

Consensus Protocol for the Collection, Processing, and Testing of Semen

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1.0 Principle and Clinical Significance

1.1 Semen is the major vehicle for the sexual transmission of HIV-1. The ability to isolate infectious HIV from the semen and to quantitate viral burden in the form of cell-free or cell-associated HIV-1 RNA in semen are important for epidemiologic and public health aspects of the epidemic. Earlier studies used viral culture to detect HIV in semen. Cell associated culturable virus recovery rates ranged from 8 to 55%(1, 10, 11, 15-19). Much lower recovery rates (3-15%) were reported by these investigators for cell-free seminal plasma. In general, subjects with lower CD4 counts, higher seminal plasma viral load (>3.5 to 4 log), and an AIDS diagnosis were more apt to have positive seminal cell HIV cultures.

1.2 More recently, quantitative HIV RNA and DNA assays have been employed (4, 5, 8, 9, 12, 14, 17, 19, 21). Overall these studies have demonstrated that 60-75% of men shed HIV RNA in the seminal plasma and that 65-80% have detectable HIV DNA in seminal cell pellets. Recent cross-sectional studies using commercially available RNA kits have concluded that seminal plasma RNA levels are significantly correlated both with blood plasma RNA levels (8, 19), and the recovery of infectious virus from seminal cells (4, 19), but not with CD4 cell count (4, 8, 19), stage of disease (4,19) or antiviral therapy (4, 8, 19).

1.3 Knowledge of the effect of topical microbicides and/or antiretrovirals on reduction of viral load in semen is necessary for the evaluation of compounds that may be useful in prevention of transmission. It has been demonstrated in longitudinal studies that the amount of HIV RNA in seminal plasma increases with time in individuals who progress to AIDS (7) and decreases with effective antiviral therapy (7, 8). Antibiotic treatment of pathogens causing urethritis, especially gonorrhea, can also reduce seminal plasma viral load (3). In addition, since the viral burden in genital secretions may serve as a reservoir in patients who have had their virus seemingly eliminated from the peripheral blood, the quantitation of virus in semen has become a major focus of clinical trials. The following procedures can be used for conducting studies involving semen.

2.0 Specimen Requirements

2.1 Patient Preparation and Specimen Collection

2.1.1 The subject should refrain from sexual activity for at least 48 hours prior to donation.

2.1.2 The subject should wash his hands and penis and then use an antiseptic towelette to wipe the head of the penis including the opening. If the subject is

uncircumcised, the foreskin should be pulled back before cleaning the head and opening.

2.1.3 The subject should masturbate and collect the specimen in a sterile container, for example a sterile urine collection container. The time that the specimen was produced should be recorded on paper work accompanying the specimen.

2.2 Specimen Transport

2.2.1 The container should be placed in a zip-lock bag and then in an appropriate transport carrier. The mode of transport will determine the type of carrier required. For example, specimens transported by cab must comply with DOT regulations for infectious substances. Effort should be made to keep specimen container upright.

2.2.2 The specimen should be rapidly transported at room temperature to the clinic or directly to the lab. The laboratory should receive the specimen within 2 hours if possible.

3.0 Culture of Seminal Cells

3.1 REAGENTS

3.1.1 Note: all reagents are prepared using TYPE 1 reagent grade dH₂O.

3.1.2 Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS): Store at room temperature. Note manufacturer's outdate or discard one month after opening.

3.1.3 Penicillin-Streptomycin: Available as 100,000 units/ml penicillin and 100,000 ug/ml streptomycin. Open bottle under laminar flow hood only. Divide into 0.6 ml aliquots in sterile 1.5 ml microfuge tubes. Store unopened bottles and aliquots at -20°C., labeled with a 1 year outdate.

3.1.4 Nystatin: Available as 10,000 units/ml suspension. Open bottle under laminar flow hood only. Divide into 2.1 ml aliquots in sterile snap-cap tubes. Store unopened bottles and aliquots at -20°C, labeled with a 1 year outdate.

