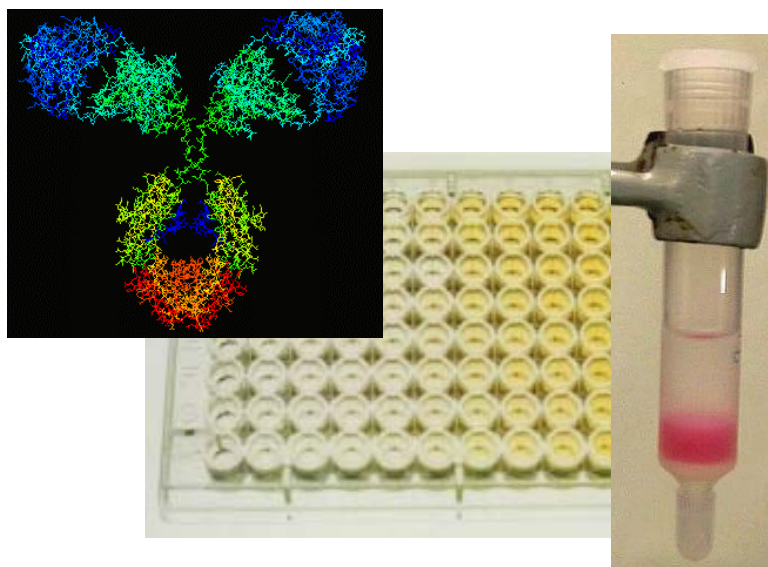


IMMUNOLOGY II

Practical Course Winter Term 2005/2006

Antibodies

Isolation, Labeling and ELISA



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Introduction

Antibodies, or immunoglobulins (Ig), are a family of structurally related glycoproteins produced in membrane-bound or secreted forms by B lymphocytes. Membrane-bound antibodies serve as receptors that mediate the antigen-triggered activation of B cells. Secreted antibodies function as mediators of specific humoral immunity by engaging diverse molecular and cellular effector mechanisms that serve to eliminate the bound antigens. Based on small differences in the heavy chain C regions antibodies are classified into isotypes and subtypes. These small differences in the heavy chain constant regions make it possible to isolate certain isotypes from blood. To isolate IgG **protein G** from *Streptococcus spp.* is used. In this practical course we will try to isolate IgG from human blood, using a protein G column and address the question how efficiently the purification procedure is.

We will analyze the isolated human IgG in an Enzyme-linked Immunosorbent Assay (ELISA). In an ELISA the antigen of interest is immobilized on a solid surface and is detected by a specific antibody covalently coupled to an enzyme. To detect our isolated human IgG we will label a commercial anti-human IgG with Horseradish peroxidase (HRP). To test the specificity of the protein G column we will use as a negative control an anti-human IgE (BSW17) conjugated to Horseradish Peroxidase.

This booklet should not only provide you with the laboratory manuals, but also help you to understand the principles and the theory underlying the practical work.

Program

First Day Isolation of IgG antibodies from human serum or Labeling of anti-human IgG with horseradish peroxidase

Second Day ELISA to test efficiency of IgG isolation and antibody labeling

Immunoglobulins

Antibody Structure

All antibodies have the same basic structure (**Figure 1**) but show remarkable variations in the regions of the antigen binding site that is formed by the variable light chain (VL) and variable heavy chain (VH) domains. Every antibody is composed of two identical light chains and heavy chains. The complement and Fc receptor binding sites are located within the heavy chain constant regions.

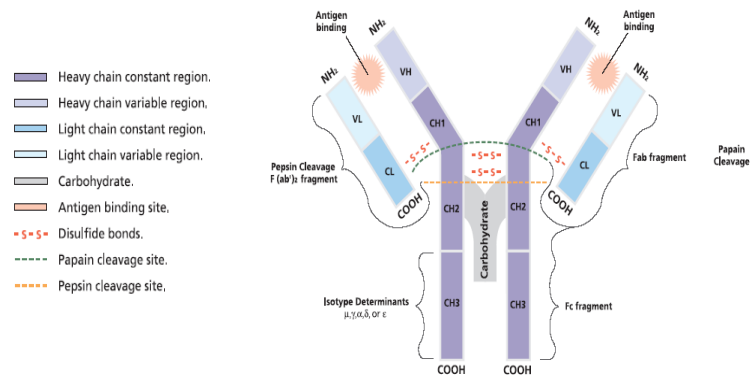


Figure 1: Structure of Immunoglobulins (from Sigma-Aldrich)

Antibody Classes

Antibody Isotype and Anti-Isotypes

An antibody isotype is determined by its heavy chain. As there are five different heavy chains available, five types of antibody classes can be defined: **IgM, IgD, IgG, IgA, and IgE**. Each antibody isotype performs a different set of effector functions (**Figure 2** and **Table 1**).

