light table (254 nm).
- The alkaline transfer procedure using 0.4 N NaOH as transfer buffer works fine for chromosomal DNA blots. We routinely set up the capillary transfer (standard molecular biology procedure) for at least 24 h. Make sure that the weight on top of the absorbent paper stack does not exceed 1 kg. There is no need to fix DNA after alkali blotting.
- For ³²P-labelled DNA probes, the random hexamer priming method in combination with the following hybridization buffer usually works well to give a good signal-to-background ratio with nylon membranes: 7% SDS, 0.5 M EDTA (pH 8.0), 1% BSA. Single-copy genes of *P. falciparum* are generally detected after 6 h to overnight exposure on X-ray film.
- Remove probes by standard protocols or as recommended by the manufacturer. Stripped blots can be stored for prolonged periods at room temperature.

Restriction enzyme digestion of embedded chromosomes

Generally, restriction enzymes diffuse into agarose blocks and are thus suited for chromosome mapping studies. The most useful restriction endonucleases are those with 8-base recognition sites which cut only a few times in chromosome-sized DNA fragments. However, in genomes which are biased in their AT content, such as *P. falciparum*, certain enzymes which recognize GC-rich 6-base pair sequences (*Sma*1, *Bgl*1) can also be used as “rare” cutters in these AT-rich genomes.

- For restriction enzyme digestion of agarose-embedded total parasite DNA, remove the lysis buffer by extensive washes in TE buffer (2 to 3 washes in 20 mL of TE).
- Inactivate any remaining proteinase K by treating the blocks with 1 mM phenylmethylsulphonyl fluoride (PMSF) in TE (10 mL for 20 blocks) for 2 h at room temperature, followed by three washes in 10 mL of TE for 30 min each.

Individual chromosomes or DNA fragments that have been cut out of a stained agarose gel after a PFGE run must be treated with 2-butanol to extract any remaining ethidium bromide prior to restriction enzyme digestion.

- Add 9 volumes of 2-butanol saturated in 1 M NaCl/TE and 1 volume of the aqueous phase to the gel block (5:1, v/v).
- Agitate the mixture at room temperature for 30 min.
- Repeat the extraction twice and wash the block five times in 10 mL of TE buffer for 30 min each.
- Store the blocks at 4 °C.
- Equilibrate the blocks with 5 volumes of restriction buffer containing 100 µg/mL of nuclease-free BSA for 30 to 60 min at room temperature. Remove the buffer and add 2 volumes of fresh buffer and restriction endonuclease (approximately 100 to 200 U/mL). Incubate the blocks at the recommended temperature for 4 h or overnight.
- Partial digestion of chromosomal DNA is useful for mapping studies. In this case, set up separate digestions of serial dilutions of the restriction enzyme for 2 h each (0.01 U/mL, 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL). Stop the reactions by adding EDTA to a final concentration of 50 mM.
- Depending on the expected fragment size, restricted DNA can be separated by PFGE according to the run conditions described in Table 1.

Two dimensional PFGE

Most chromosomes in *P. falciparum*, as in other protozoan parasites, are heterogeneous in size and can be separated from each other by PFGE. Two-dimensional (2D) PFGE studies of a karyotype can give useful information concerning genetic markers located on several chromosomes. Multigene families are often observed in pathogenic protozoa.

- Separate chromosomes in the first dimension and stain them with ethidium bromide as described above.
- Photograph the gel using longwave UV light (360 nm).
- Use a razor blade to excise strips of gel (~2 mm) that contain all chromosomes.
- Extract ethidium bromide using 2-butanol in a 15-mL Falcon tube (as described above).
- Perform restriction enzyme digestions as described above.
- Embed the slice of gel containing the digested chromosomes and size markers (λ ladder or 5 kb ladder) into a precut slot in the top of a 1.3% agarose gel and seal it with 1% low-melting point agarose.
- Carry out standard PFGE separation using the run conditions described in Table 1. For Southern hybridization, continue as described above.

References

Hernandez-Rivas R, Scherf A. 1997. Separation and mapping of chromosomes of parasitic protozoa. Mem Inst Oswaldo Cruz 92(6):815-819.

Hinterberg K, Scherf A. 1994. PFGE: improved conditions for rapid and high-resolution separation of *Plasmodium falciparum* chromosomes. Parasitol Today 10(6):225.

VII:B. PFGE Protocol 2by **Mats Wahlgren***Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden**e-mail: mats.wahlgren@mtc.ki.se*Equipment

centrifuge
incubator
Plug mold (Bio-Rad)
PosiBlot 30-30 pressure blotter (Stratagene)
Stratalinker UV crosslinker (Stratagene, model 2400)
Falcon tubes (BD Labware)

Materials and reagents

10% saponin in PBS
10 mM EDTA (pH 7.6)
PBS
low-melt agarose
0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate (sarkosyl), proteinase K
chromosomal grade agarose (Bio-Rad)
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

0.25 M HCl
denaturation buffer:
0.5 M NaOH
1.5 M NaCl
neutralization buffer:
1 M Tris
1.5 M NaCl
10× SSC
nylon membrane Hybond-N (Amersham)

TSE:
10 mM Tris-HCl (pH 8.0)
100 mM NaCl
1 mM EDTA

Sample preparation

- Calculate the parasitemia of the culture to be used.
- Add 1/100 volume of 10% saponin to the culture. Incubate the culture for 5 min at room temperature.
- Pellet the free parasites by centrifuging them at ~1,000 rpm for 5 min (i.e., a “gentle spin”).
- Dissolve the pellet in PBS and spin it again. When dissolving the pellet, use a broad tip.
- Wash the pellet two more times with PBS.

- After the last centrifugation, dissolve the pellet in TSE to get a concentration of 8×10^8 parasites/mL.
- Mix the parasites with an equal volume of 1.6% low-melt agarose to get a final concentration of 4×10^8 parasites/mL. The agarose should be between 37 and 42 °C.
- Dispense the mix into plug molds and let the agarose solidify, first at room temperature and then at 4 °C for 20 to 30 min.
- Put the DNA/agarose plugs in 0.5 M EDTA, 1% *N*-lauryl sodium sarcosinate, 2 mg/mL of proteinase K; 10 mL of solution will be enough for 20 DNA plugs in a Falcon tube.
- Incubate the plugs at 37 °C for 48 h.
- Store the DNA plugs at 4 °C in the same solution as above (or possibly in TE).

Electrophoresis conditions

See also the section on electrophoresis conditions in *MOLECULAR BIOLOGY*, section VII:A, for details.

Separation		Pulse time (ramping)	Voltage	Run time	Included angle	Buffer/ agarose *
Quick separation 500–2500 kb	Blk 1	5–12 min	2.0 V/cm	30 h	53°	1× TAE/
	Blk 2	3–5 min	4.0 V/cm	17.5 h	53°	0.7%
Good separation 500–1800 kb	Blk 1	6–8 min	2.5 V/cm	80 h	60°	0.5× TBE/
	Blk 2	4–6 min	2.5 V/cm	64 h	60°	1%
	Blk 3	2–4 min	2.5 V/cm	20 h	60°	

* The agarose used is chromosomal grade agarose (Bio-Rad). Buffer temperature is 14 °C for quick separation and 10 °C for good separation.

Southern blot

- Stain the gel for 30 min in 1 µg/mL of ethidium bromide. Destain the gel for 10 min in distilled water. Look at the gel under UV light and take a photograph. Destain the gel for another hour in distilled water.
- Prepare the gel for Southern transfer by depurinating it in 0.25 M HCl for 30 min.
- Rinse the gel in distilled water and denature it in denaturation buffer for 30 min.
- Rinse the gel in distilled water and neutralize it in neutralization buffer for 30 min.
- Rinse the gel in 10× SSC. Wet the nylon membrane in distilled water and let it soak for 5 min in 10× SSC.
- Transfer the chromosomes from the gel to the nylon membrane in a PosiBlot 30-30 pressure blotter. Assemble the pressure blotter according to the manual and use 10× SSC as the transfer buffer. One hour at 75 to 80 mm Hg is enough to transfer the chromosomes to the membrane.
- Quickly rinse the filter in 2× SSC to get rid of any gel pieces sticking to the membrane. Put the membrane on a Whatman paper to dry off a little.

- While the membrane is still moist, cross-link the DNA to the membrane by exposing it to UV light in a crosslinker. Use setting “auto cross-link”.

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Chen Q, Fernandez V, Sundström A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of *var* gene expression in *Plasmodium falciparum*. *Nature* 394(6691):392-395.

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VII:B. PFGE Protocol 3

by Alan Cowman, Brendan Crabb, Alexander Maier, Chris Tonkin, Julie Healer, Paul Gibson and Tania De Koning-Ward

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see: Transfection IV, B, page 286

VIII. Recombinant expression of *Plasmodium falciparum* var- gene domains

by **Qijun Chen**

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Equipment

bacterial culture flask
bacterial culture incubator
sonicator/French press
centrifuge (4 °C)
spectrophotometer

Materials and reagents

pGEX vector (Pharmacia)
BL21-CodonPlus-RIL competent cells (Stratagene) or BL21 competent cells (Pharmacia Biotech)
LB plates (ampicillin 100 µg/mL)
PBS (pH 7.2)
PBS plus 0.2 M NaCl and 0.05% Tween-20
2× YT/amp medium:
 16 g/L tryptone
 10 g/L yeast extract
 5 g/L NaCl
 ampicillin 100 µL/mL
EDTA
PTG (1 M)
Pefabloc (Roche Applied Science)
lysozyme (Pharmacia Biotech)
Triton X-100
glutathione-Sepharose (Pharmacia Biotech)
glutathione solution
thrombin (Pharmacia Biotech)

Procedure

- Clone the sequence of var-domains into the pGEX vector in the correct reading frame.
- Transform BL21-CodonPlus-RIL competent cells or BL21 competent cells and select the positive transformed bacteria with ampicillin in LB (100 µg/mL) using manufacturer's conditions. The former strain appears to result in less degradation during expression.
- Pick several colonies and culture them separately overnight in 20 mL of LB medium with 100 µg/mL of ampicillin at 37 °C.
- Inoculate the overnight culture in prewarmed (room-temperature) 2× YT/amp medium in a ratio of 1:100. Use 20 mL of medium for the initial expression test, and 1 to 10 liters for large scale expression. Continue shaking the culture at room temperature until the OD₆₀₀ of the culture is 0.4 to 0.6. Precool the centrifuge to 4 °C.

- Check the induction system for your vector. If you are using the pGEX system and thus the *tac*-promoter, add IPTG to 0.1 mM (using a 1 M stock) and induce the expression at a temperature lower than 30 °C:
 - overnight for the ATS-region
 - less than 4 h for DBL-domains
- Sediment the bacteria by centrifugation at $1,500 \times g$ for 20 min at 4 °C if using large-volume centrifuge flasks. For small volumes, sediment for 10 min.
- Resuspend the bacteria with cold PBS (20 times the pellet volume), then add:
 - EDTA to 0.5 mM (do NOT use if using a his-tag vector)
 - Pefabloc to 1 mM (or 1 mg/mL)
 - lysozyme to 1 mg/mL
 - (DTT to 5 mM, optional)
- Mix completely and sonicate the bacteria five times for 18 s each, or use a French press to release the fusion protein from the bacteria. You may have to pass the bacterial solution through the press twice to release protein.
- Add 20% Triton X-100 to a final concentration of 1% and shake it at 4 °C for 30 min. If using a GST-vector, prepare glutathione–Sepharose according to manufacturer's instructions and follow the protocol below. Otherwise, refer to the manufacturer's recommendations for the expression system you have chosen.
- Centrifuge bacterial lysis at $2,000 \times g$ for 15 min at 4 °C.
- Remove and save the supernatant, add to it at least 0.5 mL of bead volume of glutathione–Sepharose per liter of original culture.
- Allow the binding of the fusion protein and matrix to proceed for 1 h with gentle shaking at 4 °C.
- Wash the adsorbed beads twice with cold PBS plus 0.2 M NaCl and 0.05% Tween-20 and once with cold PBS only.
- Sediment the suspension by centrifuging ($2,000 \times g$). Remove the supernatant.
- Add an equal volume of glutathione solution to the beads and release the protein for 60 min at room temperature, or overnight at 4 °C with slow shaking.
- Sediment the beads and measure the yield of fusion protein in the supernatant with the spectrophotometer at 280 nm.
- The fusion protein can be digested either directly on the glutathione–Sepharose beads or in the eluent solution. Use 30 U of thrombin per 500 μ L of bead volume of beads or 1 mL of eluent solution. The digestion is usually carried out overnight at 4 °C.
- Aliquot, then store the fusion protein at -70 °C.

Reference

Chen Q, Heddini A, Barragan A, Fernandez V, Pearce FA, Wahlgren M. 2000. The semiconserved head structure of *Plasmodium falciparum* erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. *J Exp Med* 192(1):1-9

IX. Telomere repeat amplification protocol in *Plasmodium falciparum* (PfTRAP)

by **Luisa M. Figueiredo**¹, and **Artur Scherf**²

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Equipment

homogenizer (Kontes; size 19)
slides and coverslips
optical microscope
centrifuges (15-mL tube and microfuge)
PCR machine
vertical electrophoresis system (10 × 8 cm)

Materials and reagents

parasite culture of 10% parasitemia containing ~10⁹ parasites
(mostly 16- to 30-h cultures)

Buffer B:

10 mM Tris–HCl (pH 7.5)
1 mM MgCl₂
1 mM EGTA
5 mM 2-mercaptoethanol
10% glycerol
10 µg/mL leupeptin
10 µg/mL pepstatin

TRAP buffer:

200 mM Tris–HCl (pH 8.3)
15 mM MgCl₂
630 mM KCl
0.05% Tween 20
10 mM EGTA
500 µM dNTP
1 mg/mL BSA

Oligonucleotides:

PfTS (5' AATCCGTCGAGCAGAGTTCA 3')
PfCX (5' GGCGCGT G/A AACCCCT G/A AACCCCT G/A AACCC 3')
a GC-clamp followed by 3 telomere repeats)

Preparation

- Wash the homogenizer carefully and rinse it with 0.4 M NaOH, 0.25 M HCl, 0.4 M NaOH, and distilled water successively. Put it on ice before use.
- Use only RNase-free solutions and materials for the TRAP assay.
- Wear gloves all the time and keep parasite extracts on ice.

Preparation of a semipurified protein extract (~3 h)

- Do a saponin lysis of a freshly recovered culture (described elsewhere in this book) containing $\sim 10^9$ parasites.
- Wash the pellet in 5 volumes of PBS to eliminate the majority of erythrocyte contaminants.
- Wash the pellet in Buffer B.
- Resuspend the parasite pellet in 200 μL of Buffer B.
- Transfer parasites to the homogenizer and lyse the parasitophorous vacuole and cytoplasmic membrane by applying 80 strokes on ice.
- Carefully, take $\sim 2 \mu\text{L}$ of the lysate and put it between the slide and a coverslip.
- Using a phase contrast optical microscope, check that the parasitophorous vacuole and cytoplasmic membrane have been disrupted (resulting in debris in the suspension). If not, apply 80 more strokes. Repeat this procedure until more than 90% of the parasites have been lysed.
- Transfer the lysate to a prechilled 1.5-mL tube.
- Centrifuge the lysate for 1 hr at $17,600 \times g$ at 4°C .
- The supernatant consists of a semipurified cytoplasmic fraction at a concentration of 5×10^6 parasite equivalents per microliter. Aliquot it in 2-, 4-, and 12- μL amounts and store it at -80°C .
- The pellet containing the nuclei can be resuspended in 200 μL of Buffer B, lysed by sonication, and centrifuged as described above.
- Both cytoplasmic and nuclear fractions contain telomerase activity. For practical reasons we usually use the cytoplasmic fraction.

TRAP assay (~6 h)

The TRAP assay consists of an elongation and an amplification step. In the elongation step, an oligonucleotide (PfTS) that mimics a chromosome extremity is used as a substrate for telomerase. The amplification step consists of a PCR in which the products from the elongation step are amplified and labelled with ^{32}P .

Elongation step:

- Use enough DNA in the reaction (equivalent to about 10^7 parasites) to get a positive reaction.
- In an RNase-free PCR tube, prepare the following mix:
 - 2 μL of protein extract
 - 5 μL of TRAP buffer
 - 100 ng of PfTS oligonucleotide
 - H_2O to 48.5 μL
- Incubate the mix at 37°C for 1 h.

Amplification step:

- To each tube containing the elongation products add:
 - 100 ng of PfCX oligonucleotide
 - 2.5 μCi ^{32}P -dNTP
 - 2.5 U Ampli *Taq* Gold DNA polymerase (Applied Biosystems)
 - H_2O up to final volume of 50 μL
- Amplify as follows:
 - 1 cycle: 95°C for 10 min
 - 35 cycles: 95°C for 10 s, 55°C for 30 s, 72°C for 1 min

- Run 2 controls for nontelomerase-mediated incorporation. Before starting the elongation step, (i) pretreat semipurified protein extract for 30 min at 37 °C with 10 µg of RNase A and (ii) denature it at 95 °C for 10 min.
- Resolve PCR products on a 15% nondenaturing 8 × 10-cm polyacrylamide gel, at 150V (~2 h), in 1× TBE.
- At the end of electrophoresis, disassemble apparatus, leaving the gel stuck to one of the glass plates. Cover it with Saran wrap and expose it (without drying) to Kodak film for a few hours.

Reference

Bottius E, Bakhsis N, Scherf A. 1998. *Plasmodium falciparum* telomerase: de novo telomere addition to telomeric and nontelomeric sequences and role in chromosome healing. Mol Cell Biol 18(2):919-925.

X. Genotyping of *Plasmodium falciparum* parasites

X.A. Genotyping of *Plasmodium falciparum* parasites by PCR: *msp1*, *msp2*, and *glurp* by Georges Snounou¹ and Anna Färnert²

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Equipment

micropipettes and tips
thermal cycler
apparatus for agarose gel electrophoresis
photographic equipment and UV transilluminator
refrigerator (4 °C) and freezer (−20 °C) for reagent storage
microcentrifuge

Materials and reagents

Taq polymerase (store at −20 °C) with appropriate buffer (store at 4 °C)
MgCl₂ stock solution (often provided by enzyme supplier)
dNTP: a working solution with a concentration of 5 mM for each of dATP, dCTP, dGTP and dTTP (store at −20 °C)
oligonucleotide primers (sequences are given below): a working solution with a concentration of 2.5 μM for each oligonucleotide primer (store at −20 °C)
mineral oil
loading buffer (5×):
50 mM Tris (pH 8.0)
75 mM EDTA (pH 8.0)
0.5% SDS
30% w/v sucrose
10% Ficoll (w/v, average molecular weight 400 000)
0.25% orange G dye (w/v approximately)
10× TBE buffer:
1 M Tris
1 M boric acid
50 mM EDTA
A pH of approximately 8.3 should be obtained without any adjustment.
Agaroses:
normal agarose
high resolution agaroses (such as NuSieve or MetaPhor, Cambrex)
ethidium bromide solution containing 10 mg per mL of water
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood. Store in the dark at 4 °C.
pure water

Genotyping of *P. falciparum* infections permits characterization of distinct subpopulations present in an isolate. The genetic markers merozoite surface protein (*msp1*) (block2), *msp2*, and glutamate rich protein (*glurp*) have been chosen due to their extensive polymorphism in size and sequences. The different allelic types, i.e., families, identified for *msp1* (K1, MAD 20, and RO33 types) and *msp2* (FC27 and IC/3D7) are detected with specific primers in a second nested PCR.

The amplification strategy used for genotyping *P. falciparum* parasites is the nested PCR. In the first amplification reaction oligonucleotide primer pairs, which will hybridize to conserved sequences flanking the repeat polymorphic regions of the genes, are used. The product of this first reaction is then used as a DNA template for separate second amplification reactions in which the oligonucleotide primers used recognise sequences contained within the DNA fragment amplified in the first reaction.

Setting up the first amplification reaction

- Calculate the total volume of reaction mixture required: 20 μ L per reaction \times (total number of reactions + 1).
- Calculate the amount of:
 - buffer (final concentration 1 \times)
 - MgCl₂ (final concentration 1.5 mM)
 - dNTP (final concentration 125 μ M)
 - oligonucleotides (final concentration 250 nM)
 - Taq* polymerase (final concentration 2 units per 100 μ L)
 - and water to make up the total volume

NB: If all three genetic markers (*msp1*, *msp2*, and *glurp*) are to be analysed, then a mixture of the corresponding 3 oligonucleotide primer pairs can be added to the reaction. Thus one first amplification reaction only is required for each sample.

- Add, in order, the correct volumes of: water, buffer, MgCl₂, oligonucleotide primers, dNTP, and the *Taq* polymerase. Mix by a short vortex pulse.
- Aliquot 20 μ L of the reaction mixture per labelled tube.
- Add 40 μ L of mineral oil overlay to each tube.
- Add 1 μ L of DNA template to each tube (water for the negative controls).
- Place in the cycler and run the PCR reaction.

Setting up the second amplification reactions

Steps 1 – 5 as above.

- Remove 1 μ L from the first amplification reaction tube from under the oil overlay and add it to the oil overlay of the second amplification reaction tube.
- Place the tube in the cycler and run the PCR reaction.

NB: Each second amplification reaction must be performed with a single oligonucleotide pair. Thus, per sample, 3 separate reactions will be required for the analysis of *msp1*, two for *msp2*, and one for *glurp*.

Cycling parameters

Step 1	95 °C for 5 min	Initial denaturation
Step 2	X °C for 2 min	Annealing
Step 3	72 °C for 2 min	Extension
Step 4	94 °C for 1 min	Denaturation
Step 5	Repeat Steps 2–4 a total of 25 cycles (Nest 1) or 30 cycles (Nest 2)	
Step 6	X °C for 2 min	Final annealing

- Step 7 72 °C for 5 min Final extension
Step 8 The reaction is completed by reducing the temperature to 25 °C.

X = 58 °C for Nest 1 (M1-OF/M1-OR; M2-OF/M2-OR; G-OF/G-OR) and the Nest 2 reactions of *glurp* (G-NF/G-OR).

X = 61 °C for Nest 2 reactions of *msp1* (M1-KF/M1-KR; M1-MF/M1-MR; M1-RF/M1-RR) and of *msp2* (M2-FCF/M2-FCR; M2-ICF/M2-ICR).