3.1.5 Benzidine stock solution: Dissolve 125 mg benzidine (Sigma Chemical Co., St. Louis, MO) in 50 ml 96% ethanol. This may take as long as 24 hours. Add 50 ml distilled water. Store in a brown bottle at room temperature. Label with a one year outdate and appropriate hazardous materials identification. **CAUTION: BENZIDINE IS A CARCINOGEN AND CONSIDERED HIGHLY TOXIC. AVOID INHALATION, INGESTION AND CONTACT WITH SKIN. WEAR PROTECTIVE CLOTHING, GLOVES, AND EYE/FACE PROTECTION.**

3.1.6 Working benzidine solution: To 2 ml stock benzidine solution add 25 μ l of 3% hydrogen peroxide. If kept in a brown bottle, this will last for one month at room temperature. However, since contamination with peroxidase can frequently occur, it is probably best to make this up fresh daily. **DISPOSE OF THE BENZIDINE STOCK SOLUTION BY DILUTING IN BLEACH (TO DILUTE THE ALCOHOL) AND POURING DOWN THE SINK. (Contact your Environmental Health & Safety Department to be sure this disposal method is complies with local regulations.)**

3.1.7 Fetal Bovine Serum (FBS): available in 500 ml sterile bottles from various manufacturers. Store frozen at -20°C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37°C water bath, then heat-inactivate in a 56°C water bath for 30 minutes with occasional shaking. The level of the water in the water bath should be as high as the level of the serum in the bottle. Store at 4°C after thawing. Heat-inactivated FBS has a one month outdate. (Note: FBS lots should be pretested at the site for toxicity to normal human lymphocytes.)

3.1.8 RPMI 1640 medium with L-glutamine (2mM): Store at 4°C and observe manufacturer's outdate.

3.1.9 IL-2 (interleukin-2): Note the manufacturer's outdate and storage requirements. As needed, thaw a 50 ml bottle and freeze the remaining unused portion.

3.1.10 Viral transport medium (VTM) (final concentration 1000 units/ml penicillin, 1000 ug/ml streptomycin, 200 units/ml nystatin).

To make 100 ml:

(a). To 97 ml of RPMI 1640 medium with L-glutamine, add 1 ml penicillin/streptomycin.

(b). Add 2 ml nystatin solution.

(c). Dispense in 10 mL aliquots. Store at 4°C for up to a month.

3.1.11 Seminal cell culture medium (final concentration = 500 units/ml penicillin, 500 μ g/ml streptomycin, 100 units/ml nystatin, 20% FBS, 5% IL-2)

To make 100 ml:

(a). To 73.5 ml RPMI 1640 medium with L-glutamine, add 0.5 ml penicillin/streptomycin.

(b). Add 1 ml nystatin solution.

- (c). Add 20 ml heat inactivated fetal bovine serum.
- (d). Add 5 ml interleukin-2.
- (e). Store at 4°C for up to 30 days.

3.1.12 Trypan Blue Stain - This stains non-viable cells dark blue and is used to determine viable cell count (Viable cells will be clear).

- (a) Prepare a 0.4% solution by adding 0.4 g Trypan Blue and 1 ml Glacial Acetic Acid to 9 ml distilled water or saline.
- (b) After dissolving, filter solution through Whatman filter paper or a 0.45 u filter
- (c) Store at room temperature for 6 months.
- (d) Alternatively, trypan blue solution can be purchased from Sigma .

3.1.13 PHA-stimulated uninfected donor PBMCs - See ACTG Virology Manual.

3.2 EQUIPMENT AND SUPPLIES

Gloves
 Lab coat or gown
 Sterile cup (such as a sterile urine container)
 Sterile 15 ml conical centrifuge tubes
 Sterile 1, 5, 10, and 25 ml pipettes
 Sterile 5 ml tubes
 Sterile 100 ml brown bottle or foil wrapped bottle
 Hemacytometer
 Sterile 24 well tissue culture plate
 Sterile 100 ml bottle
 200 ul and 1000 ul pipette tips
 Bleach (household bleach diluted 1/100 with tap water)
 Laminar flow hood (Class 2 biosafety hood)
 Centrifuge capable of speeds up to 800 x g and equipped with a horizontal rotor and O-ring sealed safety cups
 Vortex
 Microscope
 CO₂ incubator (37 +/- 1°C with humidity)
 37°C and 56°C water baths
 Pipette aid
microcentrifuge tubes
microcentrifuge

fume hood (for preparation of Benzidine solution)