Anti-isotypic antibodies are directed against the Fc portion of an antibody. One example of an anti-isotypic antibody is the mouse monoclonal anti-human IgE antibody BSW17 which binds to the constant region of IgE immunoglobulins (see chapter below).

	<p>IgG 80% of the total Ig. Found in blood and can cross blood vessel walls to enter tissues. Can pass freely across the placenta thus providing immunity to the fetus. Protects against bacteria, viruses, and toxins.</p>
	<p>IgA 10-15% of the total Ig in the blood. More abundant in mucus secretions - saliva, tears, breast milk. Too large to cross placenta. Prevents attachment of pathogens to mucosa. Protects neonates from NEC</p>
	<p>IgM 5-10% of Ig in the blood. Does not pass freely into tissues. Appears quickly after infections. Opsonizing Ig, triggers complement cascade, ABO incompatibility.</p>
	<p>IgD 0.2% in blood. Found on immature B cells. Probably plays role in regulation</p>
	<p>IgE 0.002% in blood normally. Increase during allergic response. Fc region binds to mast cells and causes degranulation.</p>

Figure 2: Antibody Classes

(<http://www.utc.edu/Faculty/Becky-Bell/210-Ab-classes.pdf>)

Antibody	Subtypes	Heavy Chain (Designation)	Heavy Chain Constant Domains (Number)	Hinge	Tail Piece	Serum Concentration (mg/mL)	Serum Half-Life (Days)	Secretory Form	Molecular Size of Secretory Form (kD)
IgA	IgA1	α 1	3	Yes	Yes	3	6	Monomer, dimer, trimer	150, 300, or 400
	IgA2	α 2	3	Yes	Yes	0.5	6	Monomer, dimer, trimer	150, 300, or 400
IgD	None	δ	3	Yes	Yes	Trace	3	—	180
IgE	None	ϵ	4	No	No	0.05	2	Monomer	190
IgG	IgG1	γ 1	3	Yes	No	9	23	Monomer	150
	IgG2	γ 2	3	Yes	No	3	23	Monomer	150
	IgG3	γ 3	3	Yes	No	1	23	Monomer	150
	IgG4	γ 4	3	Yes	No	0.5	23	Monomer	150
IgM	None	μ	4	No	Yes	1.5	5	Pentamer	950

Table 1: Properties of Human Immunoglobulins (from “Cellular and Molecular Immunology”, Abbas et al., 4th edition)

Idiotype and Anti-Idiotype

The idiotype constitutes the totality of antigen receptor determinants that distinguish each lymphocyte clone from all the others. These determinants are located in the Fab region of an antibody and are dependent on the variable regions of both heavy and light chains, which form the antigen binding site (**Figure 3**). An antibody directed against the antigen specific part of another antibody is called an anti-idiotypic antibody. In principle anti-idiotypic antibodies have the potential both to inhibit or to promote a specific immune response and are thus thought to be crucial in the regulation of the humoral immune system (network hypothesis of Jerne).

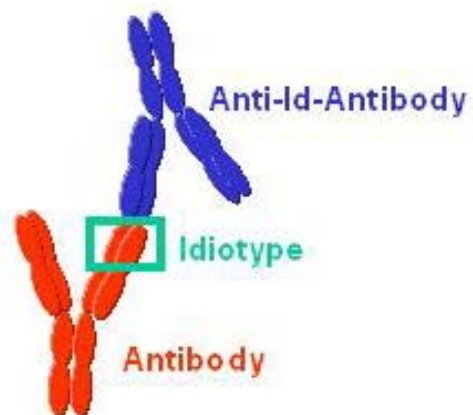


Figure 3: Idiotype and Anti-Idiotype

Links on the Internet:

<http://ntri.tamuk.edu/immunology/classes-ab.html>

<http://www.isat.jmu.edu/users/klevicca/isat351/immunology/antibodies.ppt>

Anti-IgE-Treatment in Allergies: BSW17

IgE antibodies play a central role in allergic reactions. Allergies are IgE mediated hypersensitive immune responses to relatively harmless environmental antigens. IgE binds to receptors (FcεRI and FcεRII) on mast cells and basophils via the Fc portion of the antibody. Cross linking of these membrane bound antibodies by allergens leads to mediator release and allergic reactions.