Sensitivity and specificity

- The parameters provided above (cycling parameters and final concentrations) may have to be altered in order to obtain optimal sensitivity and specificity. The use of different temperature cyclers and enzymes has an influence on the efficiency of the PCR reaction. The nested PCR for the 3 genetic markers should be capable of detecting about 10 parasite genomes per microliter of blood.
- Optimization of the PCR conditions requires the preparation of a standard set of genomic DNA templates. These are prepared from a defined quantity of in vitro cultured cloned parasite lines. Cloned lines must be used since the haploid genome contains a single copy of each of the 3 genes. If needed the conditions are varied in such a way that a single band is obtained for each of the specific second amplification reactions for a range of parasite genomic DNA concentrations.
- Sensitivity is mainly dependent on the number of cycles, thus these can be increased if needed.
- Specificity is most affected by the annealing temperature and eventually the MgCl₂ concentration. A common problem is the generation of two PCR products for the specific second amplification reactions. This is due to carryover of oligonucleotide primers and PCR product from the first reaction, which can be minimized by reducing the oligonucleotide concentrations and/or the number of cycles in the first amplification reaction.

Minimizing contamination

- The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. In this context the oil that is used to overlay the reaction mixtures acts as a very efficient contamination barrier, and it is strongly suggested to retain the practice even when a heated lid is available.
- Ideally, setting up the first and second amplification reactions should be performed in a separate room from the one where the gels are migrated. Moreover, the transfer of the template from the first to the second amplification reactions must be performed with a dedicated pipette in yet another room, preferably with filter tips.

Analysis of the PCR product

- Add 5 µL of the loading buffer to the PCR product.
- Load 12 µL of sample on a suitable agarose gel and migrate (1× TBE buffer).
- Stain the gel in TBE buffer containing ethidium bromide (final concentration 1 µg per mL) for 30 min.
- Destain in TBE or water for 5 min.

- Visualise on a UV transilluminator.

Normal agarose is suitable for the analysis of all 3 genetic markers. However, given the small size of the bands which will result from the amplification of *msp1*, and the small variations in the sizes of the different allelic variants, the use of agarose type which give higher resolution is advised. The best results are obtained if the gel is kept cold before and during electrophoresis. The high cost of such agarose types (NuSieve or MetaPhor) can be compensated by the fact that the gels can be reused at least 10 times without significant loss of resolution. Used gels can be stored in TBE buffer, and when needed for further use, they are just reboiled. To compensate for condensation, add up water to the original gel volume when reboiling.

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Snounou G, Zhu X, Siripoon N, Jarra W, Thaitong S, Brown KN, Viriyakosol S. 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg* 93(4):369-374. (NB: The order of the *msp1* K1 and MAD20 oligonucleotide was inverted in Table 1).

Oligonucleotide sequences

Merozoite Surface Protein 1: *msp1*

First reaction: M1-OF 5'- CTAGAAGCTTTAGAAGATGCAGTATTG -3'
M1-OR 5'- CTAAATAGTATTCTAATTCAAGTGGATCA -3'

Second reaction:

K1-type M1-KF 5'- AAATGAAGAAGAAATTAACAAAAGGTGC -3'
M1-KR 5'- GCTTGCATCAGCTGGAGGGCTTGCACCAGA -3'
MAD20-type M1-MF 5'- AAATGAAGGAACAAGTGGAAACAGCTGTTAC -3'
M1-MR 5'- ATCTGAAGGATTTGTACGTCTTGAATTACC -3'
RO33-type M1-RF 5'- TAAAGGATGGAGCAAATACTCAAGTTGTTG -3'
M1-RR 5'- CATCTGAAGGATTTGCAGCACCTGGAGATC -3'

Merozoite Surface Protein 2: *msp2*

First reaction: M2-OF 5'- ATGAAGGTAATTAACATTGTCTATTATA -3'
M2-OR 5'- CTTTGTTACCATCGGTACATTCTT -3'

Second reaction:

FC27-type M2-FCF 5'- AATACTAAGAGTGTAGGTGTCARATGCTCCA -3'
M2-FCR 5'- TTTTATTTGGTGCATTGCCAGAACTTGAAC -3'
IC/3D7-type M2-ICF 5'- AGAAGTATGGCAGAAAGTAAKCCTYCTACT -3'
M2-ICR 5'- GATTGTAATTCGGGGGATTCAGTTTGTTCG -3'

Glutamate Rich Protein: *glurp*

First reaction: G-OF 5'- TGAATTTGAAGATGTTCACTGAAC -3'
G-OR 5'- GTGGAATTGCTTTTTCTTCAACTAA -3'

Second reaction:

G-NF 5'- TGTTCACTGAACAATTAGATTTAGATCA -3'
G-OR 5'- GTGGAATTGCTTTTTCTTCAACTAA -3'

X:B. *msp2* genotyping of *Plasmodium falciparum* by capillary electrophoresis and GeneMapper® Program

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Background

Genotyping by capillary electrophoresis and GeneMapper program.

The genotyping technique presented in the following characterises different *P. falciparum* clones by sizing the PCR product of the polymorphic marker gene merozoite surface protein 2 (*msp2*) using the GeneMapper® (formerly GeneScan®) program. This technique provides higher resolution of length polymorphic fragments and thus optimal discrimination of genetic diversity of the marker gene *msp2*.

The GeneMapper® program operates with data obtained with automated sequencer (3730x1s DNA Analyser; Applied Biosystems Ltd). It measures the size of labelled DNA fragments by automated fluorescence detection after separating them by capillary electrophoresis technology. To overcome the problem of varying migration times between samples, an internal size standard is added, which is labelled with a different fluorescent dye than the sample PCR products. The size standard can be used to determine the length of the sample DNA fragments. It also serves as a positive control for the scanner analysis. The GeneMapper® system calculates the length of sample fragments based on the size standard length. Using the running times of fragments with known length, the program creates a calibration curve. For this the Local Southern Method is applied, where each point depends on the next two faster and the next two slower size standard fragments. The results of size calling can be displayed as electropherogrammes, as tabular data or as a combination of both. An alternative commercial program GeneMarker is available from <http://www.gatc-biotech.com>. The latter can be used for analysing data from capillary electrophoresis or gels (e.g. PCR-RFLP.)

Previously reported applications of GeneScan®/GeneMapper® genotyping in *P. falciparum* includes microsatellite typing (Anderson et al. 2000) and *msp2* genotyping used to assess clonal population dynamics during malaria treatment (Jafari et al. 2004). Further discrimination of both allelic families of *msp2* has been described (Falk et al. 2006).

Msp2 genotyping.

Msp2 is the most size-polymorphic single copy locus of *P. falciparum*. In order to reach highest sensitivity of the assay, a primary PCR is followed by a nested PCR. For nPCR, fluorochrome-labelled reverse primers are used that are specific for either the 3D7 allelic family or the FC27 allelic family of *msp2*. Genotypes are distinguished by their fluorescent dye (indicating the allelic family) and by their size which is determined by an automated sequencer and GeneMapper® software.

Fluorescently labelled Primers.

As in most GeneMapper® applications, ROX-labelled size standards are used. The size standard used in this study consisted of 16 fragments ranging from 50 to 500 bp in length (Applied Biosystems). 6-FAM has been chosen for labelling

the FC27 family-specific primer M5. The 3D7 specific primer N5 was labelled with VIC. These two colours have been chosen because they have similar intensities and a big difference in emission wavelength.

Tailed Primers.

The tendency of the Taq polymerase to add a non-template nucleotide (usually an A) to the 3' end of the double-stranded DNA leads to PCR fragments which are 1 bp longer than expected. Because this happens only in a fraction of the reaction, a mixed population of fragments arises, and the precision of sizing is corrupted. A tailed forward primer (S_{Tail}) was used, which should promote the A-addition in nearly 100 percent, leading to a homogeneous population.

Equipment

thermocycler: any company such as MJ Research
(Optional: centrifuges for pre-PCR quick spin to collect mixture of master mix puls template at bottom of tube/plate: minifuge for tubes; if plates are used, a centrifuge with adaptor for plates is needed.)

Materials and reagents

PCR Reagents and materials

ddH₂O, home made, store at RT
10x Buffer B, Solis BioDyne, store at -20 °C
dNTPs (Nucleotides) 2 mM, Qiagen, 201913, store at -20 °C
MgCl₂ 25mM, Solis BioDyne, store at -20 °C
FIRE Pol® DNA Polymerase I, Solis BioDyne, store at -20 °C
Specific Oligos (Primer), ABI & Operon, store at -20 °C
Tris EDTA concentrate (100x TE buffer), Fluka, 86377, store at RT
mineral Oil¹, SIGMA, M5904, store at RT (not required if thermocycler with heated lid is used)
pipettes (1000 µL, 200 µL, 20 µL), Gilson, F267630, store at RT
500 µL reaction tubes, Sarstedt, 72.735.002, store at RT
filter tips 2-30 µL, F161933
filter tips 2-200 µL, F161934
filter tips 200-1000 µL, F161673, Gilson, store at RT
twin.tec PCR Plate 96, Eppendorf, 0030 128.575, store at RT
ROX-500 Size Standard, Applied Biosystems, 401734, store at -20 °C
deionised formamide (Hi-Di)
(Proposed supplier for reagents and consumables. Other products might be equally suitable.)

2. PCR primers

Primers used for primary PCR (according to Foley et al. 1992)

Primer	Sequence (5' → 3')	Manufacturer
S2-fw	GAA GGT AAT TAA AAC ATT GTC	Operon
S3-rev	GAG GGA TGT TGC TGC TCC ACA G	Operon

PCR primers (Operon) are dissolved in 1x TE.

Prepare stock solution with a concentration of 100 µM.

For working solution, dilute stock solution to a concentration of 50 µM. Keep aliquots at -20 °C.

Primers used for nested PCR (labelled and unlabelled oligos according to Falk et al. 2006)

Primer	Sequence (5'→ 3')	Manufacturer
S1 _{Tail} -fw	7bpTail - GCT TAT AAT ATG AGT ATA AGG AGA A	Applied Biosystems
M5-rev	6FAM - GCA TTG CCA GAA CTT GAA	Applied Biosystems
N5 -rev	VIC - CTG AAG AGG TAC TGG TAG A	Applied Biosystems

Primers used for nested PCR (labelled and unlabelled oligos according to Falk et al. 2006)

Primer	Sequence (5'→ 3')	Manufacturer
S1 _{Tail} -fw	7bpTail - GCT TAT AAT ATG AGT ATA AGG AGA A	Applied Biosystems
M5-rev	6FAM - GCA TTG CCA GAA CTT GAA	Applied Biosystems
N5 -rev	VIC - CTG AAG AGG TAC TGG TAG A	Applied Biosystems

Post-PCR reagents and materials

ddH₂O, home made, store at RT

ROX-500 Size Standard, Applied Biosystems, 401734, store at -20°C

pipettes (1000µL, 200 µL, 20µL), Gilson, F267630, store at RT

1.5 mL Safe-Lock tubes, Eppendorf, 0030 120.086, store at RT

filter tips 2-30 µL, F161933

filter tips 2-200 µL, F161934

filter tips 200-1000 µL, F161673, Gilson, store at RT

twin. tec PCR Plate 96, Eppendorf, 0030 128.575 , store at RT.

PCR film, Eppendorf, 951023019, store at RT

Procedure

- Master mix primary PCR and primary PCR conditions
- Primary Mix:
 - ddH₂O 34.2 µL
 - 10x Buffer B (Solis) 5 µL
 - dNTPs (2 mM each) 5 µL
 - MgCl₂ (25mM) 3 µL
 - S2 Primer 50µM 0.25 µL
 - S3 Primer 50µM 0.25 µL
 - Taq polymerase (FirePol, Solis Biodyne, 5U/µl) 0.3 µL
 - total volume 48 µL, add DNA 2 µL**

Thermoprofile primary PCR

94 °C - 2 min

94 °C - 30 sec

45 °C - 45 sec 25 cycles*

70 °C - 1 min 30sec

70 °C - 10 min

* depending on efficacy of pPCR in a particular lab setting, number of cycles can be modified.

Master mix nested PCR and nested PCR conditions

• Nested Mix:

- ddH₂O 31.6 µL
- 10x Buffer B (Solis) 5 µL
- dNTPs (2 mM each) 5 µL
- MgCl₂ (25mM) 3 µL
- Primer S1_{Tail} (10 µM) 2 µL
- Primer N5 (10 µM) 1 µL
- Primer M5 (10 µM) 1 µL
- Taq polymerase (FirePol, Solis Biodyne, 5U/µl) 0.4 µL
- total volume 49 µL,**
- add pPCR product 1 µL,**

Thermoprofile nested PCR
94°C - 2 min

94 °C - 30 sec
50 °C - 45 sec 25 cycles*
70 °C - 1 min 30 sec

70 °C - 10 min

* depending on efficacy of nPCR in a particular lab setting, number of cycles can be modified.

- Prepare master mix for all samples to be amplified (plus positive and no template control) according to 3. or 4. in a template-free room dedicated to PCR with dedicated no template pipettes; use aerosol protected pipette tips. Aliquot master mix to reaction tubes or 96 well plate.
- Add DNA template or ddH₂O in case of no template control to master mix. Use a separate tip for each DNA sample. The template-adding step should be performed at a location different from that used for master mix preparation.
- If the thermo cycler used lacks a heated lid, overlay aliquoted master mix with 2 drops of mineral oil. In this case, the DNA template or pPCR product is added through the oil layer to the reaction mixture.
- Optional: To decrease risk of contamination, quick spin all tubes and plates containing extracted DNA or PCR product before opening tubes or removing caps or PCR film from plates.
- Post-PCR procedures
- Sample preparation for capillary electrophoresis:
- nPCR products (positivity checked on an agarose gel) are prepared for capillary electrophoresis as follows:
- 975 µl of ddH₂O are mixed with 25 µL of 8 nM size standard ROX-500 (1:40 dilution) and vortexed well. 10 µL of this solution are pipetted into each well of a 96 well plate, resulting in 0.25 µL of size standard per well.
- In a new 96 well plate 22.5 µL of water are pipetted in each well and 2.5 µL of nPCR product that have been found positive by gel electrophoresis are added in each well (1:10 dilution), the mixture is pipetted up and down several times to assure it is mixed well.

- 2.5 μL of the diluted PCR product are added to the size standard. The final amount of sample DNA corresponds to 0.25 μL PCR product per well and a size standard concentration of 0.16 nM. The solution is mixed by pipetting up and down several times.
- Prior to loading the automated sequencer 10 μL highly deionised formamide (Hi-Di) is added per well and carefully mixed. Then the plates are run on a 3730xIs DNA Analyser (Applied Biosystems Ltd). The output files of the sequencer are in .fsa-format.
- Shipment of samples:
- If fragment sizing by capillary electrophoresis is not available, the plates are shipped to a provider of this service. The plate containing the correct dilutions of size standard and PCR product are put for air-drying in a dark box or drawer overnight. The next day, after all the liquid has evaporated, the plate is sealed with PCR film, labelled, covered with aluminium foil for protection against light and sent by mail.
- At the receiving end, the plates are spun down at 1650 g for a minute and stored at $-20\text{ }^{\circ}\text{C}$ until further processing. Prior to loading the automated sequencer, 10 μL highly deionised formamide (Hi-Di) is added per well and the samples are incubated at room temperature for 45 min.
- Analysis of samples with GeneMapper® Software:
- Electropherogrammes are analysed using the GeneMapper® program (Applied Biosystems Ltd.). Peaks are identified using the following parameters: Minimal peak width is set to 2 measurement points, the polynomial degree of the fitting curve is 3, and the peak window size is 15 measurement points. The sizes of the fragments are calculated by the local southern method, where each point is analysed with help of the next two points in either direction. The peak list generated by GeneMapper® is exported as tab delimited text for further analysis.

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XI. Monitoring of malaria drug resistance associated SNPs in *P. falciparum* on microarray

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Equipment

PCR Thermocycler
incubator
centrifuge
microarray reader (microscopic slide format)
computer
12 or 8 channel pipette

Materials and reagents

QIAamp 96 DNA Blood Kit

96 well plates
10 x PCR Buffer
EDTA
dNTP mix containing all four nucleotides at 2mM conc.
25 mM MgCl₂
primer mixes for primary and nested PCR reactions
Taq Polymerase
thermostable DNA polymerase
H₂O
ddNTP mixes (labelled with Cy3 and Cy5)
extension primer mix
Shrimp Alkaline Phosphatase
2x SSC + 0.1% SDS
2x SSC
2xSSC + 1% EtOH
10% SDS
custom made microarrays for drug resistance associated SNPs

Principle of procedure

In order to analyse all known single nucleotide polymorphisms associated with antimalarial drug resistance we have developed this microarray platform. The microarray is currently set up for the following SNPs:

Pfdhps 436, 437, 540, 581, 613, 640 645

Pfdhfr 16, 51, 59, 108, 164

Pfmdr1 86, 184, 1034, 1042, 1246

Pfcrt 72, 74,75, 76, 97,152, 163, 220, 271, 326, 356, 371

Pfatpase6 538, 574, 623, 683,769

This is essentially a nested PCR amplification of target sequences containing the SNPs in question, followed by shrimp alkaline phosphatase digestion to eliminate all dNTPs for the subsequent step of a one tube primer extension reaction with fluorochrome labelled ddNTPs over the SNP sites. Because most affordable microarray readers can only satisfactorily discriminate two fluorochromes simultaneously, two assays with different dye combinations have to be set up. After the extension reaction, extended primers are hybridized to antisense primers deposited on glass microarrays. Slides are scanned with

a microarray reader and dedicated software which identifies wildtype, mutant or mixed infection for each of the SNP sites.

This technique allows the analysis of several hundred samples within a time period of few days at very affordable costs. This technique is not suited for individual sample analysis, and therefore this protocol is set up for at least 48 samples.

Procedure (Cramer et al. 2007)

- Prepare DNA from whole blood (anti-coagulated with EDTA) or red blood cell pellets using QIAamp[®] 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions (http://www1.qiagen.com/literature/protocols/QIAamp96_DNABlood.aspx)

Amplification of target sequences

- Primary PCR: set up reactions in 96 well plates to 50 µL final volume per well
- prepare per well:
 - 30.0 µL H₂O
 - 5.0 µL 10 x PCR buffer (without MgCl₂)¹
 - 5.0 µL dNTP mix (2mM each)
 - 6.0 µL 25mM MgCl₂
 - 1.0 µL Primary PCR primer mix (10 µM each)²
 - 0.5 µL Taq polymerase 5U/µl
- distribute 47.5 µl of the mix into each well
- add 2.5 µl DNA
- run thermocycler program
 - 96 °C 180 sec
 - 96 °C 30 sec
 - 52 °C 90 sec
 - 72 °C 90 sec

20 cycles for symptomatic samples

25 cycles for asymptomatic samples

¹ see buffers and reagents

² Primary PCR primer mixes (sequence information: see primer table):

- 1.P 1-1(*pfmdr1* PCR I:P 1-1 for / P1-1 rev, 10 µM each in TE buffer)
- 2.P 3-1(*pfmdr1* PCR II:P 3-1 for / P3-1 rev, 10 µM each in TE buffer)
- 3.P 5-1(*pfdhfr* PCR:P 5-1 for / P5-1 rev, 10 µM each in TE buffer)
- 4.P 8-1(*pfdhps* PCR:P 8-1 for / P8-1 rev, 10 µM each in TE buffer)
- 5.P 10-1(*pfcr1* PCR I:P 10-1 for / P10-1 rev, 10 µM each in TE buffer)
- 6.P 11-1(*pfcr1* PCR II:P 11-1 for / P11-1 rev, 10 µM each in TE buffer)
- 7.P 12-1(*pfcr1* PCR III:P 12-1 for / P12-1 rev, 10 µM each in TE buffer)
- 8.P 16-1(*pfcr1* PCR IV:P 16-1 for / P16-1 rev, 10 µM each in TE buffer)
- 9.P 17-1(*pfATPase6* PCR:P 17-1 for / P17-1 rev, 10 µM each in TE buffer)
- 10.P 18-1(*pfcr1* PCR V:P 18-1 for / P18-1 rev, 10 µM each in TE buffer)

Nested PCR:

- transfer into new 96 well plate to 100 µl final volume
- prepare per well:
 - 60.0 µL H₂O
 - 10.0 µL 10 x PCR buffer (without MgCl₂)

10.0 μ L dNTP mix (2mM)
 12.0 μ L $MgCl_2$ (25mM)
 2.0 μ L Nested primer mix (10 μ M each)³
 1.0 μ L Taq polymerase 5U/ μ l

- distribute 95 μ L of the mix into each well
- add 5 μ L DNA Primary PCR product
- run thermocycler program
 96 °C 180 sec

96 °C 30 sec
 52 °C 90 sec
 72 °C 90 sec

20 cycles for symptomatic samples

25 cycles for asymptomatic samples

³ Nested PCR primer mixes (sequence information: see primer table):

11.P 1 (*pfmdr1* PCR I: P 1 for / P1 rev, 10 μ M each in TE buffer)
 12.P 3 (*pfmdr1* PCR II: P 3 for / P3 rev, 10 μ M each in TE buffer)
 13.P 5 (*pfdhfr* PCR: P 5 for / P5 rev, 10 μ M each in TE buffer)
 14.P 8 (*pfdhps* PCR: P 8 for / P8 rev, 10 μ M each in TE buffer)
 15.P 10 (*pfcr1* PCR I: P 10 for / P10 rev, 10 μ M each in TE buffer)
 16.P 11 (*pfcr1* PCR II: P 11 for / P11 rev, 10 μ M each in TE buffer)
 17.P 12 (*pfcr1* PCR III: P 12 for / P12 rev, 10 μ M each in TE buffer)
 18.P 16 (*pfcr1* PCR IV: P 16 for / P16 rev, 10 μ M each in TE buffer)
 19.P 17 (*pfATPase6* PCR: P 17 for / P17 rev, 10 • M each in TE buffer)
 20.P 18 (*pfcr1* PCR V: P 18 for / P18 rev, 10 • M each in TE buffer)

SAP (Shrimp Alkaline Phosphatase) digest of PCR products

- pool 10 μ L of each nPCR reaction into a single well of a new 96 well plate
- mix and centrifuge briefly (PCR pool plate).
- transfer 10 μ L of each well of PCR pool plate into a new 96 well plate and add 90 μ L of H₂O to each well.
- mix and centrifuge briefly. (PCR pool plate 1:10).
- add 7 μ L SAP mix to each well of a new 96 well plate.
- add 5 μ L DNA from PCR pool plate 1:10 in duplicate (thus each plate holds now DNA in duplicate from 48 samples).
 SAP mix:
 4.0 μ l H₂O
 1.0 μ l 10 x SAP buffer
 2.0 μ l Shrimp Alkaline Phosphatase (SAP) 1U/ μ L
- run thermocycler program: SAP digest: 1 hour at 37 °C, inactivation of SAP digest: 15 min at 90 °C