3.3 PROCEDURE

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING THE USE OF GLOVES AND LAB COATS)

3.3.1 Allow liquefaction to occur. This typically occurs within 20-45 minutes of specimen collection

3.3.2 Transfer the sample to a conical centrifuge tube using a pipette. Measure and record the volume of semen.

3.3.3 Count the cells using the Endtz test (6,13) to document inflammation:

(a). Vortex sample briefly. Place 20 μ l of liquefied semen in a microfuge tube. Add 20 μ l phosphate buffered saline and 40 μ l of working benzidine solution.

(b). Vortex briefly and allow to sit at room temperature for 5 min.

(c). Count brown staining (peroxidase positive) cells in the four large squares of a hemacytometer.

(d). Calculate and record the number of peroxidase positive cells/ejaculate = Brown cells in all 4 squares $\times 10^6$. Inflammation is considered present when there are 1 million peroxidase positive cells/ml of semen.

3.3.4 Centrifuge semen at 600-800 \times g for 10 minutes.

3.3.5 Remove supernatant, divide into 0.5 ml aliquots, and freeze at -70°C .

3.3.6 Resuspend cells in the pellet from step (5) in 5.0 mL Viral Transport medium, if culture is anticipated, or in Hank's balanced salt solution or PBS (Do not use fetal calf serum or amphotericin as these reagents will inhibit the HIV culture). Centrifuge at 400-600 \times g for 10 min.

3.3.7 Note: At this point some investigators remove spermatozoa from the cellular fraction using a standard ficoll-hypaque gradient. This may be important for studies in which HIV proviral DNA is investigated. DNA found in sperm may interfere with the analysis of HIV DNA. If this proves to be a problem, layer the seminal cells onto 4mL of ficoll-hypaque, then centrifuge at 600 \times g for 20 minutes. Carefully remove the non-spermatozoal cells found at the interface and resuspend in 10mL HBSS or PBS. Proceed with step 3.38 below. If sperm cells do not interfere with your assay, this ficoll separation does not need to be performed.

3.3.8 Centrifuge at 400-600 x g for 10 min.

3.3.9 Repeat the step (6) once and re-suspend in 3 ml seminal cell culture medium that contains high doses of antibiotics (penicillin @ 500 u/ml, streptomycin @ 500 ug/ml, and nystatin @ 100 u/ml). (Note: Gentamicin may be substituted for the streptomycin.)

3.3.10 Determine the viability of the cells using the trypan blue dye exclusion method (See ACTG Virology Manual:http://www.niaid.nih.gov/daids/vir_manual/). An accurate cell count of the white blood cells will be difficult as they cannot be readily distinguished from immature germ cells.

3.3.11 Use half of the suspension (i.e. 1.5 ml) from step (7) for the quantitative microculture by adding it to 1.5 ml seminal cell culture medium and then making five 5-fold dilutions in seminal culture medium. The number of cells will vary with each specimen. Record this number such that it may be retrieved if needed.

3.3.12 Proceed with the quantitative culture using the ACTG consensus protocol (except calculate the Infectious Units per Ejaculate by multiplying the IUPML by 6, since 1/6 of the total ejaculate cells were used in each duplicate A well of the quantitative culture).

3.3.13. Alternatively, use the 1.5 ml of diluted semen from step 3.3.7 to set up a qualitative HIV microculture using the ACTG consensus protocol. Adjust the volume of donor cell solution to 1.25 mL per well.

3.3.14 If a culture is positive, save viral isolates as described in the ACTG Virology Manual under Specimen Processing .

3.3.15 Freeze 2 aliquots of primary seminal cells/ patient ejaculate:

(a) Centrifuge the remaining primary seminal cells 10 minutes at 600-800 x g.