Most therapies are aimed at inhibiting these immediate effects and the subsequent inflammation. New treatment strategies focus on the neutralization of IgE antibodies and the inhibition of IgE-receptor interaction by means of non-anaphylactogenic (no mediator release) anti-IgE autoantibodies.

We have described a mouse monoclonal anti-human IgE antibody (BSW17) that is capable of recognizing receptor-bound IgE without triggering mediator release. BSW17 induces a conformational change of the IgE molecule, which results in dissociation from the receptor. BSW17 can also neutralize soluble IgE preventing its binding to the FcεRI and is able to inhibit IgE synthesis as a divalent molecule. This unique capacity to recognize and to remove receptor-bound IgE without triggering mediator release renders BSW17 a candidate antibody for clinical use. A derivate of BSW17 is already used for the treatment of allergies.

The administration of anti-IgE antibodies represents a passive immunization, which temporarily neutralizes IgE. An alternative strategy for the neutralization of IgE is active immunization using IgE epitopes in order to induce a natural autoimmune response. Peptides that mimic the epitope recognized by BSW17 on the IGE molecule, so called mimotopes, were used for immunization, which led to the production of anti-IgE-antibodies. It was shown that the mimotopes-immunization could protect against hypersensitive reactions upon allergen challenge (Vogel *et al.*, 2000).

Another possibility for active immunization is the administration of anti-idiotypic antibodies. The binding site of an anti-idiotypic antibody represents the image of an epitope on a foreign or a self-antigen. Anti-idiotypic antibodies with structural homology to mimotopes of IgE were tested for active immunization in animals. They could induce the production of antibodies that had biological activity similar to BSW17. Clinical trials will show, whether an anti-IgE immune response will be induced and allergic reactions can be treated with this method.

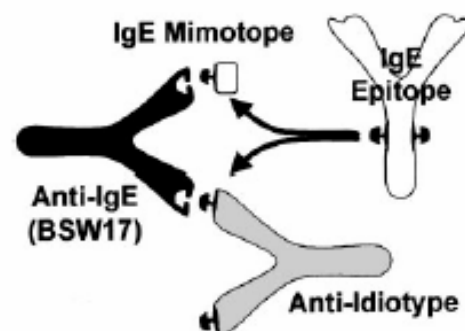


Figure 4: IgE mimotopes and anti-idiotypic antibody mimicking IgE epitopes can be used for active immunization

First Course Day

Antibody and Protein Purification

Precipitation with Ammonium Sulfate

Protein precipitation achieves separation by the conversion of soluble proteins to an insoluble state, which subsequently can be removed by various means. Ionic precipitation, utilizing inorganic salts like ammonium sulfate, is the most common precipitation method. Ammonium sulfate has several advantages:

- At saturation, it is of sufficiently high molarity that it causes the precipitation of most proteins.
- Its saturated solution has a density that does not interfere with the sedimentation of most precipitated proteins by centrifugation.
- Its concentrated solutions are generally bacteriostatic.
- In solution, it protects most proteins from denaturation.

The concentration of ammonium sulfate required for precipitation varies from protein to protein and should be determined empirically.

Affinity Chromatography using Protein A/ Protein G

Protein A is a cell wall component produced by several strains of *Staphylococcus aureus*. It has the ability to specifically bind to the Fc region of immunoglobulin molecules, especially IgG (**Figure 5**). The interaction between Protein A and IgG is not equivalent for all species, and even within a species, Protein A interacts with some IgG subgroups and not with others. For example, human IgG1, IgG2 and IgG4 bind strongly, while IgG3 does not bind.

Protein G is a bacterial cell wall protein isolated from group G streptococci. Like Protein A from *Staphylococcus aureus*, Protein G binds to most mammalian immunoglobulins through their Fc regions. The unique immunoglobulin binding characteristics of Protein G can be used for the purification of mammalian monoclonal and polyclonal antibodies that do not bind well to Protein A. Protein G is reported to bind with greater affinity to most mammalian immunoglobulins that does Protein A. It also binds with significantly greater affinity to several immunoglobulin subclasses such as human IgG3 and rat IgG2a. Another significant difference is that it does not bind to human IgM, IgD and IgA.