Primer extension

- Preparation of ddNTP mix (2 combinations):
 Combination 1: ddATP Cy3, ddCTP Cy3, ddGTP Cy5, ddUTP Cy5
 Combination 2: ddUTP Cy3, ddCTP Cy3, ddATP Cy5, ddGTP Cy5

- Stock concentration of labelled ddNTPs is 100 μM (Perkin Elmer, Boston, USA)
- combine 25 μl 100 μM of each ddNTP stock (final conc of each ddNTP is 25 μM)
- add 900 μL TE buffer (final concentration of each ddNTP is 2.5 μM)
- make aliquots and store as 2.5 μM ddNTP mix at $-20\text{ }^{\circ}\text{C}$

Preparation of extension primer mix (2 combinations)

- Combination 1: *Pfdhps* 437, 540, 581, 613, 640
Pfdhfr 16, 51, 59, 108, 164
Pfmdr1 86, 184, 1034, 1042
Pfcrt 72, 75B1, 152, 271, 326, 326B, 356, 356B
Pfatpase6 538, 769, 769B
- Combination 2: *Pfdhps* 436, 613B, 645
Pfdhfr 108B, 164B
Pfmdr1 1246
Pfcrt 74, 76, 97, 163, 220, 371
Pfatpase6 574, 623, 683
- Primer stock concentration is 10 μM in TE:
- add 2 μl of each primer stock to the respective combination
- add TE accordingly to obtain a 62.5 nM conc. of each primer
for Combination 1 (25 x 2 μl = 50 μl): add 270 μl TE buffer
for Combination 2 (15 x 2 μl = 30 μl): add 290 μl TE buffer
- store extension primer mixes at $+4\text{ }^{\circ}\text{C}$

Extension reaction

- set up 2 primer extension mixes (final volume of 20 μL)
- prepare per well:
 - 1.6 μL H_2O
 - 2 μL Sequenase Buffer (Solis Biodyne Buffer + 2,5mM MgCl_2)
 - 2 μL Extension primer mix Combination 1 or Combination 2 (62.5 nM)
 - 2 μL ddNTP mix Combination 1 (2.5 μM)
 - 0.4 μL Thermo Sequenase (5U/ μl)
- add 8 μl of extension mix (Combination 1 and Cobination 2) to the SAP digested PCR products in the SAP plate to a final volume of 20 μl (fill one row with Combination mix 1, and fill the next row with combination mix 2)
- run thermocycler program
 - 94 $^{\circ}\text{C}$ 60 sec
 - 94 $^{\circ}\text{C}$ 10 sec
 - 50 $^{\circ}\text{C}$ 40 sec
 - 35 cycles

Hybridization to microarray⁴

- pool extension reaction mixes Combination 1 and Combination 2 from each sample (final volume = 40 μL)
- add 6 μl of denaturing solution to a final volume of 46 μl :
 - 0.5 μL 0.5M EDTA pH 8.0
 - 2.0 μL 10% SDS

3.5 μL H_2O

- incubate at 94 °C for 60 sec
- keep on ice for 2 min
- add 23 μL of combined extension reaction into 1 well of the microarray
- add 6 μL 20 x SSC to each well of the slide
- incubate microarray in a dark humid chamber at 50 °C for 60-90 min
- wash microarray with:
 - 2x SSC + 0.2% SDS: 10 min at room temperature (RT)
 - 2x SSC: 10 min at RT
 - 2x SSC + 2% EtOH: 2 min at RT
- dry microarray with compressed air
- store at RT in the dark until read
- ⁴ arrays are spotted on patterned microscopic slides (12 or 16 well slides) with aldehyde activated surfaces (Array-It, Telechem, Sunnyvale, USA). The spotting protocol is available on request from hans-peter.beck@unibas.ch

Data acquisition and analysis

- Microarrays can be scanned with any microarray scanner suitable for microscopic slides.
- acquire signals for Cy3 (wavelength: 532 nm)
- acquire signals for Cy5 (wavelength: 635 nm)
- store single signal or combined signal images as tif-files for further analysis
- name files with unique identification code for study, slide, experiment, and operator

tif-files can be analysed using commercially available software such as Axon GenePix Pro (www.axon.com).

Additional dedicated software to determine the appropriate genotypes for each SNP site including recognition of mixed infections can be obtained from hans-peter.beck@unibas.ch

Buffers, reagents, and oligonucleotides

Buffers

- 500 mM EDTA pH 8.0
- 180 mM phosphate buffer pH 8.0
- 20 x SSC pH 7.0
- 2 x SSC
- 2 x SSC + 0.2% SDS
- 2 x SSC + 2% EtOH
- 10% SDS
- 1 x TE buffer (= 10 mM Tris/HCl pH 8.0)
- 10 mM Tris/HCl pH 7.4
- Prepare all buffers/solutions according to the protocols in Sambrook et al. 1989
- Store all buffers at room temperature

Reagents

- 10 x PCR buffer (=buffer B) (Solis BioDyne, Tartu, Estonia)
- 25 mM MgCl₂ (Solis BioDyne, Tartu, Estonia)
- *Taq* polymerase (Firepol®; 5 U/μl) (Solis BioDyne, Tartu, Estonia)
- dNTP mix (2mM each) (Qiagen, Hilden, Germany): dilute 100 mM stock solutions 1:50 in 10 mM Tris/HCl pH 7.4
- 10 x SAP buffer (Amersham Biosciences)
- Shrimp Alkaline Phosphatase (SAP; 1U/μl)(Amersham Biosciences)
- 10 x Sequenase buffer (Amersham Biosciences)
- Thermo Sequenase (Termipol®; 5 U/μl) (Solis BioDyne, Tartu, Estonia)
- Store all reagents at -20 °C

- Cy3 /Cy5 labelled ddNTP (Perkin Elmer)
- Store 100 μM ddNTP stock solutions at -80 °C
- Oligonucleotides for microarray spotting:
500 μM stock solutions in 180 mM phosphate buffer pH 8.0 (aliquots at -20°C), all spotting oligos carry an amino C-7 linker at the 3' end (Operon, Cologne, Germany)
- Oligonucleotides for extension:
100 μM stock solutions in TE buffer pH 8.0 aliquots at -20°C, HPLC purified (Operon, Cologne, Germany)

Oligonucleotide primer sequences (Operon Biotechnologies GmbH, Cologne, Germany)

Name	Locus / fragment	Sequence
	Primary PCR amplification	5' 3'
P5-for	<i>dhfr</i>	TTTATGATGGAACAAGTCTGC
P5-1 rev	<i>dhfr</i>	ATTCATATGTACTATTTATTCTAGT
P8-1 for	<i>dhps</i>	ATTTTTGTTGAACCTAAACGTGCTGTTCA
P8-1 rev	<i>dhps</i>	CTTGTCTTTCCCTCATGTAATTCATCT
P1-1 for	<i>mdr1</i> , first fragment	TTAAATGTTTACCTGCACAACATAGAAAATT
P1-1 rev	<i>mdr1</i> , first fragment	CTCCACAATAACTTGCAACAGTTCTTA
P3-1 for	<i>mdr1</i> , second fragment	AATTTGATAGAAAAAGCTATTGATTATAA
P3-1 rev	<i>mdr1</i> , second fragment	TATTTGGTAATGATTCGATAAATTCATC
P10-1 for	<i>crt</i> , first fragment	TTGTCGACCTTAACAGATGGCTCAC
P10-1 rev	<i>crt</i> , first fragment	AATTTCCCTTTTTATTTCAAATAAGGA
P18-1 for	<i>crt</i> , second fragment	ACTTTATTTGTATGATTATGTTT
P18-1 rev	<i>crt</i> , second fragment	TAACGCTCCGAGATAATTGT
P11-1 for	<i>crt</i> , third fragment	ATTTACTCCTTTTTAGATATCACTTA
P11-1 rev	<i>crt</i> , third fragment	TTATATTTTTTAAAACTATTTCCCTTG
P16-1 for	<i>crt</i> , fourth fragment	TCTGTTATTTTTATTTCTTATAGGCTAT

P16-1 rev	<i>crt</i> , fourth fragment	CTTGTATGTATCAACGTTTTTCATCC
P12-1 for	<i>crt</i> , fifth fragment	AGGAAATAAATATGGGAATGTTTAATTGA
P12-1 rev	<i>crt</i> , fifth fragment	TTCTAAGATAATATTTTCTACACGGT
P17-1 for	<i>ATPase6</i>	AATATTGTTATTTCAGAAATATGATTATAA
P17-1 rev	<i>ATPase6</i>	TGGATCAATAATACCTAATCCACCTA
	Nested PCR amplification	5' 3'
P5 for	<i>dhfr</i>	ACAAGTCTGCGACGTTTTTCGATATTTATG
P5 rev	<i>dhfr</i>	AGTATATACATCGCTAACAGA
P8 for	<i>dhps</i>	TTGAAATGATAAATGAAGGTGCTAGT
P8 rev	<i>dhps</i>	CCAATTGTGTGATTTGTCCA
P1 for	<i>mdr1</i> , first fragment	TGTATGTGCTGTATTATCAGGA
P1 rev	<i>mdr1</i> , first fragment	CTCTTCTATAATGGACATGGTA
P3 for	<i>mdr1</i> , second fragment	GAATTATTGTAAATGCAGCTTTA
P3 rev	<i>mdr1</i> , second fragment	GCAGCAAACCTACTAACACG
P10 for	<i>crt</i> , first fragment	CTTGTCTTGGTAAATGTGCTC
P10 rev	<i>crt</i> first fragment	GAACATAATCATACAAATAAAGT
P18 for	<i>crt</i> , second fragment	TCCTTATTTGGAAATAAAAAGGGAAATT
P18 rev	<i>crt</i> , second fragment	TAAGTGATATCTAAAAAGGAGTAAAT
P11 for	<i>crt</i> , third fragment	ACAATTATCTCGGAGCAGTTA
P11 rev	<i>crt</i> , third fragment	CATGTTTGAAAAGCATAACAGGC
P16 for	<i>crt</i> , fourth fragment	CTTTTTCCAATTGTTCACTTCTTG
P16 rev	<i>crt</i> , fourth fragment	TCTTACATAGCTGGTTATTAAT
P12 for	<i>crt</i> , fifth fragment	ACCATGACATATACTATTGTTAG
P12 rev	<i>crt</i> , fifth fragment	TTATAGAACCAAATAGGTAGCC
P17 for	<i>ATPase6</i>	AGCAAATATTTTCTGTAACGATAATA
P17 rev	<i>ATPase6</i>	TGTTCTAATTTATAATAATCATCTGT
	Extension primer at SNP site	5' 3'
16	<i>dhfr</i> 16	GACGTTTTTCGATATTTATGCCATATGTG
51	<i>dhfr</i> 51	GAAATAAAGGAGTATTACCATGGAAATGTA
59	<i>dhfr</i> 59	TTACATATGTTGTAACCTGCAC
108	<i>dhfr</i> 108 forward	CAAAATGTTGTAGTTATGGGAAGAACAA
108B	<i>dhfr</i> reverse	AAAGGTTTAAATTTTTTTGGAATGCTTTCCCAG
164	<i>dhfr</i> 164 forward	GGGAAATTAATTAATAAATGTTTTATT
164B	<i>dhfr</i> 164 reverse	TTCTTGATAAACAACGGAACCTCCTA
436	<i>dhps</i> 436	TTATAGATATAGGTGGAGAATCC

437	<i>dhps</i> 437 reverse	TTGGATTAGGTATAACAAAAGGA
540	<i>dhps</i> 540	AGGAAATCCACATACAATGGAT
581	<i>dhps</i> 581	GGATACTATTTGATATTGGATTAGGATTTG
613	<i>dhps</i> 613 forward	GGATATTCAAGAAAAAGATTTATT
613B	<i>dhps</i> 613 reverse	ATTTTGATCATTTCATGCAATGGG
640	<i>dhps</i> 640 reverse	CAATTGTGTGATTTGTCCACAA
645	<i>dhps</i> 645	ATAAAAAATATTGTGGACAAATCAC
86	<i>mdr1</i> 86	TTTGGTGTAAATATTAAGAACATG
184	<i>mdr1</i> 184	TGCCAGTTCCTTTTTAGGTTTAT
1034	<i>mdr1</i> 1034	ATTGTAATGCAGCTTTATGGGGATTC
1042	<i>mdr1</i> 1042 reverse	AGAAGGATCCAAACCAATAGGCAAACTAT
1246	<i>mdr1</i> 1246	TAATATATGTGATTATAACTTAAGA
72	<i>crt</i> 72	TTTTAAGTATTATTTATTTAAGTGTA
76	<i>crt</i> 76 reverse	TTTGTTTAAAGTTCCTTTTAGCAAAAATT
97	<i>crt</i> 97	GTTTTGTAACATCCGAAACTCA
152	<i>crt</i> 152	CCTTCATAGGTCTTACAAGAACT
163	<i>crt</i> 163	ATCCAATCATTGTCTTCAATTAAG
220	<i>crt</i> 220	TTCTATCATATTTAATCTTGTCTTAATTAGT
271	<i>crt</i> 271	TATACACCCTTCCATTTTTAAAA
326	<i>crt</i> 326	AAACCTTCGCATTGTTTTCTTCTTT
326B	<i>crt</i> 326 reverse	ACATAGCTGGTTATTAATTATCACAAATG
356	<i>crt</i> 356	TTGTTAGTTGTATACAAGGTCCAGCA
356B	<i>crt</i> 356 reverse	GGCTAAGAATTTAAAGTAATAAGCAATTGCT
371	<i>crt</i> 371	CTTTTTAATTTTATAGGGTGATGTTGTAA
538	<i>ATPase6</i> 538	AAATGTAATAAAGCTAATTCGGT
574	<i>ATPase6</i> 574	TGAAAAAATACAACACCTGTAC
623	<i>ATPase6</i> 623	AACCATTCTAATTATACTACAGCTCAGG
683	<i>ATPase6</i> 683	TGAATGTATTTCTTCTTGAGAAA
769	<i>ATPase6</i> 769	ACTTAGCTTTGCTTATAAAAAATTAA
	Arrayed as antisense oligonucleotides	5' 3' ---- C7
16 C-7	<i>dhfr</i> 16	CACATATGGCATAAATATCGAAAACGTC
51 C-7	<i>dhfr</i> 51	TACATTTCCATGGTAATACTCCTTTATTTTC
59 C-7	<i>dhfr</i> 59	GTGCAGTTACAACATATGTGAA
108 C-7	<i>dhfr</i> 108 forward	TTGTTCTTCCCATAACTACAACATTTTG

108B C-7	<i>dhfr</i> reverse	CTGGGAAAGCATTCCAAAAAATTTAAACCTTT
164 C-7	<i>dhfr</i> 164 forward	AATAAACATTTTATAGTAATTTAATTTCCC
164B C-7	<i>dhfr</i> 164 reverse	TAGGAGGTTCCGTTGTTTATCAAGAA
436 C-7	<i>dhps</i> 436	GGATTCTCCACCTATATCTATAA
437 C-7	<i>dhps</i> 437 reverse	TCCTTTTGTTATACCTAATCCAA
540 C-7	<i>dhps</i> 540	ATCCATTGTATGTGGATTTCCA
581 C-7	<i>dhps</i> 581	CAAATCCTAATCCAATATCAAATAGTATCC
613 C-7	<i>dhps</i> 613 forward	AATAAATCTTTTTCTTGAATATCC
613B C-7	<i>dhps</i> 613 reverse	CCCATTGCATGAATGATCAAAAT
640 C-7	<i>dhps</i> 640 reverse	TTGTGGACAAATCACACAATTG
645 C-7	<i>dhps</i> 645	GTGATTTGTCCACAATATTTTTAT
86 C-7	<i>mdr1</i> 86	CATGTTCTTTAATATTACACCAAA
184 C-7	<i>mdr1</i> 184	ATAAACCTAAAAAGGAAGTGGCA
1034 C-7	<i>mdr1</i> 1034	GAATCCCCATAAAGCTGCATTTACAAT
1042 C-7	<i>mdr1</i> 1042 reverse	ATAGTTTTGCCTATTGGTTTGGATCCTTCT
1246 C-7	<i>mdr1</i> 1246	TCTTAAGTTATAATCACATATATTA
72 C-7	<i>crt</i> 72	TACACTTAAATAAATAACTTAAAA
76 C-7	<i>crt</i> 76 reverse	AATTTTTGCTAAAAGAACTTTAAACAAA
97 C-7	<i>crt</i> 97	TGAGTTTCGGATGTTACAAAAC
152 C-7	<i>crt</i> 152	AGTTCTTGTAAGACCTATGAAGG
163 C-7	<i>crt</i> 163	CTTAATTGAAGAACAATGATTGGAT
220 C-7	<i>crt</i> 220	ACTAATTAAGACAAGATTAATATGATAGAA
271 C-7	<i>crt</i> 271	TTTTAAAAATGGAAGGGTGTATA
326 C-7	<i>crt</i> 326	AAAGAAGGAAAACAATGCGAAGGTTT
326B C-7	<i>crt</i> 326 reverse	CATTTGTGATAATTTAATAACCAGCTATGT
356 C-7	<i>crt</i> 356	TGCTGGACCTTGATACAACATAACAA
356B C-7	<i>crt</i> 356 reverse	AGCAATTGCTTATTACTTTAAATTCTTAGCC
371 C-7	<i>crt</i> 371	TTACAACATCACCCCTATAAAATTAATAAG
538	<i>ATPase6</i> 538	ACCGAATTAGCTTTATTACATTT
574	<i>ATPase6</i> 574	GTACAGGTGTTGTATTTTTTTTCA
623	<i>ATPase6</i> 623	CCTGAGCTGTAGTATAATTAGAATGGTT
683	<i>ATPase6</i> 683	TTTCTCCAAGAAGAAATACATTCA
769	<i>ATPase6</i> 769	TTAATTTTTTATAAGCAAAGCTAAGT

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XII. Nuclear run-on analysis

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Equipment

refrigerated centrifuge
dounce homogenizer (B-pestle)
hybridization oven
water baths/heating blocks (30°C, 37°C, 42°C and 100°C)
liquid scintillation counter
dot blot manifold (this protocol is for the 96-well manifold from Schleicher & Schuell)
pump to apply suction to the manifold

Materials and reagents

15 mL Falcon tubes
centrifugation tubes, resistant to organic solvents and 12 000g
Eppendorf tubes, RNase free
DEPC (diethyl pyrocarbonate)
0.1% Saponin in PBS (freshly made)
Triton X-100
100 µg yeast RNA
TRIZOL (Gibco)
chloroform
isopropanol
75% Ethanol
Chromaspin columns (Chromaspin+TE-100; Clontech)
125 U/mL RnaseOut (Gibco)
100-150 µCi P³²-UTP 3000 Ci/mmol (Amersham Pharmacia)
DNase1 (RNase free!!) (Amersham Pharmacia)
DNA of interest, e.g. PCR products
Whatman papers, cut to fit the dot blot manifold
membrane, cut to fit the dot blot manifold (we recommend Hybond N⁺ from Amersham)
1 M NaOH
200 mM EDTA pH8.2
ddH₂O
0.4 M NaOH

Buffer A:
10 mM Tris-HCl pH7.4
4 mM EDTA
15 mM NaCl
60 mM KCl
1 mM DTT
1 mM PMSF
0.15 mM Spermine
0.5 mM Spermidine

Buffer B:

50 mM Hepes pH7.9
50 mM NaCl
10 mM MgCl₂
1.2 mM DTT
10 mM Creatine phosphate
0.2 mg/mL creatine kinase
1 mM GTP and CTP
4 mM ATP
25% Glycerol

HSB (high salt buffer):

0.5 M NaCl
50 mM MgCl₂
2 mM CaCl₂
10 mM Tris-HCl pH7,4

Stop Buffer:

5% SDS
0.5 M Tris pH 7,4
0.125 M EDTA

Proteinase K

20 mg/mL in HSB buffer

Prehybridization solution:

50 mM Tris pH7,4
0.3 M NaCl
10 mM EDTA
0.2% SDS
1 mg/mL tRNA
0.5 mg/mL polyA
1% sodium pyrophosphate
5x Denhardts solution

Hybridization solution:

50 mM Tris pH7.4
0.3 M NaCl
10 mM EDTA
0.2% SDS
100 µg/mL tRNA
100 µg/mL polyA
0.1% sodium pyrophosphate
1x Denhardts solution

100x Denhardts solution:

20 mg/mL ficoll
20 mg/mL polyvinylpyrrolidone

20 mg/mL BSA

Wash solution 1:
2xSSC + 0.1% SDS

Wash solution 2:
0.1xSSC + 0.1% SDS

20x SSC :
3 M NaCl
0.3 M Na-Citrate

Procedure

- **Important: keep on ice at all times!!**
- Prepare all solutions with DEPC or DEPC-treated water. Use RNase-free plastic ware. Add CTP, GTP, ATP and Denhardt's solution to their respective solutions before use. Prepare and add DTT, spermidine, spermine, PMSF, creatine kinase, creatine phosphate, Proteinase K, yeast RNA, tRNA, polyA and sodium pyrophosphate before use.
- **Nuclei isolation (all on ice!!!) and labeling**
 - Harvest cells from a *P. falciparum* culture. For the analysis of trophozoites and schizonts, 10 mL packed red blood cells with a parasitemia of > 5% are required and, for the analysis of rings, 20 mL of packed red cells with a parasitemia of > 10%.
 - Collect cells in 15 mL Falcon tubes on ice.
 - Discard supernatant.
 - Add 10 mL of 0.1% saponin (in PBS) to 1.5 mL of packed red blood cells. Erythrocytes are lysed by resuspension in saponin (0.1% w/v in PBS), permeabilizing both the host cell membrane and the parasitophorous vacuolar membrane (Benting et al. 1994; Ansorge et al. 1997; Allen and Kirk 2004).
 - Leave on ice until erythrocytes are completely lysed. It takes approximately 5 min.
 - Spin down at 2700 g for 8 min at 4 °C.
 - Discard supernatant and wash pellet in buffer A (5-6 mL).
 - Spin down at 2700 g for 8 min at 4 °C.
 - Discard supernatant and resuspend liberated parasites in 2 mL of buffer A. Transfer the cell suspension to the Dounce homogenizer - on ice.
 - Add 20 µL Triton X-100.
 - To release nuclei, apply 10 strokes with the Dounce homogenizer.
 - Spin at 12 000 g for 8 min at 4 °C.
 - Discard supernatant and wash pellet in 1 mL of buffer A.
 - Spin at 12 000 g for 8 min at 4 °C.
 - Discard supernatant and resuspend pellet in 600 µL buffer B.
 - Add 125 U/mL RNaseOut or other RNase inhibitor.
 - Add 100 – 150 µCi of α -³²P UTP.
 - Incubate for 30 min at 37°C.