(b) Remove the supernatant and then re suspend the remaining seminal cells in 2 ml Cryoprotective medium (DMSO containing freezing medium).

(c) Divide into two 1.0 mL aliquots and freeze following the procedure found in the current ACTG Virology Manual.

(d) Store in gas phase of LN2.

4.0 QUALITY CONTROL

PHA stimulated donor cells used for culture should always be checked to confirm that the donor is indeed HIV-1 negative or that contamination has not occurred.

5.0 Seminal Plasma RNA Assay

5.1 There are probably as many versions of this as there are investigators working in the field. One thing is clear: there are factors in seminal plasma that inhibit the PCR reaction unless they are removed. The standard Roche RNA and Roche ultrasensitive extraction method does not remove these inhibitors (4, 5, 8). Options are to use Boom's silica bead extraction assay (2), use Organon Teknika Nuclisens RNA assay which includes the silica bead extraction procedure, or pellet the virus using ultracentrifugation.

5.1.1 SPECIMEN PREPARATION

1. Allow liquefaction of the semen to occur. This typically occurs within 20-45 minutes of specimen collection.
2. Transfer the sample to a conical centrifuge tube using a pipette and measure and record the volume of semen.
3. Count the cells using the Endtz test (6, 13) to document inflammation.
 - (a). Place 20 ul of liquefied semen in a microfuge tube, add 20 ul phosphate buffered saline and 40 ul of working benzidine sloution.
 - (b). Vortex briefly and allow to sit at room temperature for 5 min.
 - (c). Count brown staining (peroxidase positive) cells in the four large squares of a hemacytometer.
 - (d). Calculate and record the number of peroxidase positive cells/ejaculate = Brown cells in all 4 squares x 10. Inflammation is considered present when there are 1 million peroxidase positive cells/ml of semen.
4. Centrifuge 600-800 x g for 10 minutes.
5. Remove supernatant, divide into 0.25 ml aliquots, and freeze at -70°C.

5.2 MEASUREMENT OF HIV-1 RNA IN SEMINAL PLASMA

NOTE: IF SEMEN WAS DILUTED WITH VTM OR PBS PRIOR TESTING FOR HIV RNA, THE AMOUNT OF THE DILUTION SHOULD BE NOTED AND ACCOUNTED FOR WHEN CALCULATING THE NUMBER OF HIV-1RNA COPIES/ML.

5.3. One of the following two assay options may be used:

5.3.1. Organon – Teknika.

Use Organon Teknika's Nuclisens assay following instructions found in the package insert. See the ACTG Virology Manual.

5.3.2 Roche (Boom silica bead extraction required).

DEPC distilled water
Trizma Base
Concentrated HCl
Guanidinium thiocyanate
Triton-X 100
Silicon dioxide (Sigma)
70% Ethanol (Prepare fresh: 11 mL 95% ETOH plus
4mL d2HO for 6 specimens)
Acetone (reagent grade >99% pure)

0.2 M EDTA, pH 8.0

- (1). Add 9 g EDTA to 121 ml DEPC distilled water.
- (2). Check pH

L2 buffer (2 liters)

- (1). Dissolve 24.22 g Trizma base in 1600 ml DEPC distilled water
- (2). Adjust pH to 6.4 with concentrated HCl (approximately 15.5 ml)
- (3). Cool to room temperature
- (4). Check pH and readjust to pH 6.4
- (5). Add DEPC water 2000 ml
- (6). Store at room temperature in the dark up to 6 months.

L2 Washing Buffer (approximately 2 liters)

- (1). Dissolve 1200 g guanidinium thiocyanate (GuSCN) in 1000 ml L2 buffer. Heating to 60-65°C with shaking facilitates this process.
- (2). Store at room temperature in the dark up to 6 months.

L6 Lysis Buffer (about 800 mL)

- (1). Dissolve 660 g GuSCN in 550 ml L2 buffer (NOT washing buffer). Heating to 60-65°C with shaking facilitates this process.
- (2). Add 121 ml 0.2 ml EDTA, pH 8.0
- (3). Add 14.3 g Triton-X 100
- (4). Mix and store at room temperature in the dark up to 6 months.