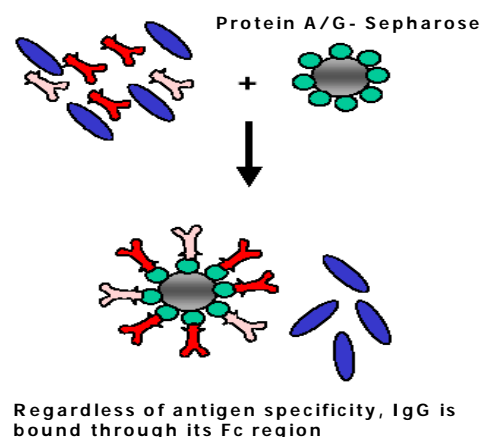


Figure 5: IgG Binding to Protein A/G- Sepharose

Agarose or Sepharose covalently coupled to Protein A or Protein G can be used as an affinity support for the isolation of IgG molecules (**Figure 5**). Serum is loaded onto a column which is filled with Sepharose coupled to protein so that the IgG present in the serum can be bound. The rest of the serum proteins will flow through and the IgG can be eluted with an acidic buffer (**Figure 6**).

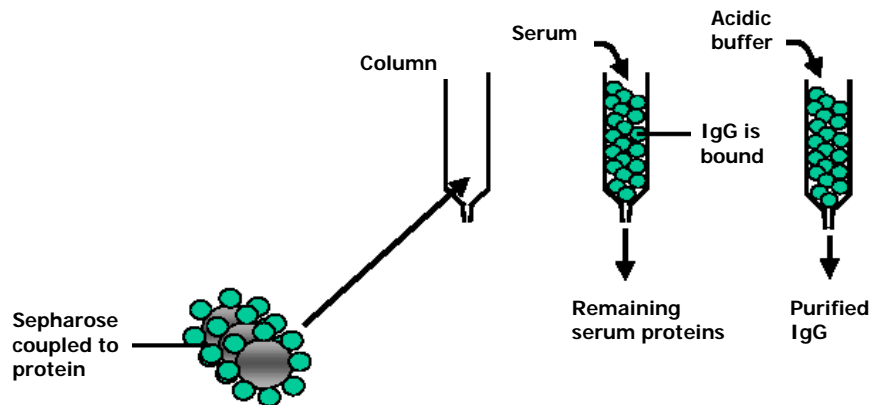


Figure 6: Isolation of IgG using a Protein A/G Column

Laboratory Manual: Affinity Purification of IgG

Material Requirements

- [Protein G Sepharose 4 Fast Flow](#) sorbent (Pharmacia), 1ml/column
- 1 Disposable polypropylene Column (Qiagen, Cat # 34924)
- 0.1M Glycine HCl, pH 3.0-2.5
- 1 M Tris-HCl, pH 9.0
- Binding buffer: 20 mM sodium phosphate, pH 7.0 (for regeneration add 0.05%NaN₃).

Method

Isolation of serum IgG by gravity affinity chromatography on a protein G column.

Preparations prior to practical course

- Equilibrate all material to the temperature at which the purification will be performed.
- De-gas the medium slurry.
- Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
- The serum was diluted 1:2 in binding buffer.
- The serum was dialysed twice against 500ml of binding (sodium phosphate) buffer before applying it to the column.
- The columns are stored at 4-8°C in 20% ethanol (to minimize bacterial growth)

Bring the columns to ambient temperature before use.

Binding:

- Wash column with 10ml 20 mM sodium phosphate, pH 7.0 as binding buffer.
- Dilute 1.2ml of the diluted and dialysed serum 1:5 in Binding Buffer and bind 5ml of it onto the column. The remaining 1ml store for further use.
- Wash column with 30ml binding buffer. Collect the Wash and store it.
- Binding capacity for Protein G Sepharose is (**Table 2**):

Species	mg/ml drained Medium
Human	17
Rat	7
Sheep	18
Rabbit	19
Goat	19
Guinea-pig	17
Cow	23
Mouse	6

Table 2: Total IgG Binding Capacity for different species

Elution:

- To elute IgG from Protein G Sepharose 4 Fast Flow it is necessary to lower the pH to about 2.5 depending on the sample.
- Elute the Immunoglobulins with 20ml 0.1M glycine buffer, pH 2.7, and collect 10 times a 0.5ml fraction.
- To preserve the activity of acid labile IgG's, add 30µl per fraction of 1 M Tris-HCl, pH 9.0, to neutralize of the eluted fractions.
- Pool the fractions containing the IgG (fractions 1-4) and transfer it to a dialysis tubing. The IgG fractions will then be dialysed three times against PBS.
- Regenerate column with 5ml binding buffer, 0.05% NaN₃.