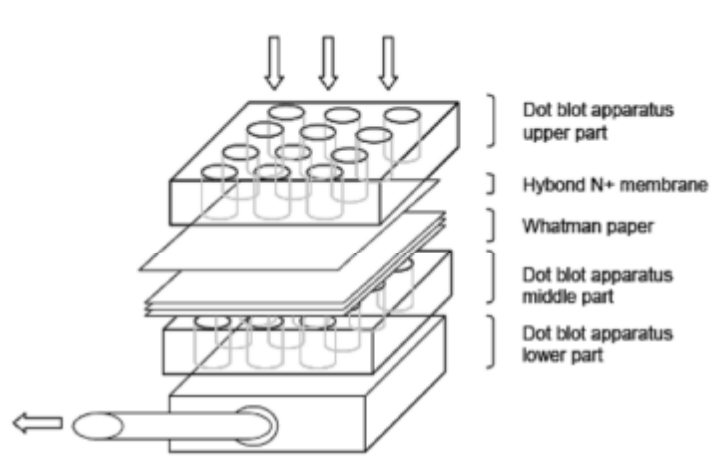
- Add 600 μL HSB and 10 U DNase.
- Incubate for 5 min at 30°C.
- Add 10 μL proteinase K from a stock of 20 mg/mL.
- Incubate for 30 min at 42°C.
- Add 200 μL Stop buffer

RNA isolation using TRIzol

- Add:
 - 100 μg yeast RNA
 - 4.5 mL TRIzol
 - 1.2 mL Chloroform
- Shake vigorously.
- Incubate for 5 min at room temperature.
- Spin down at 12000 g for 10 min at 4°C.
- Transfer aqueous phase to a new tube.
- Add 3 mL isopropanol.
- Incubate for 10 min at room temperature.
- Spin at 12 000g for 10 min at 4°C.
- Discard supernatant and wash pellet with 75% ethanol.
- Spin down at 7 000 g for 5 min at 4°C.
- Discard supernatant carefully and air dry the RNA pellet for approximately 10 min.
- Resuspend RNA pellet in 70 μL H₂O (RNase free!).
- RNA is then size fractionated on a Chromaspin 100 column, which removes unincorporated label and RNA species smaller than 100 bp.
- To prepare the columns, spin for 5 minutes at 800g. Transfer isolated RNA carefully to columns, without letting the sample touch the sides of the column. Elute by centrifuging at 800g for 5 minutes.
- Measure the radioactivity of 1 μL in a liquid scintillation counter to ensure incorporation of α -³²P UTP. Usually 20.000 to 50.000 cpm are obtained per 1 μL .

Preparation of membrane

- Resuspend 1 pmol of double-stranded DNA in 100 μL of 0.4 M NaOH and 10 mM EDTA.
- Incubate at 100°C for 10 minutes and transfer to ice.
- Pre-wet the membranes in ddH₂O for 10 minutes.
- Pre-wet the Whatman papers in ddH₂O very shortly.
- Assemble the dot blot manifold, according to the manufacturer's recommendations (see figure)
- Add 400 μl ddH₂O to all the wells and apply vacuum pressure.
- Add the DNA to the wells and let it filter through (under suction). For orientation, add some colour, e.g. bromophenol blue, to some of the wells without DNA.
- Rinse the wells and membrane twice by adding 200 μl 0.4 M NaOH
- Let the membrane air dry. In a dry state, the membranes can be kept at room temperature until needed.
- Prehybridize membrane in prehybridization solution overnight at 65°C.



Hybridization and washing

- Add the labeled RNA to the hybridization solution (the smaller the volume the better), mix and add to the prehybridized membrane. Let hybridize over night at 42°C.
- Wash membrane three times in wash solution 1 for 20 minutes at room temperature.
- Wash membrane twice for 20 minutes in wash solution 2 at 42°C.
- Analyse the results using a Phosphorimager or alternatively expose the membrane to a conventional X-ray film (at least over night exposure).

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XIII. SDS-Protein PAGE and Proteindetection by Silverstaining and Immunoblotting of *Plasmodium falciparum* proteins

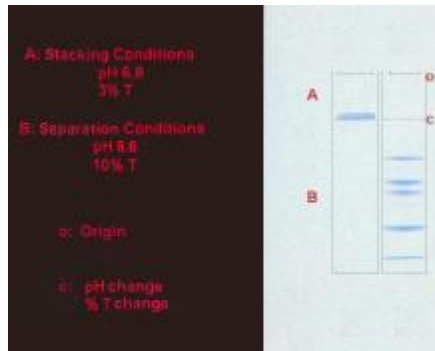
by **Ernst Hempelmann**

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Background

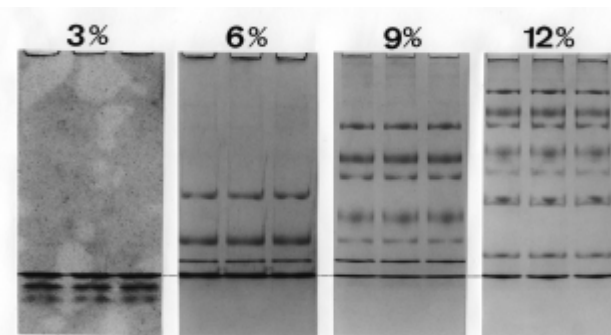
Formation of high concentrated start zones (stack formation) before the actual separation is the reason for a good separation. The SDS protein stack forms after a voltage is applied at the border between the Cl^- of the gel buffer and the Gly^- of the cathode buffer. As it migrates through a pore gradient, the protein stack gradually disperses. Unstacking occurs continuously in the gel, for every protein at a different position. For a complete protein unstacking the polyacrylamide-gel concentration must exceed 16%. The two-gel system of Laemmli (1970) is a simple gradient gel. The pH discontinuity of the buffer ("dis-electrophoresis") is of no significance for the separation quality, and a "stacking-gel" with a different pH should be avoided.



Postulated principle of the "Laemmli Gel" System : It is postulated that stacking and separation of SDS-proteins take place in different gels (Laemmli, 1970).

SDS gradient gel electrophoresis of proteins

SDS gel electrophoresis of proteins is performed in linear 6% - 20% polyacrylamide gradient slab gels of 0.75 mm thickness or in linear 10% polyacrylamide slab gels with a standard buffer system at 4°C and 15 mA [Hempelmann et al. 1987].



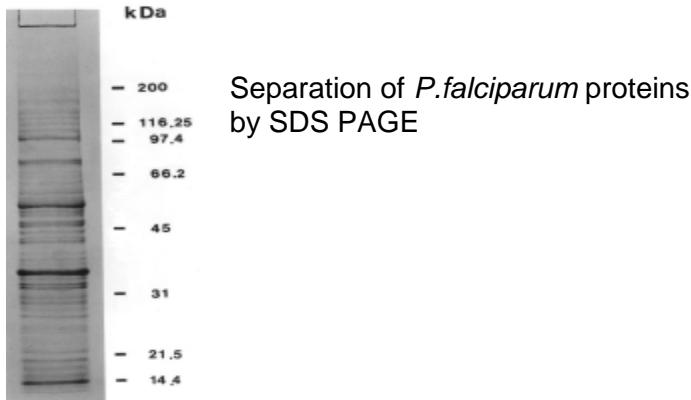
Principle of high resolution SDS gel electrophoresis: The migration of nine selected proteins ranging from 94 kD to 14.4 kD is shown. The total monomer concentration (%T) of the gel is indicated on top of the figure. Stacking and separation takes place for every protein at a different gel concentration. The dotted line indicates the voltage discontinuity at the Gly/Cl moving boundary

Pouring the gradient gel plates (6%-20%)Materials and reagents

- Solution 1: 0.8 M Tris-HCl pH 8.6
- Solution 2 : 38.9 g acrylamide and 1.1g BIS, make up to 100 mL with water
- Sample buffer: 1.2 g Urea, 1.8 mL water, trace of phenolred, 50 mg SDS
- Electrode buffer: 24 g Tris and 15 g glycine in 5 L water (anode buffer) to 500 mL cathode buffer add 500 mg SDS.
- Coomassie Blue Stain: dissolve 750 mg Coomassie Blue in 1 L methanol and 150g TCA in 1.5 L water, mix both solutions.
- Destaining solution: 30% methanol, 65% water and 5% acetic acid

Procedure

- Place 8 mL each of the gel solutions a and b into a gradient mixer and pump with a flow rate of 5mL/min into a glass cuvette (14x12x0.075 cm): this gives a gradient gel beginning with 6%.
- To obtain an even concentration, place drops at many different positions. The gels are ready to use 4h after pouring.
- 1. 3% gel: 2 mL solution 1, 0.75 mL solution 2, 7.25 mL water, 5 mg PER
- 20% gel: 2 mL solution 1, 5 mL solution 2, 3 mL water, 5 mg PER
- start the polymerisation by adding 5 ul TEMED to each mixture.

Protein Quantification by Silver Stain

Silver staining of proteins separated by gel electrophoresis has gained widespread use due to its high sensitivity, typically two orders of magnitude greater than the commonly used Coomassie Blue staining methods .

Materials and reagents

- ethanol
- acetic acid
- dichromate solution
- silver nitrate
- paraformaldehyde
- Na₂CO₃

Procedure

- Fixing: 40% ethanol, 10% acetic acid, 50% water, 30 min
- Protein-Treatment: 20% ethanol, 5% acetic acid, 75% water, 4 mg DTT, 30 min
- 0,5% dichromate solution, 5 min
- water, 5 min
- Silver Equilibration: 0,1% silver nitrate, 30 min
- water, 1 min
- Complex Formation: 0.02% paraformaldehyde, 3% Na₂CO₃ (pH 12), 8 – 10 min
- Storage: 1% acetic acid, unlimited



Long term stability of colored silver-protein complexes:
P.falciparum proteins were separated by high resolution PAGE and silverstained; left: gel stored for two days, right: same gel after storage for 15 years (storage conditions: Electrophoresis 7, 1984, 481)

Immunoblotting/Western Blotting

High resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis is done according to Hempelmann et al (1984). Protein transfer onto nitrocellulose sheets is carried out with constant current (300 mA) at 4 °C for one hour (semi dry technique).

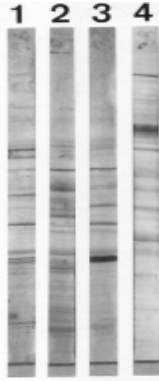
Materials and reagents

- NC paper
- PBS
- Tween
- immunsera
- anti-hu-Ig-PO conjugate
- 4-chloro-1-naphtol stock solution
- NH₄acetate
- citric acid
- acetic acid
- Amido Black

Procedure

- for a 5x7 cm NC paper (Towbin et al, 1979):
- 20 mL PBS + 10 µl Tween: 30 min
- 5 - 50 µl antiserum in 20 mL PBS Tween: 1 h
- 20 mL PBS: 15 min
- 10 µL anti-human IgG antiserum peroxidase-conjugated in 20 mL PBS: 30 min
- Rinse twice with 20 mL PBS: 15 min
- Color development: 10 min

- Mix 50 μ l 3% H₂O₂ and 200 μ l 1% 4-chloro-1-naphtol stock solution (10 mg in 1 mL methanol) in 20 mL buffer (130 mg NH₄acetate and 60 mg citric acid in 100 mL water, pH 5.0).
- Protein detection by Amido Black stain: Staining solution: 250 mL water , 200 mL methanol , 50 mL acetic acid and 0.1% (w/v) Amido Black; Destaining solution: 20% methanol, 7,5% acetic acid, 72,5% dest. water



Immunoblot: 20 μ g of *P.falciparum* proteins are separated by SDS-PAGE, blotted onto nitrocellulose paper and probed with antiserum from different donors

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TRANSFECTION

I. Transfection of *Plasmodium falciparum* within human red blood cells

by *Yimin Wu*

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There are 3 basic steps in asexual stage *P. falciparum* transfection:

- A. Preparing the materials, parasite culture, and DNA constructs
- B. Transfecting DNA into parasites by electroporation
- C. Assaying the expression of transfected genes

This protocol includes the chloramphenicol acetyl-transferase (CAT) assay, luciferase assay, and the selection of stable transformants by the anti-folate pyrimethamine.

I:A. Materials

Parasite culture

Prepare 1×10^9 to 2×10^9 parasitized RBC (PRBC) for each transfection. A synchronized ring-stage parasite (<20 h) culture with high parasitemia (>15%) is essential for an improved transfection efficiency. For cultivation, synchronization, and purification see PARASITES, sections I through IV. Because washed human RBC deteriorate after 4 weeks at 4 °C (even sooner at 37 °C), the parasite culture should be replenished with fresh RBC before transfection, especially when a stable selection is desired.

DNA constructs

The design of transfection constructs is specific to the goal of each individual experiment. Plasmodial flanking regions are required for the expression in parasites; see also “References” at the end of this protocol. Use 50 to 100 µg of DNA for each transfection. The plasmid may be dissolved in TE buffer at 1 to 3 mg/mL.

Buffers

Incomplete Cytomix:
120 mM KCl
0.15 mM CaCl₂
2 mM EGTA
5 mM MgCl₂
10 mM K₂HPO₄/KH₂PO₄
25 mM HEPES

PBS:
1.7 mM NaH₂PO₄
5 mM Na₂HPO₄
150 mM NaCl
10% saponin/PBS stock solution

Equipment

GenePulser (Bio-Rad)
Capacitance Extender (Bio-Rad)

electroporation cuvettes (0.2-cm and 0.4-cm gap)
table-top centrifuge (Sorvall)

Reagents, buffers, and equipment for CAT assay

TEN:

1 mM EDTA
150 mM NaCl
40 mM Tris-HCl (pH 7.6)
250 mM Tris-HCl (pH 7.6)
 [¹⁴C]chloramphenicol (110 mCi/mmol, Moravek Biochemicals, diluted to 2.5 nCi/μL)
acetyl coenzyme A (20 mg, Calbiochem, dissolved in 250 mM Tris-HCl [pH 7.6] to 40 mM)
ethyl acetate
chloroform
methanol
TLC plate (aluminium-backed, thin-layer silica gel 60A plates, Whatman)
glass chromatography chamber
X-ray film and film cassettes, alternatively Phosphorimager cassettes and scanner (Molecular Dynamics)

Reagents, buffers, and equipment for luciferase assay

Luciferase Assay System (Promega):
cell lysis buffer
luciferase substrate
substrate solvent
purified luciferase as positive control
luminometer (Turner)

Reagents for stable selection

Pyrimethamine stock solution is made in dimethyl sulfoxide (DMSO, 50 mg/mL).
Make the selection culture medium without gentamycin. The final content of DMSO in the culture medium should be less than 0.5%.

I:B. Transfection

Transfecting parasites, Day 1

- Change the parasite culture supernatant with fresh medium and make a smear to ensure the quality of the culture. Return the culture to the incubator for at least one h while preparing for transfection.
- Chill the electroporation cuvette and the Cytomix solution on ice. Set the GenePulser electroporator at 2.5 kV, 25 μF, 200 Ω.
- After everything is in place, spin down the cells at 1,500 rpm for 5 min in a table-top centrifuge. Resuspend the cells (about 100 to 200 μL) with 500 μL of Cytomix and not more than 100 μL of the DNA construct*. Mix well by pipetting up and down several times.

*Note: The DNA construct solution should be further concentrated if the volume to be added exceeds 100 μL in order to reach the desired final concentration in the transfection mix.

- Transfer the mixture into a 0.4-cm cuvette and execute the electroporation. Take a note of the set voltage, the actual voltage, capacitance, and time constant. A slight deviation of actual voltage is acceptable. The time constant should be 0.7 to 0.9 ms.
- Immediately return the cuvette to ice for 5 min, then transfer the cells from the cuvette to a culture flask together with 10 mL of culture medium (rinse the cuvette with culture medium to ensure a complete transfer). Return the flask to the incubator.
- An alternative electroporation setting is 0.31 kV/960 μ F with the Bio-Rad GenePulser, using a 0.2-cm cuvette. The total volume allowed in the 0.2-cm cuvette is 400 μ L. The time constant is about 12 to 15 ms.

Changing medium, Day 2

- Change the culture medium and add 50 μ L of fresh blood. Check the parasite smear. Don't panic if the smear contains lots of extracellular, dying parasites or cell debris, as many parasites normally die during this procedure. Recovering parasites should have developed to trophozoites or schizonts by now.

Starting selection for stable transformants or changing medium for transient expression assay, Day 3

- **For stable transformation**, this is the day to start drug pressure. Feed the culture with medium containing pyrimethamine (100 ng/mL). On *Day 4*, continue to feed the culture with medium containing pyrimethamine (100 ng/mL). Most parasites die after 2 days of drug treatment, as can be seen in a Giemsa-stained thin smear. The pyrimethamine pressure may thus be dropped to 40 ng/mL. Continue this pressure until transformants are detected in Giemsa-stained thin smear (usually 3 to 5 weeks). Add 25 μ L of fresh RBC into the culture each week during the selection procedure.
- **For transient CAT and luciferase expression assays**, continue to change the culture supernatant with 10 mL of fresh medium. Check the smear. New rings should emerge by *Day 3*.

I:C. Transfectant harvest and expression assays, Day 4

- **The luciferase assay** substrate is supplied as a powder and accompanied by a vial of solvent. Once the powder is dissolved, aliquot the substrate solution and store it at -20°C . Before harvesting the cells, thaw and equilibrate the substrate to room temperature. Transfer 100 μ L of the substrate into an assay tube and cover it with a piece of aluminium foil to avoid light.
- Spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it into a microfuge tube. Add 10 μ L of 10% saponin/PBS stock to bring the final concentration of saponin to 0.1%; incubate the culture at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min and aspirate the supernatant and RBC ghosts.
- Wash the pellet 2 or 3 times with PBS to remove hemoglobin which interferes with the luciferase assay. Resuspend the pellet completely in 30 μ L of $1\times$ lysis buffer by pipetting up and down. Freeze/thaw the sample for one cycle, then vigorously pipette up and down again. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a

microfuge for 3 min at top speed. Take 20 μ L of the supernatant for the luciferase assay.

- Because there is a slight delay of the photon peak after luciferase is added to the substrate, the timing of sample measurement is crucial if a quantitative comparison is desired. A practice optimized for Turner Luminometer measurements is to wait until the Luminometer is available (you are the only user at the time) and ready (the baseline has returned to zero). Set the timer for 10 s upon mixing the substrate and the lysate. Insert the tube into the chamber and start the measurement when the timer goes off. The set-up of the Turner Luminometer is usually done by a specialist from the vender company. This measuring protocol should be revised if a different luminometer is used.
- An alternative positive control for the luciferase assay is *Escherichia coli* cells carrying the tester plasmid.
- For the **CAT assay**, spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it to a microfuge tube. Add 10 μ L of 10% saponin/PBS stock to make the final concentration to 0.1% and incubate the pellet at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min, then aspirate the supernatant and RBC ghosts. Wash the pellet once with 1 mL of PBS and once with 1 mL of TEN. Resuspend the cell pellet in 120 μ L of 250 mM Tris-HCl (pH 7.6) with PMSF (1 mM); pipette up and down vigorously. Freeze/thaw the sample for 3 cycles. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Incubate the lysate for 10 min at 60 °C to inactivate cellular acetylases. Clear the lysate by centrifugation in a microfuge at 15,000 $\times g$ for 2 min.
- In a microfuge tube, mix 10 μ L of lysate, 20 μ L of acetyl-CoA (40 mM), and 1 μ L of [14 C]chloramphenicol (2.5 nCi). Incubate the mixture at 37 °C for 3 h, then add a second 20- μ L aliquot of acetyl-CoA. Continue the incubation overnight.
- Handle all the samples, reagents, and waste according to radiation safety regulations from this point onward.

Continue the CAT assay. Day 5

- Momentarily spin down condensations of the reaction mix, then add 350 μ L of ethyl acetate. Vortex to thoroughly mix and spin briefly to separate the organic and aqueous phases. Transfer 300 μ L of the upper organic layer to a clean microfuge tube and dry the extract in a speed-vac centrifuge (30 to 60 min at 45 to 60 °C).
- Redissolve the sample with 25 μ L of ethyl acetate. Mark the origin of a thin-layer chromatography (TLC) plate (Whatman) with a soft lead pencil 1.5 to 2 cm from the edge. Apply 10 μ L of sample to the origin. Prepare a TLC chamber containing 200 mL of chloroform:methanol (20:1). Place the TLC plate in the chamber and allow the solvent front to move ~15 cm. Remove the plate from the chamber and air-dry the plate.
- Place the TLC plate in a film cassette and align the plate and the X-ray film with radioactive ink. A 3- to 5-day exposure is required due to the low level expression (or low transfection efficiency). Develop the film and align it with the plate. Alternatively, expose the TLC plate to a Phosphorimager screen for overnight and scan it with the Phosphorimager. Three radioactive spots should be observed. The two faster migrating spots represent the 1- and 3-acetylated chloramphenicol.

The spot that has migrated the least distance from the origin represents the nonacetylated form.

- Liquid scintillation may be used to quantify the CAT activity. This involves cutting the radioactive spots from the plate for measurement of the amount of radioactivity they contain.

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II. Protocol using DNA-loaded red blood cells for transfection

by **Kirk Deitsch**

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Equipment

Electroporator (Bio-Rad)

Materials and reagents

Incomplete Cytomix (see Wu et al. 1995):

8.95 g KCl

0.017 g CaCl₂

0.76 g EGTA

1.02 g MgC₂

0.871 g K₂HPO₄

0.68 g KH₂PO₄

7.08 g HEPES

Dissolve ingredients in water, adjust pH to 7.6 by adding NaOH, and adjust final volume to 1 liter. Filter-sterilize and use.