Silica Reagent

- (1). Place 60 g silicon dioxide in a 500 ml glass cylinder.
- (2). Add dH₂O to 500 ml and allow to stand overnight at room temperature.
- (3). Remove supernatant (approximately 430 ml) by suction.
- (4). Add dH₂O to 500 ml and shake vigorously to resuspend silica.
- (5). Let stand 5 hours at room temperature.
- (6). Remove supernatant (approximately 440 ml) by suction.
- (7). Adjust pH to 2 with approximately 400 ul of concentrated HCl.
- (8). Dispense in glass containers and autoclave for 15 min.
- (9). Store at room temperature in the dark up to 1 year.

5.3.2.2 Roche & QC Reagents

Roche Monitor kit
VQA Standards

5.3.2.3. Equipment and Supplies

2 ml Sarstedt tubes
Vortex mixer
Microcentrifuge (12,000 x g)
Transfer pipets with thin tips
Dry heating block
Water bath
Aspiration device or transfer pipettes
pH meter
Mixer
P1000 and P200 pipettors
Aerosol barrier pipette tips

5.3.2.4. Procedure

- a. Add 100 ul of Roche QS to 12 ml L6 Lysis Buffer.
Use the QS from the kit you intend to use to assay the specimens. Do NOT mix QS lots.
- b. Mix well by vortexing for 5 sec. and tilting tube several times.
- c. Aliquot 900 ul into each labeled 2.0 ml Sarstedt tube.
- d. Resuspend silica solution by vigorous mixing.
- e. Add 40 ul to each tube of L6 Lysis buffer.
- f. Vortex each tube until silica pellet is resuspended.
- g. Add 200 ul of seminal plasma or VQA standards. Roche Monitor kit controls should be processed per Roche package insert instructions (50uL control mixed with 200uL negative human plasma).
- h. Vortex immediately until solution is homogeneous (5-10 seconds).
- i. Incubate at room temperature for 10 minutes.
- j. Vortex for 5 seconds.
- k. Centrifuge for 15 seconds at 12,000 x g. Aspirate supernatant with fine tipped transfer pipette and discard.
- l. Wash step 1:
 - (1) Add 1 ml of L2 Washing Buffer and vortex until pellet is completely resuspended.
 - (2) Centrifuge for 15 seconds at 12,000 x g.
 - (3) Use fine tipped transfer pipet to aspirate supernatant.
- m. Wash step 2: Repeat Step 1.
- n. Wash step 3:

- (1) Add 1.0 ml 70% ethanol and vortex until pellet is completely resuspended. This may be somewhat difficult--shaking vial may help.
 - (2) Centrifuge for 15 seconds at 12,000 x g.
 - (3) Use fine tipped transfer pipet to aspirate supernatant.
- o. Wash step 4: Repeat Step n.
 - p. Wash step 5:
 - (a) Add 1.0 ml acetone and vortex thoroughly.
 - (b) Centrifuge for 15 seconds at 12,000 x g.
 - (c) Use fine tipped transfer pipet to aspirate supernatant. Pellet may be slick. Re-centrifuge if pellet is accidentally aspirated.
 - q. Evaporate acetone by incubating open vials in 56°C heating block for 10-15 minutes. Pellet must be dry.
 - r. Add 400 ul Roche Monitor kit sample diluent to each vial.
 - s. Recap vials and vortex until pellet is resuspended.
 - t. Incubate 10 minutes at 56°C.
 - u. Centrifuge at 12,000 x g for 2 min to pellet silica. The supernatant contains the RNA.
 - v. Amplify immediately or store frozen at -20°C until ready to proceed with the HIV Monitor Assay. If samples are frozen prior to amplification, thaw them, vortex to resuspend the silica, heat to 56°C 10 min and centrifuge at 12,000 x g for 2 min before adding 50 ul of the RNA containing supernatant to the Roche PCR tubes.

6.0 References

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