Preparation

The procedure outlined below is a modification of the above protocol and takes advantage of the observation that malaria parasites actively take up DNA from the cytoplasm of DNA-loaded red blood cells.

- Using a stock of red blood cells stored at 50% hematocrit, transfer 350 μ L of cells to a 10- or 15-mL centrifuge tube and spin them down. Remove the supernatant and wash the pellet with 5 mL of Incomplete Cytomix. Spin down the cells again and remove the supernatant.
- Resuspend the cells in Cytomix containing 50 to 100 μ g of plasmid DNA. The final volume should be 400 μ L. Transfer the cells to a 0.2-cm cuvette and place it on ice.
- Electroporate the cells using the conditions outlined above. We are using the following conditions: voltage: 0.31 kV; capacitance: 960 μ FD; time constants should be in the 10 to 14 ms range.
- Rinse the cells from the cuvette with 5 mL of culture medium. Two consecutive washes of 2.5 mL each works well. Transfer the cells to a centrifuge tube.
- Spin down DNA-loaded red blood cells and remove the supernatant. The supernatant should be quite red from cell lysis, however if nearly all of the red cells have lysed, check the pH of the Cytomix. It should be \sim 7.6.
- Transfer the cells to a culture flask and inoculate with parasites. Typically the red cells from two electroporations are used for a 5-mL culture. Parasites will take up and express DNA as they invade the DNA-loaded red blood cells.

Advantages of using DNA-loaded red blood cells

- Flasks containing DNA-loaded red blood cells can be inoculated with 100 to 200 μ L of an established culture. Such parasites tend to be healthier because they have not been electroporated and give higher levels of transient expression compared to the same starting numbers of parasites transfected directly by electroporation.

- High levels of expression can be obtained by first loading red cells as described above, then inoculating the cells with mature stage parasites purified by Percoll/sorbitol gradients as outlined in PARASITES, section IV:D. As the cells infected by the mature stages rupture, the resulting merozoites invade the DNA-loaded red blood cells resulting in very high numbers of synchronous, transfected parasites.
- If extremely high levels of transient expression are desired, a culture of parasites can be continuously maintained with DNA-loaded red blood cells. After four generations (8 days) of parasite multiplication in DNA-loaded cells, maximal levels of expression are observed. These levels can be up to 20-fold higher than that observed after direct electroporation of parasite-infected cells.

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III. Selectable episomal transfection of asexual stage *Plasmodium falciparum* parasites

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Equipment

Gene Pulser electroporator with Capacitance Extender (Bio-Rad)
electroporation cuvettes (0.2-cm gap, Bio-Rad)
centrifuge
incubator (37 °C)
microscope with 100× lens
modular incubator chambers (Billups–Rothenberg)

Materials and reagents for plasmid preparation

electrocompetent or chemically competent *Escherichia coli* (We prefer XL10-Gold or DH5α for propagation of plasmids containing *P. falciparum* DNA.)

TE buffer:

1 mM EDTA
10 mM Tris–HCl (pH 7.6)

incomplete Cytomix buffer:

120 mM KCl
0.15 mM CaCl₂
2 mM EGTA
5 mM MgCl₂
10 mM K₂HPO₄/KH₂PO₄
25 mM HEPES

Plasmid Maxiprep columns (Qiagen)
Amicon Centricon 100 columns (Millipore)

Materials and reagents for *P. falciparum* propagation

P. falciparum strains

gas mixture of 5% O₂, 5% CO₂, and 90% N₂
human red blood cells (RBC) washed free of leukocytes
RPMI 1640 with L-glutamine (Invitrogen, Catalog No. 31800)
hypoxanthine (Sigma)
HEPES (Sigma)
sodium bicarbonate (Invitrogen)
gentamycin (Invitrogen)
Albumax (I or II, Invitrogen)
complete medium:
RPMI 1640 with L-glutamine
50 mg/liter hypoxanthine
10 mg/liter gentamycin
25 mM HEPES
0.225% NaHCO₃
0.5% Albumax
Parafilm

sterile 6-well tissue culture plates
sterile glass pipettes and other tissue culture plastic ware
WR99210 (available upon request from David Jacobus, Jacobus
Pharmaceuticals, Princeton, NJ, USA, e-mail: dpjacobus@aol.com)
blasticidin S HCl (Sigma)
pyrimethamine (Sigma)

This protocol describes fundamental steps for selecting episomally transfected asexual-stage *P. falciparum* parasite lines:

- A. Preparation of parasite cultures and DNA constructs
- B. Electroporation of circular plasmid DNA into parasites
- C. Maintenance of transfected parasite lines

III:A. Preparation of parasite cultures and DNA constructs

Parasite cultures

For each transfection, use 1×10^7 to 4×10^7 ring stage parasitized RBC (PRBC). When preparing parasite cultures for transfection, decrease the hematocrit of the culture from the normal 4% to between 2.5 and 3.0%. Reducing the hematocrit and providing regular medium changes will help obtain higher percentages of ring stage parasites in culture. Because washed human RBC deteriorate after 4 weeks at 4 °C, infuse the parasite cultures with fresh RBC shortly before transfection. Feed the parasite cultures regularly to provide nutrients and to prevent buildup of toxic wastes.

The day before transfection, adjust the parasite cultures to between 1.8 and 2.5% parasitemia, rich in trophozoite stages, with addition of fresh RBC. This parasitemia usually gives 4 to 7% ring stage cultures the next day. Feed the parasite cultures in the late evening again to get healthy ring stage parasites the next day. On the day of transfection, smear and feed the parasites early in the morning and allow them to grow for about 3 to 4 h prior to electroporation. Check that the parasite cultures provide healthy rings and RBC for transfection and determine the parasitemia (best is about 4 to 6%).

DNA constructs

Prepare pure supercoiled plasmid DNA using Maxiprep columns. Dissolve the DNA in TE buffer at a concentration of 1 to 2 mg/mL. Before transfection, exchange the TE buffer with incomplete Cytomix buffer using Amicon Centricon 100 columns (see below). Store the plasmid DNA equilibrated with incomplete Cytomix buffer at 4 °C, so that whenever parasites are ready, transfection can be performed. Typically, use 50 to 100 µg of plasmid DNA per transfection.

- To exchange TE buffer with incomplete Cytomix buffer for transfection, load approximately 300 µg of plasmid DNA onto an Amicon 100 column.
- Make the volume up to 2.0 mL with filter-sterilized incomplete Cytomix buffer. Cover the top of the column with Parafilm and puncture the Parafilm several times with a sterile 21-23 gauge needle.
- Spin the loaded column in an Amicon collection tube at 3,000 rpm at 4 °C. It usually takes about 40 to 60 min to concentrate.
- After the spin, add 100 to 200 µL of incomplete Cytomix buffer to the column membrane and gently pipette up and down with the residual incomplete Cytomix

buffer in the column, avoiding rupture of the column membrane. Remove the column from the collection tube, flip it upside down into a sterile Eppendorf tube and spin at 3,000 rpm for 5 min to recover the plasmid DNA.

- Measure the volume of eluted plasmid DNA and determine the volume required to transfect the parasites with 100 µg of plasmid DNA.

The design of the construct, the choice of selectable marker, and the selection of the initial transformation plasmid all depend upon the transfection strategy. Plasmids are most often designed to achieve gene disruption (“knockout”) or allelic replacement (e.g., to exchange polymorphic codons). Selectable markers established for stable episomal transfection of *P. falciparum* include *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase (*dhfr-ts*), human dihydrofolate reductase (*dhfr*, with or without the gene encoding green fluorescent protein as a fusion product), blasticidin S deaminase (from *Aspergillus terreus*), neomycin phosphotransferase II (transposon Tn5), and puromycin-*N*-acetyltransferase (from *Streptomyces alboniger*). These markers confer resistance to pyrimethamine, WR99210, blasticidin S HCl, geneticin/G418, and puromycin respectively. When using *T. gondii dhfr-ts*, be careful to transfect pyrimethamine-sensitive parasites. The other selectable markers listed above can be used against antimalarial drug-resistant and drug-sensitive parasite strains.

Transfection plasmids are available for both allelic exchange and transgene expression strategies. One recent addition is the development of constructs expressing thymidine kinase as a negative selectable marker. The use of this marker, in conjunction with the positive marker human *dhfr*, reportedly enables the selection of parasites in which the plasmids have integrated via double crossover events. This recent approach will help in selecting for recombination into the desired locus (as compared to recombination into genomic sequences that have homology to noncoding, regulatory elements present elsewhere on the transfection plasmids). Further, this approach should help in selecting for integration events that confer a deleterious growth phenotype relative to episomally transfected or wild-type parasites.

While not covered by this protocol, techniques and plasmids also exist for transient transfection assays in *P. falciparum*, for example to study regulatory elements. These plasmids typically use luciferase or chloroamphenicol acetyl-transferase (CAT) for signal detection.

III:B. Electroporation of circular plasmid DNA into parasites

Before starting the transfection experiment, label all electroporation cuvettes and tissue culture plates for parasite culture. Set the Bio-Rad Gene pulser to 0.31 kV, 950 µF, and infinity resistance. Prepare a stock of RBC at 4% hematocrit in complete medium. The whole transfection procedure should be finished in about 30 to 60 min from the time the parasites are harvested to the time the transfected parasites are put back into the incubator.

We note that the procedure listed below relies on transfection of parasitized RBC. An alternative approach recently published by Kirk Deitsch and colleagues involves transfecting *P. falciparum* by first electroporating uninfected RBC with plasmid DNA, then adding mature parasite stages and allowing them to invade the plasmid DNA-preloaded RBC. This is an effective means of obtaining relatively high levels of luciferase

activity and may well represent an improvement for selectable episomal transfection. Nonetheless, the protocol listed below is reliable and works well.

- For each transfection, harvest 2.5 to 3.0 mL of predominantly ring stage parasite culture into a sterile centrifuge tube and spin down the culture at 2,200 rpm for 4 min at room temperature.
- Aspirate the supernatant using a sterile glass pipette.
- Gently resuspend the parasite culture pellet in 3 mL of sterile incomplete Cytomix buffer and spin the culture again at 2,200 rpm for 2 min at room temperature. Aspirate the supernatant as described above.
- After washing the parasite culture with incomplete Cytomix buffer, resuspend the parasite pellet in the required volume of incomplete Cytomix buffer containing plasmid DNA. Measure the volume of this parasite culture–plasmid DNA suspension and make the final volume up to 400 μ L with incomplete Cytomix buffer.
- Transfer the suspension into a Bio-Rad Gene Pulser cuvette (0.2-cm), avoiding any air bubbles.
- Perform the electroporation (using the 0.31 kV, 950 μ F conditions described above). Time constants between 7 and 11 milliseconds give good parasite transfection efficiency.
- Immediately after the electroporation, aseptically add 1 mL of fresh complete medium into the Gene Pulser cuvette. Add the medium gently down the side of the cuvette as the RBC are fragile at this point.
- When all the electroporations are completed, transfer the Gene Pulser cuvettes back to the tissue culture hood. Using a 1-mL pipette, gently remove the cuvette contents into 6-well tissue culture plates (one well per transfection). Pipette 1 mL of complete medium into the cuvette and gently remove most of culture, leaving behind the lysed and dried culture that accumulates around the top of the cuvette.
- Add 3.0 mL of the pre-prepared RBC/complete medium mixture (4% hematocrit) into the well containing the 2 mL of culture from the electroporated material. Mix gently with a 5-mL pipette.
- When all the electroporations are finished, place the 6-well tissue culture plate into a modular chamber, gas the chamber with 5% O₂, 5% CO₂, and 90% N₂, and place it back into a 37 °C tissue culture incubator.
- Three to four hours after electroporation, remove the cultures from the wells and spin them at 2,000 rpm for 4 min at room temperature. Discard the supernatants.
- Resuspend each parasite pellet in 5 mL of fresh complete medium, put it back into the appropriate well of the tissue culture plate, gas, and return it to the incubator.
- The following day, feed each well with 5 mL of complete medium.

III:C. Maintenance of transfected parasite lines

- On the second day post-transfection, smear the electroporated parasite cultures to determine their parasitemia. A parasitemia of 0.8 to 2.4% at this time point is usually indicative of relatively good transfection efficiency. For most plasmids, this typically means that episomally transfected parasite lines will be first observed in 2 to 3 weeks. From this day onward, feed parasites with medium

containing the selectable agent. To select for human *dhfr*, blasticidin S-deaminase, or *T. gondii dhfr-ts*, we recommend using complete medium supplemented with 2.5 nM WR99210, 2.5 µg/mL of blasticidin S HCl, or 20 ng/mL of pyrimethamine respectively.

- Feed the parasites daily on *Days 3-5*.
- On *Day 6*, smear the lines. There should be no live asexual stage parasites. Large numbers of gametocytes are a sign of excessive stress during the transfection and/or subsequent handling procedures. Reasons for this can include poor parasite health prior to transfection, too much time spent outside of the incubator, or poor RBC condition. Similarly, it is not good to see too many “blebby” RBC. (If needed, these RBC can be diluted out by replacing 20 to 30% of the culture with fresh uninfected RBC at this time.)
- Resuspend the *Day 6* cultures in 5 mL of complete medium containing the drug and transfer them into a sterile centrifuge tube. Spin the tube at 1,800 rpm for 3 min at room temperature; this centrifugation helps to remove toxic lysis products and dead parasite material. Aspirate and discard the supernatant. Resuspend the pellet in 5 mL of medium containing both drug and an extra 0.1 mL of RBC (stock of 50% hematocrit).
- Skip *Day 7*.
- Feed again on *Day 8*.
- Skip *Day 9*.
- Feed again on *Day 10*. Repeat the smearing, centrifugation, and addition of fresh RBC procedures performed on *Day 6*. There should be minimal parasite debris left with few gametocytes and the RBC should be in good condition. Note: From *Day 10* onwards, it is possible to reduce culture volumes from 5 mL down to 1 mL per line until parasites are observed, then the lines can be expanded back out to 5 mL again for freezing.
- Feed every second day (*Days 12, 14* etc.) and smear every 4 days until parasites become microscopically detectable on Giemsa-stained slides. Typically, parasites are first visualized on *Days 14 to 24*. To prevent the parasite cultures from lysing, discard 30 to 40% of the cultures and replace them with fresh RBC every 6 or 7 days.
- Once parasites have been seen, begin feeding them daily. Freeze the transfected parasite lines as soon as the parasitemia reaches $\geq 2\%$ with $>50\%$ rings. Note the growth rate of the episomally transfected cultures, which grow slower than the parental nontransfected lines.
- Expand and harvest the remaining cultures for genomic DNA extraction from trophozoite mature stage parasites to confirm transfection by plasmid rescue. Subsequently, parasite lines can be maintained in 1-mL volumes for long-term propagation.
- Make frozen parasite stocks every month. It is also convenient to prepare genomic DNA once a month to screen for integration by PCR.
- Stable integrants resulting from homologous recombination and single-site crossover of the plasmid into the genome can often be observed between 45 and 90 days post-transfection. These stable integrant parasites typically grow faster than episomally transfected lines. PCR with primers specific to the vector and the plasmid can be used in combination to efficiently screen for the presence in the bulk culture of parasites that underwent the desired recombination event. PCR positive data can be confirmed by Southern hybridization. Once the desired

integration has been detected, the corresponding parasite lines can be cloned by limiting dilution and detected using the Malstat lactate dehydrogenase reagent.

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IV. Transfection of *Plasmodium falciparum*

(Adapted from: BIOMALPAR/HHMI/WHO Special Programme for Research and Training in Tropical Diseases (TDR) Practical Course)

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IV: A. Transfection procedure of *Plasmodium falciparum*

Background

The process that we routinely use for transfection of *P. falciparum* is summarized below. This is the standard procedure for both transient [1, 2] and stable transfection [2-4] although higher initial parasitaemias are used for transient expression. The method shown below uses electroporation of parasite-infected erythrocytes, however, an alternative method of transfection involving spontaneous uptake of plasmid DNA by *P. falciparum* has also been described [5]. Some plasmid constructs lead to the establishment of transfected parasite populations much faster than others. For example, parasites transfected with plasmids designed to express green fluorescent protein (GFP) grow considerably slower than those transfected with other vectors, especially those containing Rep20 sequence (see below). Plasmids should be transfected as undigested circular DNA and will replicate episomally in parasites following transfection and drug selection. Gene targeting by single crossover recombination to disrupt genes or for allelic replacement can be achieved using a drug cycling procedure. Furthermore, gene knockouts using double recombination crossover can be generated using the pHTK plasmid (see below). This transfection plasmid possesses a *thymidine kinase* gene cassette that allows negative selection against the presence of the plasmid backbone and hence selects for parasites that have integrated the positive selectable marker cassette via double crossover recombination. It also greatly shortens the length of time required to derive parasites that have integrated the appropriate portion of the plasmid vector.

Preparation of *P. falciparum* parasites for transfection

Materials and reagents

- Preparation of RBC for transfection: Bags of red blood cells are obtained from the Blood Bank in anticoagulant citrate phosphate dextrose solution. The red blood cells are transferred from the bags to sterile bottles for storage at 4°C and are NOT washed prior to use.
- For 100 mL of RPMI-Hepes, supplement with 5.8 mL of 3.6% NaHCO₃ and 10mL of 5% albumax.
RPMI-Hepes. For a 1 litre solution: RPMI-1640 10.44 g, 25 mM Hepes 5.96 g, 200 µM Hypoxanthine 50 mg, 20 µg/mL Gentamicin 10 mL of a 2 mg/mL stock, H₂O 960 mL, pH 6.72 with 1M NaOH, make up to 1 litre with H₂O, filter sterilise and aliquot, store at 4°C

- 5% albumax: Dissolve 5 g of albumax in 100 mL of RPMI-Hepes at 37 °C, filter sterilise and store at 4°C.
- CytoMix: 120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 25 mM Hepes, pH 7.6.
For 100 mL:
6 mL 2M KCl
7.5 µl 2M CaCl₂, 1 mL 1M K₂HPO₄/KH₂PO₄, pH7.6 (8.66 mL 1M K₂HPO₄ + 1.34 mL 1M KH₂PO₄ = 10 mL 1M phosphate buffer, pH7.6), 10 mL 250 mM Hepes/20 mM, EGTA, pH7.6 with 10M KOH, 500 µl 1M MgCl₂, to 90 mL with ddH₂O, pH to 7.6 with 0-350 µl 1M KOH, add ddH₂O to 100 mL, Filter-sterilise, store in 3 x 33mL aliquots at 4°C.
- Cytomix stock buffers:
(a) 10M KOH= 5.61 g/10 mL DDW
(b) 250 mM Hepes/20 mM EGTA
5.96 g Hepes (Free acid)
0.76 g EGTA
To 80 mL with ddH₂O
pH to 7.6 with 10M KOH(~1.4 mL)
To 100 mL with ddH₂O.
- Pyrimethamine: 200 µM Pyrimethamine (10 mL), add 0.012 g to 5 mL 1% glacial acetic acid (in ddH₂O). Have 1% acetic acid at RT. Dilute 200 µl into 10 mL HTPBS (fresh sterile bottle from media each time). Filter-sterilise & store at 4°C. Stable only for 1 month.
- WR99210 (Jacobus Pharmaceuticals): dissolve 8.6 mg WR99210 in 1 mL of DMSO (=20mM). (This may be stored long term at -70 °C). Dilute 1/1000 in RPMI-Hepes (=20µM). Filter sterilise and store at 4°C. (Stable for 1 month at 4°C)
- Ganciclovir: (Cymevene® for intravenous infusion - Roche). Stock: 51.04 mg in 1 mL ddH₂O (=200mM). Filter sterilize (0.2µm). Store 50 µl aliquots at -70°C. Working solution: dilute stock 1:10 in H₂O (=20mM) (stable for 4 weeks at 4 °C)
- 0.15% Saponin: Dissolve 0.15g saponin in 100mL RPMI-Hepes, filter sterilise and store at 4°C
- TE buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA
- 5% Sorbitol: Dissolve 5g sorbitol in 100mL H₂O, filter sterilise and store at 4°C
- gas mixture of 5% carbon dioxide, 1% oxygen and 94% nitrogen
- GenePulser (BioRad) cuvettes
- GenePulser (BioRad)
- Centrifuge
- 10 cm petri dishes

Procedure

- Synchronise *P. falciparum* parasites at 1-2% ring stages using 5% sorbitol two days before transfection.
- Feed synchronised parasites again 1 day before transfection.
- It is important to use fresh human erythrocytes to ensure that they support growth of parasites during the lengthy initial selection process. The erythrocytes are not washed prior to use
- On the day of transfection parasites should be approximately >5% parasitemia.
- 5 mL of culture (at 4% haematocrit) will be required for each transfection.

Preparation of DNA for transfection

- Prepare plasmid DNA for transfection by ethanol precipitating at least 50 µg of the vector (usually 50-100 µg).
- Allow the pellet to dry for 5 min in a laminar flow hood. Resuspend DNA in 15-30 µl of sterile TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). It is essential that the DNA be fully dissolved in the buffer before adding further solutions.
- Add 370-385 µl of sterile Cytomix to each plasmid DNA pellet. Cytomix consists of 120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM Hepes pH 7.6.

Electroporation and plating

- Centrifuge 5 mL of culture/transfection at 1,500 x g for 5 min and remove supernatant.
- Add the Cytomix/plasmid mixture to the parasitised erythrocyte pellet and pipette up and down gently to mix (avoid bubbles!).
- Transfer the parasitised erythrocyte/DNA mixture to a GenePulser (BioRad) cuvette (0.2 cm gap). Electroporate at 0.310 kV and 950 µF. The resulting time constant should be between 7 and 12 msec.
- Immediately add the electroporated sample to a labelled 10 mL petri dish containing 3-4% erythrocytes in complete RPMI/Hepes medium with 10% albumax. Grow parasites at 37°C in a gas mixture of 5% carbon dioxide, 1% oxygen and 94% nitrogen.

Positive drug selection

- 5-8h after transfection change medium and add WR99210 at 2.5-10 nM when using the human *dihydrofolate reductase (dhfr)* gene as the selectable marker (see below for other positive selectable marker).
- At day 2, add WR99210 to 2-10 nM when using the human *dihydrofolate reductase (dhfr)* gene as the selectable marker or 0.2 µM pyrimethamine when using the *Toxoplasma gondii dhfr* gene as the selection system (see other positive selectable markers below).
- Fresh media and the appropriate drug are added to cultures daily for the first 4 days then every 2 days until parasite establishment.
- Smear parasite culture on day 2 post transfection to check for the presence of rings (there should be almost no rings present).
- Add fresh RBC (~100 µl) once a week.
- Parasitised erythrocytes should be detectable in Giemsa stained smears of erythrocytes after 7-75 days (average 21-30 days).
- To select parasites with the plasmid vector integrated by homologous recombination, grow the parasites for 3 weeks without drug selection then reapply drug pressure and continue to culture until parasites (rings) appear in blood smears.
- The parasites should be analysed by Southern blotting (or alternative means) to determine if integration into the relevant gene has been obtained (see details below).
- Continue drug cycling until no death observed after addition of drug.

Negative selection using Thymidine kinase vectors

- For selection of transfected parasites using vectors containing the thymidine kinase gene for negative selection the parasites should be transfected as described above and selected with WR99210.
- Once stable transfected parasites are established on WR99210 add ganciclovir to 20 μ M to a synchronised culture of 2% rings. (Important: WR99210 selection continues during ganciclovir treatment). During this period there may be substantial parasite death.
- Grow the culture until parasites re-appear and until parasite growth is firmly established.
- Analyse chromosomes and genomic DNA of the parasites using PFGE and Southern blotting to determine if integration into the appropriate gene has occurred in these parasites.

IV: B. Monitoring transfectants: genetic analysis

Background

It is particularly important to genetically monitor stable transfectants once a drug-resistant population emerges post-transfection and during the drug cycling process. It is important to do this for three key reasons:

- To ensure that transfected populations do not represent naturally drug-resistant mutants, such as those with a mutation in the endogenous DHFR-TS gene, but are instead transformed with the desired plasmid,
- To determine if the transfected populations possess episomally replicating and/or integrated copies of the transfection plasmid and
- To examine the nature of the integration event (ie, homologous vs non-homologous; single vs double crossover recombination). A combination of three approaches can be used for this analysis:

PCR

This approach can be used for all requirements. However, for a number of reasons we believe that its use is limited and that the technique should be used as a guide only and results have to be confirmed using other techniques. For example, detection of the transfection plasmid by PCR using oligonucleotides specific for a unique sequence (such as a targeting sequence) is confounded by the presence of residual DNA left over from the original transfection. This DNA can be destroyed by pre-digestion of the gDNA with Dpn I, a restriction enzyme with a frequently found recognition sequence that cleaves only methylated (such as that replicated in *E.coli*) and not un-methylated (parasite replicated) DNA, although *DpnI* digestion is unlikely to be 100% efficient. PCR is particularly useful to detect the presence of homologous integration events using a combination of a plasmid-specific oligonucleotide (not specific to the gene targeting sequence) and one directed to genomic sequence located immediately outside of the gene targeting fragment found in the plasmid. The presence of such a product (which should be sequenced for confirmation) demonstrates that homologous integration has indeed occurred. Using this approach, however, it is not possible to determine the proportion of the parasites that possess integrated forms of the plasmid.

Pulsed-field gel electrophoresis (PFGE).

The separation of *P. falciparum* chromosomes by PFGE followed by Southern blotting is a powerful approach to monitor the genotype of transformants [6, 7]. By hybridizing identical blots with a probe to detect the presence of the transfected plasmid (eg. plasmid backbone or selectable marker gene) and a probe to detect the targeting sequence present in the plasmid, the progress of episomally replicating to integrated plasmid can be followed. While this approach demonstrates plasmid integration and the chromosome into which this has occurred, it does not reveal the specific nature of the integration event. Good examples of the use of PFGE to analyse transfectants is shown in papers by Baldi *et al* [6], Gilberger *et al* [8] and Maier *et al* [9].

During pulsed field electrophoresis (PFGE) macromolecules are subjected to alternately pulsed electric fields and results in the separation of double stranded linear DNA molecules up to 3 megabases, a size range that includes the chromosomes of *P. falciparum*. The resolution of different size DNA molecules can be optimised during PFGE by varying the applied voltage and the pulse time of the alternating electric field.

The separation of *P. falciparum* chromosomes by PFGE can provide a powerful tool in the analysis of transfected parasites in a number of different ways:

- **Detection of true transfectants**
Hybridization of plasmid backbone or the selectable marker sequence to southern blots of pulsed field gels allows the differentiation of true transfectants from drug resistant mutants. These probes will not hybridize to DNA from a drug resistant mutant.
- **Differentiation between episomal and integrated plasmid**
Circular DNA molecules migrate in an aberrant manner during pulsed field electrophoresis that differs dramatically from the migration of linear molecules of the same molecular weight. Therefore electrophoresis conditions can be chosen that allow easy differentiation between episomal and integrated plasmids following hybridization of plasmid backbone and target sequence to Southern blots of pulsed field gels.
- **Chromosomal localisation of integration events**
Hybridization of duplicate Southern blots with either the plasmid backbone or selectable marker sequence and a probe that detects the targeting sequence will reveal whether an integration event has occurred on the correct chromosome. This approach will not reveal the specific nature of the integration event. Successful targeting of the endogenous locus must be confirmed by Southern hybridization of genomic DNA that has been digested with appropriate restriction enzymes.
- **Differentiation of integration by single and double crossover recombination**
Transfection plasmids containing a marker for negative selection such as *thymidine kinase* integrate into the genome via double crossover recombination events that integrate the positive selectable marker without the plasmid backbone. Therefore hybridization of duplicate Southern blots of a pulsed field gel with the plasmid backbone and the positive selectable marker sequence will differentiate between integration by single and double crossover recombination. Only the positive selectable marker will hybridize to double crossover integration events and the plasmid backbone will not.
- **Molecular karyotype analysis of parent and transfectant**
It can take several months to generate a gene knockout parasite so before any phenotype analysis is carried out it can be worthwhile to confirm that the parent and the knockout have an identical karyotype. Karyotype analysis can be done by ethidium bromide staining chromosomes that have been resolved by PFGE. This will also detect any chromosomal rearrangements that have occurred during the transfection experiment. Further comparison of the genome of the parent and knockout parasites can be done by hybridization of the repetitive probe rep20 to genomic DNA digested with HindIII.

Southern blotting of digested gDNA.

This is the ultimate confirmation of an integration event. To determine if your plasmid has integrated into the intended locus by homologous recombination, gDNA (~1 µg) is digested by appropriate restriction endonucleases, separated by agarose gel electrophoresis and Southern blotted to a transfer membrane. The enzymes to be used should be ones that are intended to reveal a distinct difference in size of the fragments representing wild-type locus, integrated locus and episomal plasmid when the blot is hybridized to a targeting sequence probe. It should be noted that plasmids that integrate by cross-over recombination event sometimes insert a number of head to tail plasmid copies into the locus. If this has occurred a band corresponding to that expected for the episomal plasmid will be observed. Southern blots can be detected using ³²P- or Digoxigenin labelled probes.

Materials and reagents

- 10 x Maleic Acid Buffer (500mL):
58 g Maleic Acid (final conc. 10M), 43.8 g NaCl (final conc. 1.5M), adjust the pH to 7.5 with NaOH (pH changes quickly), autoclave.
- Washing Buffer: 1 x Maleic Acid, 0.3 % Tween 20.
- 10 x Blocking Solution: Dissolve Blocking Reagent 10% (w/v), (bottle 4 of DIG kit, Roche Catalogue # 1 636 090) in 1 x Maleic Acid Buffer at 65°C. Place on stirrer and mix, autoclave. Store at 4°C.
- Detection Buffer: 0.1M Tris HCl, 0.1M NaCl pH 9.5
- CSPD (vial 5) 100 x. Thaw 100 x CSPD (vial 5 from DIG kit) when the kit arrives and make 20 µL aliquots. Freeze these aliquots. Avoid repeated freeze/thaw cycles. A 20 µL aliquot diluted 1:100 with Detection Buffer makes up 2mL (enough for the chemiluminescent detection of a 10 x 10 cm membrane).
- Denharts Hybridization Solution: 6 x SSC, 5 x Denharts, 0.1% SDS
- Saponin: Dissolve 0.15 g saponin in 100 mL RPMI-Hepes. Sterilise by filtration through a 0.2 µm filter.

Preparation of *P. falciparum* genomic DNA (A-Phenol/Chloroform precipitation)Material and reagents

- 3M Na Acetate pH5.0
- 95% and 70% ethanol
- TE-buffer
- 18% SDS
- phenol/chloroform
- chloroform
- alt. DNeasy tissue kit (Qiagen)

Procedure

- Spin culture at 1200 rpm for 5 minutes to pellet parasitised red blood cells and remove supernatant.
- Gently resuspend pellet in 4 volumes of buffer A (1.6 mL).
- Add 1 volume of 18% SDS (0.4 mL) and mix thoroughly then let sit for 2-3 minutes.
- Add 8 volumes of phenol/chloroform (2.4 mL) and mix thoroughly
- Spin at 3500 rpm for 10 minutes.

- Remove aqueous phase into a clean corex tube and ethanol precipitate by adding 1/10 volume of 3M Na Acetate pH5.0 (250 μ L) and 2.5 volumes of ethanol (6.5 mL).
- Leave at -20 °C for at least 1 hour, but the preparation could be stored overnight at -20 °C at this stage.
- Pellet DNA by spinning at 10,000 rpm for 10 minutes.
- Drain off the ethanol.
- Dissolve DNA in 600 μ L of TE and transfer to an Eppendorf tube.
- Extract twice with phenol/chloroform by adding 600 μ L of phenol/chloroform, mixing and spinning at 4,000 rpm for 3 minutes then removing aqueous phase to a clean tube.
- Extract once with chloroform by adding 600 μ L of chloroform, mixing and spinning at 4,000 rpm for 3 minutes then removing aqueous phase to a clean tube.
- Ethanol precipitate DNA by adding 50 μ L of 3M Na Acetate pH5.0 (250 μ L) and 1 mL of ethanol.
- Leave at -20 °C for at least 1 hr, but the preparation could be stored overnight at -20 °C at this stage.
- Pellet DNA by spinning at 10,000 rpm for 10 mins in a microcentrifuge.
- Remove supernatant and wash DNA pellet with 1 mL of 70% ethanol
- Dissolve DNA in 50 μ L TE and store at 4 °C.
- Run 2 μ L on a 1% agarose gel to check.

alternatively: Preparation of *P. falciparum* genomic DNA with the B-DNeasy tissue kit, or DNeasy tissue kit (Qiagen)

making a digoxigenin-labelled hybridization-probe:

PCR DIG probe synthesis kit Roche Catalogue # 1 636 090

Southern blot digoxigenin

Material and reagents

- 0.125M HCl (5 mL conc. HCl/400 mL DDW)
- 0.5M NaOH/1.5M NaCl
- 0.5M Tris/1.5M NaCl pH8.0
- DDW
- paper and Hybond-N
- 2 x SSC buffer
- UV cross-linker

Equipment

- Hybridization oven
- water-bath

Procedure

- Run digested genomic DNA (1.0-5.0 μ g) on a 0.8% agarose gel o/n at 17V.
- Take UV picture of gel with ruler next to it.
- Depurinate DNA in 0.125M HCl (5 mL conc. HCl/400 mL DDW) for 20min.
- Denature DNA in 0.5M NaOH/1.5M NaCl for 30min.

- Neutralize DNA in 0.5M Tris/1.5M NaCl pH 8.0 for 30 min.
- Cut paper and Hybond-N.
- Build up blot, pre-wet Hybond-N and 1st sheet in DDW (evenly), Blot O/N (or at least 4h).
- Rinse the membrane briefly in DDW.
- Cross-link membrane while still damp: place the membrane on Whatman paper soaked with 2 x SSC.
- UV cross-link wet membrane (700 x 100 $\mu\text{J}/\text{cm}^2$ on cross-linker).
- Rinse membrane briefly in DDW and allow to air dry (membrane can be stored dry at 4 °C).
- Pre-hybridization: (do not allow the blot to dry out once you start)
- Calculate $T_{\text{hyb}} : T_{\text{m}} = 49.82 + 0.41 (\% \text{ G+C}) - 600/\text{length of probe in bp}$, $T_{\text{hyb}} = T_{\text{m}} - (20 \text{ to } 25^\circ\text{C})$, assuming 100% homology.
- Determine how much DIG Easy Hyb will be used:

	Prehyb. -solution	Hyb. -solution
100cm ² membrane	10 mL	10 mL
Mini-gel (6.5 x 10 cm)	6.5 mL	6.5 mL
Half-gel (15 x 10 cm)	15 mL	15 mL

- Place correct amount of DIG Easy Hyb in tube and place tube in water-bath set at hybridization temperature.
- Transfer membrane in hybridization bag, add pre-warmed DIG Easy hybridization solution, remove air-bubbles, seal bag (enough buffer that it looks slightly puffy) and perform pre-hybridization at hyb temp. for at least 30 min under slight agitation.

Hybridization

- Prepare hyb solution: add appropriate amount of labelled probe (0.5-1 μL per mL final hyb solution) + 50 μL H₂O in Eppendorf tube, heat to 95°C for 5 min, cool quickly in an ice-bath.
- Immediately add denatured probe to a tube containing appropriate amount of pre-warmed DIG Easy hyb solution and mix by inversion.
- The Denharts Hybridization solution (6 x SSC, 5 x Denharts, 0.1% SDS) can also be used with the DIG system. Use with the addition of 10x Blocking Solution (9 parts Denharts Hyb solution to 1 part 10 x Blocking Solution). Using the Denharts Hyb solution can sometimes reduce background with probes that have a high amount of unspecific binding. Add 100 μL of boiled salmon sperm DNA per 25 mL prehyb solution. Hybridize at 62°C. Use less of the DIG labelled PCR probe, ~1-3 μL /hybridization.
- Pour out pre-hybridization buffer, add immediately hyb solution containing probe to bag, remove air-bubbles and seal bag.
- Immediately add denatured probe to a tube containing appropriate amount of pre-warmed DIG Easy hyb solution and mix by inversion.
- Incubate bag O/N at appropriate hyb temp, agitate blot gently.
- Pour hybridization solution off (store in Falcon tube at -20°C/can be reused 3-5 times).

Washing the membrane

- Wash membrane twice with 2 x SSC at RT for 5 min in a shaking container (make sure membrane does not dry out).

DIG Chemiluminescent Detection

- Equilibrate membrane in 15 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) for 1 min.
- Incubate membrane in 100 mL 1% blocking solution (2 mL 10% Blocking solution + 18 mL maleic acid buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5) at room temperature for 30min.
- Centrifuge anti-Digoxigenin-AP antibody at 10000rpm for 5 min (to remove aggregates), dilute AB 1:10000 in 1% Blocking solution (2µl in 20 mL) and incubate membrane in it at room temp for 30 min.
- Wash membrane in 100 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) at RT for 15 min.
- Wash membrane in 100 mL washing buffer (0.1M maleic acid, 0.15M NaCl buffer, pH7.5, 0.3% Tween-20) at RT for 15 min.
- Equilibrate membrane in 20 mL detection buffer (100mM Tris-HCl, 100mM NaCl pH 9.5) for 2 min.
- Remove excess liquid from membrane by dripping one corner dry onto a tissue, transfer into hybridization bag, add 2 mL CSPD onto membrane, remove air-bubbles by stroking it with a wet tissue, cover immediately.
- Incubate at RT for 5 min.
- Squeeze out excess liquid, remove air-bubbles, seal bag and put in exposure cassette and place at 37°C for 10 min.
- Expose film (first one for 5 minutes).

Stripping Probe from Membrane

- Rinse the membrane in DDW for 1 minute.
- Wash membrane at 37°C in 0.2M NaOH containing 0.1% SDS. (2 x 15 min).
- Rinse the membrane in 2 x SSC for 5 minutes.
- Store membrane in a hybridization bag at 4°C.

Preparation of *P.falciparum* chromosome blocksMaterial and reagents

- LOW MELTING POINT agarose
- Saponin
- lysis buffer 0.5M EDTA, 10mM Tris pH 8.0, 1% sarkosyl, 2 mg/mL proteinase K (proteinase K added fresh just prior to use)
- 50 mM EDTA, 10 mM Tris pH 8.0

Procedure

- For best results use a culture containing 6-10% trophozoites
- Chromosome blocks are agarose plugs that contain chromosomal DNA molecules that can be resolved by pulsed field electrophoresis. Standard procedures for DNA preparation do not yield chromosome-sized DNA molecules because high molecular weight DNA is sheared by mechanical forces during

- preparation. *P. falciparum* chromosome blocks are prepared by embedding parasites in agarose followed by *in situ* lysis and deproteinisation.
- Chromosome blocks are stable at 4°C for many years.
 - before start: 1. Warm PBS to approximately 50°C.
 - 2. Make up 2% LOW MELTING POINT agarose in PBS and keep at approximately 50°C while you prepare the chromosome blocks.
 - Pellet parasitised red blood cells 1200 rpm 5 minutes and discard supernatant.
 - Saponin lyse the red blood cells in 1.5 volumes (600 µl) of 0.15% saponin in RPMI-Hepes on ice for 5 minutes. (The volumes in brackets are appropriate for a 10 mL culture)
 - Pellet the parasites 2800 rpm for 10 minutes.
 - Carefully discard ALL the supernatant.
 - Resuspend parasites in approximately 3x the pellet volume (50 µl) of warm PBS.
 - Add an equal volume (50 µl) of 2% agarose in PBS and mix.
 - Pipette mixture into block cast and allow the agarose blocks to set on ice.
 - Push the blocks into lysis buffer 0.5M EDTA, 10mM Tris pH 8.0, 1% sarkosyl, 2 mg/mL proteinase K (proteinase K added fresh just prior to use). Allow approximately 1 mL of lysis buffer for up to 500 µl of blocks.
 - Incubate at 37°C for two days.
 - Store blocks at 4°C in 50mM EDTA, 10 mM Tris pH 8.0

Running a pulsed field gel

Material and reagents

- 0.5x TBE/1x TAE in MilliQ H₂O
- 1% agarose in 0.5x TBE
- 0.5 – 1 µg/mL ethidium bromide solution in H₂O
- BioRad CHEF pulsed field electrophoresis apparatus
- UV transilluminator

Procedure

The following protocol is suitable for a BioRad CHEF pulsed field electrophoresis apparatus. Gels are run at 13°C.

- Set up the gel casting stand with an appropriate comb.
- Prepare 2 litres of 0.5x TBE* in MilliQ H₂O.
- Prepare 100 mL of 1% agarose in 0.5x TBE* (make the volume up to 100 mL with H₂O after boiling in a microwave oven to melt the agarose).
- Allow the agarose solution to cool to approximately 60 °C then pour into the casting stand.
- Allow the gel to set at room temperature then carefully remove comb.
- Equilibrate the electrophoresis samples (1mm slices of chromosome blocks) in 0.5x TBE* for 30 minutes at room temperature.
- Load the samples into wells and seal with 1% LMA agarose in 0.5x TBE* (which has been melted and cooled to approximately 50 °C).
- Pour the remainder of the 0.5x TBE* buffer into the tank and turn on cooling system.
- Place gel in the tank.

- Set the appropriate running conditions (see below) and run the gel.
- To visualise the chromosomes, remove the gel into a suitable container and stain in a 0.5 – 1 $\mu\text{g}/\text{mL}$ ethidium bromide solution in H_2O or 0.5x TBE for at least 30 mins.
- Photograph the gel on a UV transilluminator.
- DNA fragments larger than 20kb must be cleaved for efficient transfer to hybridization membranes. Prior to transfer, DNA fragments separated by pulsed field electrophoresis are nicked by acid treatment or UV irradiation (5 minutes on a short wavelength UV transilluminator).
- The DNA can then be transferred and hybridized using standard procedures

Comments

*Use 1x TAE for resolution of the four largest chromosomes 11-14.

For resolution of chromosomes 1-5:

1% agarose in 0.5 x TBE, 60 – 120 s pulse, 6 V/cm (200 volts), 24 hour run.

Episomes generally migrate around 35 mm from the wells. These conditions can be useful for detecting the presence or absence of episomes and integration events into loci on chromosomes 1 and 2.

For resolution of chromosomes 1-10:

1% agarose in 0.5 x TBE, 225 sec. pulse, 4.2 V/cm (140 volts), 60 hour run.

Episomes generally migrate in a smeary pattern around 35 mm from the wells although some will migrate at the compression zone about 10 mm from the wells. These conditions are useful for detecting the presence or absence of episomes and integration events into loci on chromosomes 1 –10. However, it can be difficult to differentiate between episomes and integration if the target chromosome co-migrates with the episomes.

In addition, these conditions can be sensitive to different batches of agarose and TBE buffer.

For resolution of chromosomes 11-14:

1% agarose in 1x TAE, 360 – 800 s pulse, 3 V/cm (100 volts), 96 hour run.

A high gel strength agarose such as BioRad Chromosomal Grade Agarose is required. Episomes generally run off these gels so these conditions are only useful for detecting integration events into loci on chromosomes 11 –14.

IV: C. Monitoring transfectants: phenotypic analysisBackground

Prior to embarking on the transfection experiments, you will have already given some thought as to what possible phenotypic changes may result from the genetic manipulation you will make to the parasites. Obviously, phenotypic analyses will depend on which gene you are studying. Below is a method we use to assess whether knockout of putative invasion-related genes results in an altered erythrocyte receptor use [10],[8], [11], [12], [13].

Erythrocyte invasion assay

It is critical that parasite lines are tightly synchronised and growing well before an invasion assay is set up.

The protease enzyme treatments remove different classes of erythrocyte surface proteins, whereas neuraminidase removes specific sialic acid glycans from surface proteins such as the glycoporphins. It is advisable to treat parasitised cells at the early ring stage, since these suffer less adversely from the various treatments and washes.

Material and reagents

- sorbitol
- wash buffer
- Neuraminidase
- Trypsin
- Chymotrypsin
- Trypsin/Chymotrypsin inhibitor

Procedure

- Day 1
- Smear cultures and synchronise using 5% sorbitol 2 days before the assay set up. Adjust parasitaemia to 1%.
- Day 3
- On the morning of the assay set up, synchronise parasite stocks with sorbitol and smear cultures. Adjust parasitaemia to 1% and haematocrit to 4% with fresh erythrocytes. From this point on, it is important to take care when removing supernatants, as we want to maintain the same parasitaemia and haematocrit across different parasite lines and subsequent treatments.

ENZYME	Stock Concentration	Final Conc.
Neuraminidase	1U/mL	0.067U/mL
Trypsin	1.7 mg/mL	1 mg/mL
Chymotrypsin	1.7 mg/mL	1 mg/mL
Trypsin/Chymotrypsin inhibitor	1 mg/mL	0.5 mg/mL

- Treat iRBC with enzymes as follows: Centrifuge the entire 10 mL of culture from both dishes in separate tubes at 1200 rpm for 5 mins. For each parasite line, there should be 400 μ L RBC, enough for 4 treatments, 100 μ L per treatment. Wash cells by resuspending in 10 mL wash buffer. Spin cells at 1200 rpm for 5 mins and remove supernatant. Add 4 mL wash buffer, resuspend pellets and aliquot 1 mL into 4x 1.5 mL tubes, spin and remove supernatant, resuspend cells gently in 200 μ L enzyme solutions comprising:
 1. 200 μ L wash buffer,
 2. 20 μ L neuraminidase, 180 μ L wash buffer
 3. 180 μ L trypsin, 20 μ L buffer
 4. 180 μ L chymotrypsin, 20 μ L buffer
- Incubate 1 hour at 37°C with gentle shaking. While cells are incubating, label 10 mL tubes with parasite line and treatment. After 1 hour, resuspend trypsin and chymotrypsin-treated cells in 100 μ L inhibitor solution. Incubate at 37°C for 10 mins. Resuspend all cells and transfer to labelled 10 mL tubes for washing steps. Add 5 mL wash buffer (WASH 1). Spin down and remove medium (WASH 2). Resuspend again in 5 mL medium, spin and remove medium (WASH 3). Resuspend to exactly 2.5 mL COMPLETE medium.
- Label a sterile 96-well U-bottom plate, leaving outside wells free, these will be filled with medium to prevent drying out of assay. Set up each parasite line in columns, with triplicate wells for each treatment (4 rows). There will be a total of 12 wells for each parasite line, each with 4 treatments: no enzyme, neuraminidase, chymotrypsin and trypsin. In addition set up a couple of control wells with untreated W2mef parasites. Place plates back in incubator until day 5.
- Day 5
- Smear control wells, if parasites have reinvaded (rings), smear all wells, Giemsa stain slides and count invasion events. Percentage invasion of each parasite line into each population of rbc should be calculated by counting invasion events per 1000 erythrocytes. Compare parental and mutant parasite lines for a switch in receptor usage.

IV: D. Vector constructionBackground

Construction of appropriate vectors for stable or transient transfection using some of the available plasmids can be problematic due to instability and poor growth in *E. coli*. The main reason for this instability appears to be the high AT composition of the genes and in particular the extragenic region that can be >90% AT. Interestingly, once a construct is obtained it usually remains stable henceforth. Therefore, the problems encountered can usually be overcome by testing a number of *E. coli* strains with different genetic backgrounds to identify one that provides a stable vector. The *E. coli* strains PMC103 and XL10-Gold have proven to be very useful for this problem but can provide poor yields of the plasmid.

Subcloning of fragments into the various transfection vectors such as pHH1, pHH2, pHTK and pCC can be very inefficient and it may be necessary to screen large numbers of *E. coli* colonies to identify those that contain the correct plasmid in an un-rearranged state. This can more easily be achieved by using screening by polymerase chain reaction (PCR), picking a portion of each colony directly into the PCR reaction mixture. This facilitates screening of large numbers of colonies to identify those that have the appropriate structure required.

Common *P. falciparum* transfection plasmids

Stable transfection of *P. falciparum* has used primarily two types of vectors containing either *Toxoplasma gondii dhfr* [2, 3] or human *dhfr* [4] as the gene for selection of transfected parasites. More recently, other genes such as blasticidin, neomycin and puromycin resistance genes have been used successfully for selection of *P. falciparum* transfectants [14, 15]. The structure of some commonly used vectors are shown in the Appendix and are described below.

pHH1

- The pHH1 vector [16], its parent vector pHC1 and derivatives [17, 18] have been useful for gene targeting and for transgene expression to analyse protein trafficking, merozoite invasion and drug resistance. This vector allows integration of the plasmid into the genome of *P. falciparum* by single-crossover recombination. Although very useful, this strategy has a major drawback in that it does not allow selection of gene disruptions that are not lethal but are deleterious to parasite growth. This is because of the persistence of episomal plasmid in some parasites despite growth of transfectants in the absence of drug selection. Reapplication of drug pressure selects for parasites that have the plasmid integrated but also for those that contain the plasmid as an episome. If the parasites containing the integrated form of the plasmid grow more slowly they will be lost in the parasite population and parasites with episomal copies of plasmid will predominate.

pTK vectors

- To overcome the problem of persisting episomal plasmid in transfected *P. falciparum* we developed a new vector (pHTK and derivatives) that utilises the *thymidine kinase* gene to negatively select against its maintenance [19]. This has been very successful as it allows disruption of genes not previously obtained using pHH1 and also significantly decreases the length of time required to select

the *P. falciparum* parasites that have integrated the plasmid. Importantly, this vector allows selection of parasites that have integrated a region of the transfection plasmid by double crossover recombination. This is an important advance for reasons described above but also allows more defined deletions and mutations in the *P. falciparum* genome and will also facilitate the production of double mutations and knockouts into the genome.

pCC vectors

- pCC vectors allow - like the pTK vector - the integration via double crossover recombination [20]. Instead of the *thymidine kinase* gene they use *cytosine deaminase* gene as a negative selectable marker. This selection is more stringent allowing a faster and more reliable selection procedure. In addition the pCC vector series is modular making the exchange of the positive selectable marker cassettes easier (see below) and therefore are used for subsequent 'knockouts' of different genes in the same parasite line.

pHH2

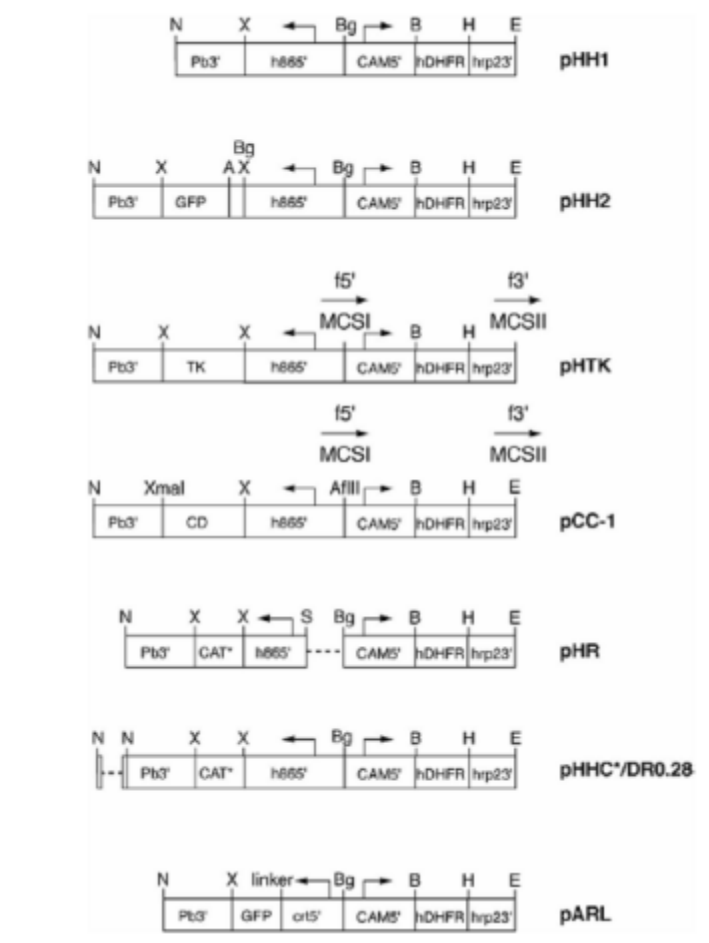
- The use of GFP tagged proteins has been an important application to follow the trafficking pathway of proteins in live *P. falciparum*-infected erythrocytes [18, 21]. The transfection vector pHH2 allows cloning of sequences into a gene cassette to obtain expression of proteins to GFP. This vector uses the promoter from the *hsp86* gene, which allows a broad expression of the GFP in *Plasmodium* blood stages.

pARL-1a

- This vector is mainly used for expression of GFP-tagged proteins. It uses a tail-to-head orientation of the expression cassettes to avoid the bi-directional influence of the *cam* promoter on the expression of the gene of interest. Additional restriction sites facilitate cloning. In contrast to the *hsp86* promoter driven expression in pHH2, expression in this vector is driven by the *P. falciparum crt* promoter. The distinct *crt* promoter activity has been successful in avoiding cytotoxic levels of GFP expression. For this application the GFP expression cassette of the pHH2 vector is transferred into the *XhoI* site of the pARL-1a vector. The *crt* promoter element can be exchanged to modify the expression profile of the gene of interest.

Rep20 plasmids

- It has recently been shown that the inclusion in transfection plasmids of stretches of the *P. falciparum* subtelomeric repeat sequence Rep20 confers improved plasmid maintenance in transfected parasites [22]. This occurs because Rep20 sequence allows transfected plasmids to tether to *P. falciparum* chromosomes and as a result plasmids are segregated efficiently between daughter merozoites. The primary advantage of this for transfection technology is that drug resistant parasite populations are established much more rapidly if Rep20 is included in the transfection plasmid; some 1-2 weeks before the appearance of parasites transfected with control plasmids.



Other positive selectable markers

In addition to the *dhfr* selectable markers described earlier, 3 other positive selectable markers have been successfully used to derive drug-resistant parasite populations. These markers, blasticidin S deaminase (BSD) [14], neomycin phosphotransferase II (NEO) [14] and puromycin-*N*-acetyltransferase (PAC) [15], confer resistance to blasticidin S, geneticin (G418) and puromycin respectively. Although all three selectable markers have been used to derive drug-resistant parasite populations harbouring episomally replicating plasmids, to date only PAC and BSD has been successfully used for gene targeting.

IV: E. Analysis of transient transfectants

Transient transfectants

Background:

Because of the low efficiency of *P. falciparum* transfection, highly sensitive reporter systems are required for use in transient transfection. Two such reporter genes, *chloramphenicol acetyl transferase (CAT)* and *luciferase (LUC)*, have been successfully used for promoter analysis following transient transfection. *LUC* is the more versatile system, but requires more elaborate equipment.

Procedure

See methods for “Preparation of *P. falciparum* parasites for transfection”, “Preparation of DNA for transfection” and “Electroporation and Plating”. These methods have the following exceptions:

- Ethanol precipitate 75 µg of the plasmid pPf86 (firefly luciferase) and 75 µg of pPfluc (*Renilla* luciferase) in the same tube.
- Allow pellet to dry in a laminar flow hood. Resuspend completely in 30 µl of sterile TE. DNA may be left at 4°C for several hours prior to transfection to facilitate resuspension.
- Add 370 µl of sterile Cytomix to each plasmid DNA pellet.
- 5h after transfection change the medium.
- Fresh media is added to the cultures 24h post-transfection.

Luciferase assays in *P. falciparum*

Background:

Both firefly and *Renilla reniformis* luciferase genes can be expressed in *P. falciparum* and their respective enzyme activities measured in the same parasite sample [25]. When the *R. reniformis* gene is transfected in conjunction with the firefly luciferase gene, one can be used to control transfection efficiency.

Material and reagents

- saponin
- PBS
- 1x Passive Lysis Buffer (PLB) (Promega)
- Promega’s Luciferase Assay Reagent (LAR II) (firefly substrate)
- Promega’s Stop and Glo Reagent (Renilla substrate)
- assay tubes/96 well plates (for tube luminometers)

Equipment

- microcentrifuge
- plate luminometer

Procedure:

Preparation of lysates for luciferase assays

- At 48h post-transfection, red blood cells are collected by centrifugation at 1500 rpm for 5 min.

- Prepare desired quantity of 1x Passive Lysis Buffer (PLB) (Promega) by diluting the provided 5x solution with distilled water. Leave on ice.
- Add 1.5x pellet volume of saponin and place on ice for 10 min.
- Centrifuge parasites at 2800 rpm for 5 min.
- Remove supernatant and add 5 mL PBS. Re-centrifuge.
- Remove supernatant and add 1 mL PBS. Transfer to small tubes (Eppendorf). Re-centrifuge to pellet parasites.
- Remove supernatant. Add 50 μ l 1x PLB to parasite pellet. Mix well by pipetting or vortexing.
- Incubate on ice for 10 min. Keep these lysates on ice.

Luciferase assay

- Prepare Promega's Luciferase Assay Reagent (LAR II) (firefly substrate) according to the instructions. The efficiency of this reagent decreases with freeze-thaw cycles. It is therefore suggested to prepare all of the provided amount at once and freeze in 1 mL aliquots at -70°C . For subsequent assays, these aliquots can be thawed at room temperature.
- Prepare desired amount of Promega's Stop and Glo Reagent (Renilla substrate). 100 μ l is required for each sample.
- Thaw the dilution solution in a room temperature waterbath. *Renilla* substrate (Stop and Glo) is provided as a 50x solution. Dilute required amount in appropriate amount of Stop and Glo dilution solution.
- Once prepared these substrates must be kept at room temperature and protected from light.
- For each sample 20 μ l of parasite lysate is aliquotted into assay tubes (for tube luminometers) or 96 well plates (for plate luminometers). Follow manufacturer's instructions for operating protocols.
- Add 100 μ l LARII. Luminescence is measured for 24-45s.
- Add 100 μ l of Stop and Glo and read luminescence for 24-45s. Addition of the *Renilla* reagent quenches the firefly luminescence and simultaneously activates *R. reniformis* activity.
- Normalise the level of firefly luciferase activity to that of *R. reniformis* luciferase activity.

IV: F. Inducible gene expression system for *P. falciparum*

Background:

Manipulations of *P. falciparum* blood-stage parasites that have even slight deleterious effects on growth rates are very difficult to derive with the current transfection methods. In order to examine the effect of deleting 'essential' genes or expressing 'dominant-negative' transgenes a conditional mutagenesis system is required in *P. falciparum*. In collaboration with Dominique Soldati's laboratory (Geneva), we have recently developed such a system [26]. This system, which is based on that developed in the related apicomplexan parasite *T. gondii* [27], allows the efficient switching on and off of transgenes using the tetracycline analogue anhydrotetracycline (ATc), in blood-stage *P. falciparum* parasites. The system involves expression of a tetracycline repressor (TetR) protein fused at its C-terminus to different *T. gondii* transactivators (termed TATi1 and TATi2). When bound to tetracycline operator sequences (TetO) placed upstream of minimal (silent) promoters [2] the transactivators facilitate transcription in a manner that is rapidly and efficiently reversed by the addition of ATc. ATc is not toxic for parasites at the levels used, indeed the GPI-anchored GFP transgenic line was generated by 3 weeks culture in the continual presence of ATc. Although much can be done with the system as it stands now in this proposal, we intend to improve the system's useability and adapt it to investigating protein function.

In this course we will inducibly express two proteins tagged with GFP from the constructs pTGFP-GPI and pTGFP19 (Figure 1). The pTGFP-GPI fusion protein contains an endoplasmic reticulum (ER) signal sequence followed by GFP and is terminated with a signal directing the attachment of a glycosylphosphatidylinositol (GPI) anchor (Figure 1A). This protein should traffick to the surface of the *P. falciparum* schizonts/merozoites and remain attached to the outside of the plasma membrane. The second fusion pTGFP19, is the same as the first except that C-terminal fragment of merozoite protein 1 (MSP-1₁₉) has been inserted in frame, in between the GFP and the GPI-attachment signal (Figure 1B).

Procedure:

Tet-inducible GFP expression in *P. falciparum*

- Day1
- starting material is *P. falciparum* blood stage parasites transfected with pTGFP-GPI and pTGFP19. These parasites should have been grown in the presence of 2.5 nm WR99210 (to select for the maintenance of the plasmids) and 0.5 µg/mL ATc (to keep the expression of the GFP fusion protein off).
- To turn expression of the GFP fusion protein on, the ATc must be removed. This is done by first harvesting the parasite culture and transferring it to a 10 mL tube. The red blood cells infected with ring-stage parasites (iRBC) are then pelleted by spinning the culture at 1500g for 5 mins. To simultaneously synchronize the parasites and remove the ATc, the culture media is removed and replaced with 5 pellet volumes of 5% sorbitol (5% sorbitol in water). The iRBC are resuspended and incubated for 5 mins at 37°C. The iRBC are then pelleted as above and rinsed in 5 volumes of culture media. Finally the iRBC are resuspended in 10 mL of media and 1.25 µL of 20 µM WR99210 is added before the culture is returned to a 10 mL Petri dish.

- Days 2 and 3
- It takes some time for the ATc to diffuse out of the RBC and for its concentration to drop sufficiently so that the parasites can express the GFP fusion protein. For this reason it takes 3 days for the expression of the GFP fusion protein to reach a maximum. To facilitate maximum expression it is important to change the parasite's media each day.
- Day 4
- When the parasites have become schizonts for a second time since having the ATc removed (approx. 3 days later) it is time to look at them under the microscope. Remove 1 mL of iRBC resuspended in their culture media and add 2-5 μL DAPI (50 $\mu\text{g}/\text{mL}$) to stain the parasite nuclei.
- Spin parasites at 1500g for 5 mins and remove 400 μL media leaving 100 μL behind in which the iRBC should be resuspended. Drop 5 μL of iRBC onto a microscope slide and gently place a 22 x 64 mm coverslip on top of the cells. Allow the cells to spread out and place under the microscope.

Figure 1A. pTGFP-GPI

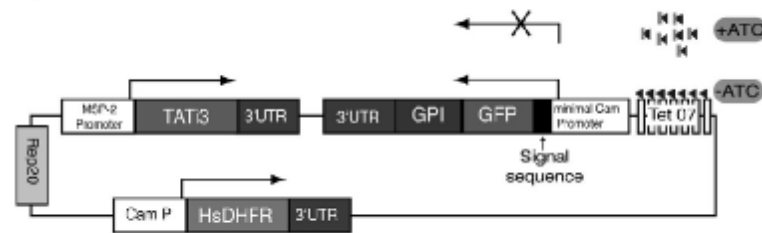
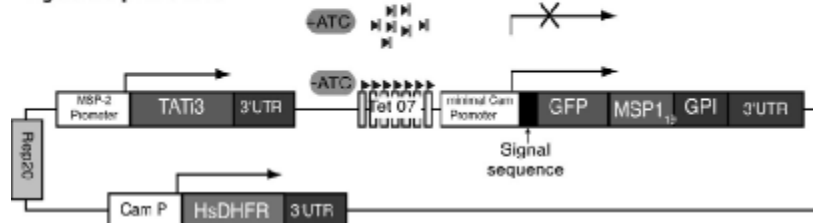


Figure 1B. pTGFP-M19



V. Transfection of *Plasmodium berghei*

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Background:

In this section, the methods that are typically used to stably transfect *P. berghei* are described. Details have been extracted from the following website (www.lumc.nl/1040/research/malaria/model.html) and more comprehensive information can be found on this site.

Since *P. berghei* cannot be readily cultured *in vitro* for more than one cycle, rats or mice infected with *P. berghei* are used as a source of bloodstage parasites for the culture and purification of mature schizonts. Introduction of DNA into these mature stages of *P. berghei* has so far proven to be more successful than into ring and trophozoite stages. The most widely used DNA constructs contain the pyrimethamine resistant form of the *T. gondii* DHFR/TS gene as a selectable marker. This enables transfected parasites, which are injected back into mice, to be selected by treating mice with pyrimethamine. Depending on the desired outcome, the plasmid DNA is either transfected as undigested circular DNA (for episomal replication within the parasite) or as linearised DNA. In the latter scenario, the DNA construct can either be linearised at a unique site located within the target sequence if attempting to integrate plasmid DNA into the parasite genome via a single crossover event, or alternatively, the DNA is digested at the ends of the 5' and 3' target sequence (and preferably also within the vector backbone) so as to remove the plasmid backbone from the rest of the construct in order to drive a double crossover event.

Recently a new method of transfection of *P. berghei* has been described using electroporation with the Amaxa device [Janse et al., in press]. The efficiency of transfection for both episomal and targeted integration into the genome is much higher using this device (10^{-2} - 10^{-3}) than the BioRad Gene Pulser (frequency of transfection = 10^{-6} - 10^{-9}). Therefore, electroporation with Amaxa is more preferable since the high transfection efficiencies obtained with this machine significantly reduces the time, number of laboratory animals and amount of materials required to generate transfected parasites.

Materials and reagents

- Complete culture medium:
Culture medium: RPMI1640, with L-glutamine and 25mM HEPES, without NaHCO_3 (if no HEPES is present, add 4.95g HEPES per litre culture medium)
Preparation of medium:
 - dissolve 10.41g RPMI1640 medium in 1 L water (add powder slowly under continuous stirring).
 - add 2 g NaHCO_3 (and HEPES if necessary)
 - add 50.000 I.U. Neomycin (stock-solution of 10.000 I.U./mL; Gibco)
 - Sterilise by filtration through a 0.2 μm sieve
 - Store at -20°C in 100-200mL bottles
 - Immediately prior to use, Foetal Calf Serum (FCS) is added at a final concentration of 25% (v/v) to give complete culture medium.

- Heparin: Dissolve the content of 1 ampoule heparin (DBL, 5000 I.U per 1 mL) in 25 mL RPMI1640 culture medium (pH 7.2) without fetal calf serum.
- 10 × PBS (Phosphate buffered saline): 0.01 M KH_2PO_4 , 1.37 M NaCl, 0.027 M KCl, pH 7.0, Working solution: Dilute the stock 10 × with demineralised water and adjust the pH to 7.2 with 1 M HCl. Autoclave.
- Nycodenz: Nycodenz powder (Lucron Bioproduct BV) is obtained from Life Technologies. Store at room temperature.
- Nycodenz buffered medium: 5 mM TrisHCL, pH 7.5, 3 mM KCl and 0.3 mM CaNa_2EDTA .
- Nycodenz stock solution: Dissolve 27.6 g solid Nycodenz in 60 mL buffered medium and make up to 100 mL with that medium (density (20°C) 1.15g/mL). Autoclave and store at 4°C.
- Pyrimethamine-solution in drinking water.
Dissolve pyrimethamine in DMSO to a final concentration of 7 mg/mL (stock solution). Dilute this stock solution 100 times with tap water and adjust the pH of the water to 3.5-5.0 using 1 M HCl.
- *P. berghei* freezing solution: 30 % glycerol in PBS. Autoclave.
- Parasite lysis buffer: 10 mM Tris pH 8, 0.4 M NaCl, 1 mM EDTA, 1 % SDS.

Procedure:

Preparation of *P. berghei* parasites for transfection

- Infecting donor rats/mice for *P. berghei* transfection (Day 0)
- Day1
- For rats, inject around 1.5×10^7 parasites intraperitoneally (i.p) per rat and these will be ready for harvest around 4 days later (day 4). The parasitemia at harvest should be between 1-4% (no more). 1 rat (approx 5-8 mL heartblood) will be sufficient for up to 12 transfections.
- For mice, inject around 2.5×10^6 parasites i.p into 7-8 new donor mice (or sufficient mice so as to get around 5 mL of heartblood). Parasitemias should be between 1-4 % on day 3. This amount of heartblood will be sufficient for 12 transfections.
- Day 3: In vitro culture of *P. berghei*
- When the parasitemias of the animals reach between 1-4 % heart bleed animals in the afternoon, using a 23 -G needle with attached syringe containing 0.1 mL heparin stock solution. Pool blood from all mice/rat into a 50 mL tube containing 5-10 mL complete culture medium.
- Spin blood at 1500 rpm, 10 min
- Resuspend parasites in complete culture medium such that the haematocrit is between 2-4%, i.e. for 5 mL heartblood of 3% parasitemia culture in around 120 mL complete culture medium. Culture infected RBC in 500 mL erylenmyer or plastic tissue culture flasks. If culturing in flasks without continuous gassing, use around 60-80 mL/flask otherwise can culture 120-180 mL per 500 mL flask.
- Gas flasks and incubate at 37°C overnight. Flasks can either be continuously gassed using an 'automatic' continuous gassing system whereby the cultures are continuously gassed throughout the complete culture period using 5% CO_2 , 5% O_2 , 90% N_2 . Alternatively cultures can be maintained in closed plastic 500 mL culture flasks that have been gassed once for 2 minutes at the beginning of the

culture period. (optional – place flasks on a shaker at minimal speed to keep cultures in suspension).

- (Day 4) At around 9.00 am the following day smear parasites by taking out 300 μ l of gently resuspended parasites, spinning briefly in 1.5 mL microcentrifuge tube, removing supernatant and resuspending parasites in residual medium. Make smear and stain. Check that the schizonts are nice and healthy.
- Day 4: Purification of mature *P. berghei* schizonts
- Split parasite cultures into 50 mL tubes so that have 30-35 mL culture in each. Prepare 55% Nycodenz solution and gently layer 10 mL of this very carefully underneath the suspension. For a culture suspension of 150 mL a total volume of 50 mL of 55% Nycodenz is used (=27.5 mL nycodenz stock solution, 22.5 mL PBS)
- Spin 20-30 min at 1200 rpm in a swing out rotor at room temp, NO BRAKE
- Carefully collect the brown layer containing schizonts (and gametocytes and old trophozoites if present) at the interface with pasteur pipette into new 50mL tube. Uninfected red blood cells will pellet at the bottom of the tube. In general a total volume of about 30-40 mL is collected. Add around 20 mL of culture medium from the top of this nycodenz density gradient to help wash away the Nycodenz.
- Centrifuge parasites at 1500 \times g, 8 min to pellet the schizonts.
- Gently remove the supernatant and resuspend pellet volume in 1 mL culture medium for each transfection. Therefore if performing 6 transfections, resuspend in 6 mL of culture medium. Split into microcentrifuge tubes so 1 mL/tube.

Preparation of DNA for *P.berghei* transfection

- Plasmids which are to be maintained episomally in transfected parasites require no digestion and thus 5 μ g of DNA can be concentrated for transfection by ethanol precipitation. For single and double crossovers, digest DNA with appropriate restriction enzyme(s) overnight to ensure complete digestion and purify the targeting DNA by gel-electroelution or by a gel-purification kit. Ethanol precipitate 5 μ g of linearised targeting DNA.
- Allow the pellet to dry and resuspend each DNA construct in 5-10 μ l water or TE buffer (10 mM Tris, 1mM EDTA, pH 8).

Electroporation of *P.berghei*

- Add 100 μ l of the 'Human T cell NucleofectorTM' solution from the Amaxa kit to the resuspended DNA.
- Pellet the schizonts by centrifugation (5s, max speed in microcentrifuge tube). Discard the supernatant.
- Resuspend each parasite pellet gently in the DNA/nucleofector mix.
- Transfer the parasite/DNA/nucleofector mix to a cuvette (0.2 μ l) and transfect using the Amaxa gene pulser, protocol U33.
- Add 50 μ l complete culture medium to the cuvette immediately after transfection (try to avoid bubbles) and then inject into 1 mouse intravenously using an insulin 27-Gauge needle. Make sure the veins of the mice are swollen first by placing the mice at 37°C for 10 min before electroporation of the parasites.
- Day 5: Drug selection of transfected *P. berghei* parasites

- To select for parasites harbouring transfected DNA provide the animals with drinking water containing pyrimethamine 24-30 hrs after injection of transfected parasites and treat for 4-7 days. Smear the mice every 2nd day from day 5-6 onwards. The parasitemia the day after transfection usually ranges between 0.05-3%. After the first 2 drug treatments a rapid drop in parasitemias occurs to undetectable levels indicating that most of the parasites do not contain the DNA constructs. In successful experiments using the Amaxa electroporator the parasitemia increases to levels of 0.1-5% between days 4 and 7 after transfection. In unsuccessful experiments parasites are often detected between day 13 –15 after the injection of transfected parasites. These parasites are usually non-resistant wildtype parasites that survived the drug treatment protocol.
- At a parasitemia of between 1-5% parasites are collected for storage in liquid nitrogen and for collection of DNA for genotype analysis (see below).

Freezing down of *P. berghei* parasites

- Collect heart blood from 1 mouse with parasitemia around 1-5% using 23-G needle containing around 0.05-0.1 mL heparin solution in the syringe.
- Gently mix blood with an equal volume of sterile freezing solution (30% glycerol made up in PBS).
- Aliquot between 300-500 μ l per cryovial. Leave cryovial at 4°C for 5 min then store in liquid nitrogen.

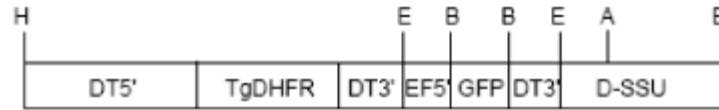
Extracting *P. berghei* genomic DNA from infected mice

- Heart bleed mice displaying parasitemia of between 5-15% using 23-G needle containing around 0.05-0.1 mL heparin solution in the syringe and suspend the blood in 5 mL PBS.

Remove leukocytes from the blood by passing the blood suspension through a Plasmodipur filter (Euro-Diagnostica, www.eurodiagnostica.com) or through CF11 powder. For removal of leukocytes through a CF11 column:

- Add 3 x blood volume of CF11 powder (Whatman) to a column.
- Run through 2 x column of PBS to pack the column.
- Add infected red blood cells (RBC)/PBS mix and collect flow through in a 50 mL tube. Then add a further 2 x column volume and continue to collect flow through. The leukocytes remain bound to the column while the infected RBC will flow through.
- Centrifuge the RBC - 1500 x g, 5-10 min.
- Resuspend the RBC pellet in 1.5 x volume of 0.15% saponin. Leave on ice for 10 min and then centrifuge 1500 x g /10 min.
- Remove supernatant, wash pellet twice with PBS and then store at -20°C until further manipulation for collection of genomic DNA. (Note: this material can also be used to extract RNA or protein).
- FOR DNA EXTRACTION: Resuspend the parasite pellet in 1 mL parasite lysis buffer and mix well
- Add 100 μ g RNase and incubate at 37°C for 30 min. Add 100 μ g proteinase K and incubate at 37°C for 45 min.
- Do 3 extractions with phenol/chloroform
- Precipitate the upper aqueous phase (DNA) with 95% ethanol and 1/10 volume sodium acetate pH 5.2 at -20°C for > 1 hr.

- Centrifuge at 13 000 × g for 20 min and then wash the DNA pellet with 70% ethanol
- After drying the pellet at room temp for 5 min, carefully dissolve the DNA pellet in 50-100 µl sterile TE (10 mM Tris, 1 mM EDTA pH 8).



- The transfection plasmid PbGFP_{CON} is used for the stable expression of GFP. This construct contains the pyrimethamine-resistant *T. gondii* DHFR-TS gene for selection of transgenic parasites and an incomplete copy of the *D-SSU-rRNA* as a target region for integration. The vector can be linearised at the unique *Apal* site for integration. The *GFP* gene is flanked by the *EF-1αa* promoter and the 3' UTR of *P. berghei* DHFR-TS. B: *Bam*HI, E: *Eco*RI and H: *Hind*III. Extracted from [28].

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FIELD BLOOD SAMPLING

I. Finger prick blood sampling

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I:A. Blood collection on filter paper

Equipment

heating block (Techne)

Materials and reagents

heparinized capillary tubes, 75- μ L (Drummond Scientific)

chromatography filter paper, ET31CHR (Whatman).

EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw finger prick blood samples into 1 to 3 heparinized, 75- μ L capillary tubes and transfer them to chromatography filter paper.
- Alternatively, allow 1 drop of blood to fall onto a filter paper directly from the finger. The finger must not touch the filter paper (see the photo).
- Soak the drop into the filter paper until the paper is evenly red without spreading to the underlying surface.
- Dry the filter-paper samples completely in air (see the photo) and place them in small plastic bags.
- We can keep the dried filter-paper samples at room temperature for at least several weeks in field conditions before extracting antibodies, human and parasite DNA, and postdose drugs.
- Store the filter-paper samples at -20°C for long term.



I:B. Blood collection in EDTA tubes

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A better quality and quantity of DNA can be obtained by EDTA tubes than by filter paper.

Materials and reagents

EDTA tubes (Microtainer, Becton Dickinson)

Procedure

- Draw blood samples taken from the finger tip into 2 to 5 heparinized capillary tubes as mentioned above and transfer into an EDTA tube.
- We can keep the EDTA-tube samples at room temperature for at least one week in field conditions before extracting DNA.
- Store the EDTA-tube samples at -80°C for long term.

II. Serum extraction from filter-paper samples

by **Akira Kaneko**

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Equipment

paper punch
shaker (Vortex)

Materials and reagents

10-mL tubes
PBS containing 0.05% Tween and 0.5% BSA
Pasteur pipettes

Procedure

- Use a paper punch with a diameter of about 6 mm for punching out discs. Be careful to use areas in the middle of the spot where the blood is evenly spread.
- Put the discs in 10-mL tubes.
- Add 500 μ L of PBS containing 0.05% Tween and 0.5% BSA.
- Incubate the discs for 2 h at room temperature on a shaker.
- Vortex them for a few seconds.
- Withdraw the liquid with a Pasteur pipette and aliquot it.
- Store at -20 °C until analysis.
- The extract corresponds to a serum dilution of $\sim 1:100$. The dilution factor is however dependent on the quality of the filter paper.

III. DNA extraction

III: A. DNA extraction from filter-paper samples

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Microscale isolation of *Plasmodium falciparum* or human DNA from filter-paper samples has been conventionally done by brief boiling (Wooden et al. 1992; Uchida et al. 1995), which is sufficient for obtaining short DNA.

Equipment

centrifuge

Materials and reagents

sterile water containing 15 μ L of Chelex-100 (Bio-Rad)
HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin (Merck)
1.5-mL tube
QIAamp DNA Mini Kit (Qiagen)
ethanol
DNA elution buffer AE:
0.5 mM EDTA
10 mM Tris-HCL (pH 9.0)

Procedure

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 μ L of blood, into 3 \times 3-mm pieces.
- Heat the pieces in 205 μ L of sterile water containing 15 μ L of Chelex-100 at 100 °C for 8 min.
- After cooling them on ice for 2 min, collect the supernatant containing DNA (200 μ L) by centrifugation and store them at -20 °C.

However, the DNA samples thus obtained show a limited PCR template activity with little amplification of DNA longer than 1 kb. Considering the extremely high A + T content of *P. falciparum* DNA, boiling may cause fragmentation of DNA and would no longer be a method of choice for DNA isolation when a long PCR product covering an entire gene is required. To solve this problem the following alternative method has been developed (Sakihama et al. 2001):

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 μ L of blood, into 3 \times 3-mm pieces.
- Incubate the pieces in 1 mL of HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin at room temperature for 1.5 h in a 1.5-mL tube.
- Wash them twice with 1 mL of HBS.
- Use a QIAamp DNA Mini Kit to isolate DNA remaining on the filter paper, according to the manufacturer's instructions with the following modifications.
- To avoid shearing DNA, minimize vortexing and substitute by repeated inversions.

- Before the addition of ethanol to the extract, thoroughly remove the filter-paper debris by centrifugation at $9,000 \times g$ for 5 min.
- Reduce the volume of DNA elution buffer AE to 50 μ L.
- Prolong the preincubation before DNA elution to 5 min to maximize the elution recovery.
- Store the eluted DNA at 4 °C until PCR amplification.

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Wooden J, Gould EE, Paull AT, Sibley CH. 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method differentiating strains. *Exp Parasitol*. 75(2):207-212.

III:B. DNA extraction from filter-paper discs previously used for serum extractionby **Manijeh Vafa, Masashi Hayano, and Marita Troye-Blomberg***Immunology Department, The Wenner-Gren Institute, Stockholm University,
SE-106 91 Stockholm, Sweden**e-mail: manijeh@imun.su.se, mhayano@imun.su.se, marita@imun.su.se***Equipment**

Vortex
Heating block
Eppendorf centrifuge

Materials and reagents

Eppendorf tubes
Saponin
PBS
Chelex-100
Distilled water

Procedure

- After serum extraction as described in FIELD BLOOD SAMPLING, section II, transfer the discs to new Eppendorf tubes containing 1 mL of 0.5% saponin in PBS.
- Incubate the tubes at 4 °C overnight.
- Discard the solution.
- Wash the discs with 1 mL PBS at 4 °C for 15-30 min.
- Discard supernatant.
- Transfer the discs into new tubes containing 100 • l of 5% Chelex-100 in water.
- Vortex the tubes for 30 sec.
- Heat at 95 °C for 15 min.
- Vortex the tubes for 30 sec. NOTE: Watch your hand! Caps of tubes are loose and the buffer is hot.
- Centrifuge at 10,000 rpm for 2-3 min.
- Collect the supernatant (using pipette) in new Eppendorf tubes.
- Use 2.5 µl in a 10 µl total PCR reaction mix, store at -20 °C.

III:C. DNA extraction from EDTA-tube samples

by **Akira Kaneko**

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Equipment

water bath
Generation Capture Plate kit (Gentra Systems)

Materials and reagents

whole blood frozen in EDTA tube
EDTA tubes

Procedure

- Use 150 to 375 μL of frozen ($-80\text{ }^{\circ}\text{C}$) whole blood collected in an EDTA tube.
- Thaw it quickly in a $37\text{ }^{\circ}\text{C}$ water bath and keep it on ice until use.
- Use Generation Capture Plate kit according to the manufacturer's instructions.

III:D. Fast methanol-based DNA extraction from blood spots in filter paper.*by Pedro Gill and Sandor Bereczky**Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-171 76 Stockholm, Sweden**e-mail: pedro.gill@ks.se, sandor.bereczky@ks.se***Equipment**

heating block (Techne)
micropipette, 1000- μ L

Materials and reagents

blood spots on filter paper (Whatman 3MM)
new surgical blades
tips
1.5-mL Eppendorf tube
methanol
distilled, sterile water

Procedure

- Cut a blood spot (about 3×5 mm) from the filter paper. (Note: Not all filter papers work when making the blood spot collection. Make sure you are using the right one, usually Whatman 3MM.) Do not touch the blood with the fingers when manipulating the filter paper. Use new surgical blades, one for each sample, to avoid contamination. The cuts should be done on a disposable surface, the best choice being small yellow stickers (note pads).
- Use the blade also for transferring the cut-out to a 1.5-mL Eppendorf tube. The filter papers with the remaining blood spots should be protected (e.g., in separate plastic bags) until a new DNA extraction. After each blood sample, remove 2 or 3 note pad sheets (due to the fact that the blade usually cuts through at least two sheets of the note pad).
- Add 125 μ L of methanol (room temperature) and wait 15 min. Make sure that the whole paper-cut is soaked in the methanol. Keep at room temperature until further processed (at least 15 min).
- Remove the methanol with a 1000- μ L micropipette (using a new tip for every sample).
- Let the paper dry at room temperature for 15 min with the lid open. Close the lid and rotate the tube gently. If the paper moves freely it is dry, if it sticks even slightly, dry it for 5 to 10 more minutes.
- Add 65 to 75 μ L of distilled, sterile water and smash the paper in the water with the micropipette. The water will become slightly red.
- Heat the tube at 95 to 100 °C in a heating block or a water bath for 15 min.
- Ready for PCR! Use 3 to 10 μ L in a 50- μ L total reaction volume. (Attention: This extraction is rather dirty. Too much template can give rise to inhibition of polymerase.)

Note: FIELD BLOOD SAMPLING, section III:A for DNA extraction is suitable for long, intact fragments over 700 bp, and is particularly efficient for fragments more than 1000 bp, while FIELD BLOOD SAMPLING, section III:D is particularly efficient for short fragments up to 500 bp and when the filter paper cut is small (2×2 mm).

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IV. Postdose drug level monitoring

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Using the filter-paper samples, chromatographic analytical methods were developed to monitor capillary blood levels of the drug and its metabolites after administration by Bergqvist and colleagues. Currently methods for the following drugs are available:

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Suppliers

5 Prime® 3 Prime
(see **Eppendorf – 5 Prime**)

ABgene
www.abgene.com

Agar Scientific
<http://www.agarscientific.com>

Amersham Biosciences
(also referred to as **Amersham**,
Pharmacia, and **Pharmacia Biotech** in this
manual)
www.amershambiosciences.com

Applied Biosystems
www.appliedbiosystems.com

ATCC
www.atcc.org

Axis-Shield PoC AS
www.axis-shield-poc.com

Bachem
www.bachem.com

Baxter Healthcare
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manual)
www.baxter.com

BD Biosciences
www.bdbiosciences.com

BD Immunocytometry Systems
www.bdbiosciences.com

BD Labware
www.bdbiosciences.com

BD Vacutainer Systems
www.bd.com/vacutainer

BDH Laboratory Supplies
www.bdh.com

Beckman Coulter
(referred to as **Beckman** in this manual)
www.beckmancoulter.com

Becton Dickinson
www.bdbiosciences.com

Billups-Rothenberg
www.brincubator.com

Bio-Rad Laboratories
www.bio-rad.com

BOC Specialty Gases
<http://www.boc-gases.com/>

Brinkmann Instruments
www.brinkmann.com

Calbiochem
www.emdbiosciences.com

Cambrex
www.cambrex.com

Clontech
www.bdbiosciences.com/clontech/

CML
<http://www.cml.fr/uk/accueil-cml-uk.htm>

Cole-Parmer
www.coleparmer.com

Corning Life Sciences
www.corning.com/lifesciences/

Dako Corp
www.informagen.com

Drummond Scientific
www.drummondsci.com

Electron Microscopy Sciences:
<http://www.emsdiasum.com/default.htm>

Eppendorf
www.eppendorf.com

Eppendorf – 5 Prime
(referred to in this manual as **5 Prime® 3 Prime**)
www.5prime.com

Fluka

http://www.sigmaaldrich.com/Brands/Fluka___Riedel_Home.html

Fresenius France

www.biam2.org

Gentra Systems

www.gentra.com

Gibco

www.invitrogen.com

HCM-Hygenic Corporation

products available from:
www.e-dental.com/buyersguide/

HD supplies

<http://www.tcsbiosciences.co.uk>

ICN Biomedicals

www.icnbiomed.com

Invitrogen

(also referred to as **Gibco** in this manual)
www.invitrogen.com

Iwaki

products available from
<http://www.barloworld-scientific.com/>

Janssen Pharmaceutica

www.janssenpharmaceutica.be

Joseph Long, Inc.

Phone: 1-973-759-5331

Kontes

www.kimble-kontes.com

Leigh Labs

www.leighlabs.com

Leo Pharma

www.leo-pharma.com

Lillie Glassblower

Phone: +1-404-436-8959

Life Technologies

products available from:
<http://www.invitrogen.com/>

Mabtech

www.mabtech.com

Merck

www.merck.com

Millipore

www.millipore.com

Miltenyi Biotec

www.miltenyibiotec.com

MJ Research

<http://www.gmi-inc.com/BioTechLab/mjresearchptc100.htm>

Molecular Dynamics

(see also Amersham Biosciences)
www.mdyn.com

Molecular Probes

www.probes.com

Moravek Biochemicals

www.moravek.com

Nalge Nunc International

(referred to as **Nalgene** and **Nunc** in this manual)
www.nalgenunc.com

Narishige

www.nuhsbaum.com

Nikon

www.nikon-instruments.com

Novakemi

www.novakemi.se

Olympus

<http://microscope.olympus.com>

Operon Biotechnologies

<http://www.operon.com/>

PerkinElmer

(referred to as **Wallac** in this manual)
www.perkinelmer.com

Pharmacia (see **Amersham Biosciences**)

Pharmacia Biotech (see **Amersham Biosciences**)

Pierce Chemicals
www.piercenet.com/

Promega
www.promega.com

Qiagen
www.qiagen.com

R&D Systems
www.rndsystems.com

Roche
www.roche.com

Roche Applied Science
www.roche-applied-science.com

Savant Speed Vac Systems
<http://www.gmi-inc.com/Products/Savant%20Speed%20Vac.htm>

Sigma (see **Sigma–Aldrich**)

Sigma-Aldrich
www.sigmaaldrich.com

Solis BioDyne
<http://www.sbd.ee/>

Sorvall
www.sorvall.com

Sterilin
<http://www.barloworld-scientific.com/>

Stratagene
www.stratagene.com

Sutter Instrument
www.sutter.com

Syntesome
products available from
www.lectinity.com

Techne
www.techne.com

Terumo
www.terumo.co.jp

Thermo Electron
www.thermo.com

TomTec Cell Harvester
www.tomtec.com

Turner BioSystems
www.turnerbiosystems.com

Vector Laboratories
www.vectorlabs.com

Wallac (see **PerkinElmer**)

Wardley
www.wardleyfish.com

Waters
www.waters.com

Whatman
www.whatman.com

World Precision Instruments
www.wpiinc.com

ZeptoMetrix
www.zeptometrix.com

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