Labeling of antibodies

In the sixties of the last century a new Immunoassay was developed, in which radioactive markers coupled to antibodies or antigens were used for the detection of antibody-antigen interactions. During the next decades other markers for the labeling of antibodies were developed because of safety concerns and because of emerging technologies (e.g. FACS, ELISA). Because of their good sensitivity, many antibodies are labeled with enzymes which render a colorless substrate into a easily detectable product. As the amount of colored product is proportional to the enzyme concentration and at the same time to the antigen concentration, it is possible to

quantify antibody or antigen concentration and to characterize binding affinities. The basic principle of the method is shown in the figure below (**Figure 7**):

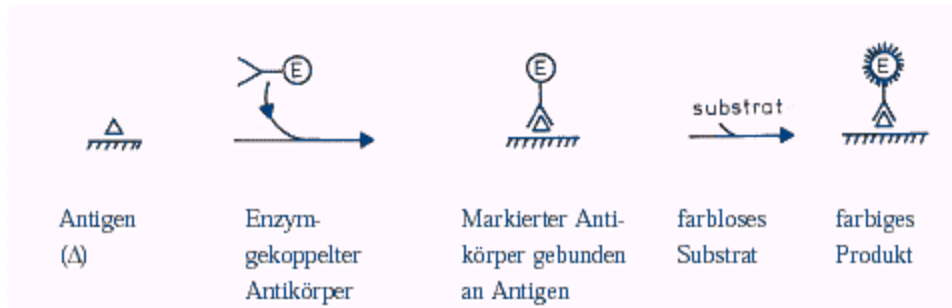


Figure 7: Basic principle of a Enzyme-linked Immunoassay

Antibodies conjugated with Horseradish Peroxidase (HRP)

Horseradish Peroxidase (HRP) coupled to immunoglobulin G (IgG), has proven to be a useful marker for Immunohistochemistry. In contrast to other immunohistochemical markers, such as fluorescein and ferritin, HRP may be used for both light and electron microscopy. It is especially suitable for the intracellular localization of antigens at the ultrastructural level since HRP (Molecular weight: 40kD) is considerably smaller than ferritin (Mw: 650kD); thus, HRP-labeled IgG (IgG HRP) has superior penetration properties.

Peroxidase labeling has the advantage of being sensitive and permanent. Various substrates for Peroxidase have been described – precipitating substrates for Immunohistochemistry or Western Blot (DAB, AEC, 4CN, TMB), soluble substrates for ELISA (TMB, OPD, ABTS) and chemiluminescent substrates (Luminol). However, HRP labeling has some major disadvantages:

- Peroxidase is rendered inactive by oxygen, hypochloric acid and sodium azide. It is therefore advisable to use highly purified water.
- The presence of endogenous peroxidase in certain tissues like macrophages, red cells, bone marrow cells and plants could increase the background.

Selected Antibody and Antigen Markers

Coupling agent	Preferentially used in	Positive	Negative
Fluorescein (FITC)	FACS	<ul style="list-style-type: none"> - easily detectable under a microscope - multiple labeling possible in Histochemistry or Cytochemistry 	<ul style="list-style-type: none"> - may lose rapidly its fluorescence
Alkaline Phosphatase	ELISA Immunohistochemistry	<ul style="list-style-type: none"> - high sensitivity - is not rendered inactive by certain inhibitors - has various substrates 	<ul style="list-style-type: none"> - restricted Buffer compatibility
Rodamine (TRITC)	FACS	<ul style="list-style-type: none"> - less susceptible to fading than fluorescein 	<ul style="list-style-type: none"> - comparatively higher background
Beta galactosidase	ELISA	<ul style="list-style-type: none"> - good sensitivity 	
Glucose Oxidase	Immunohistochemistry		<ul style="list-style-type: none"> - medium sensitivity
Biotin	Two-step detection system in concert with conjugated Avidin	<ul style="list-style-type: none"> - allows the detection of low quantity of antigen 	
Radio labeling	Radioimmunoassays, Radioautography, Immunoblots, Receptor-Antigen cell binding	<ul style="list-style-type: none"> - sensitivity in certain assays 	<ul style="list-style-type: none"> - safety concerns
Colloidal Gold	Scanning EM		

Table 3: Selected Markers of Antibody and Antigen

References

“Peroxidase-labeled antibody; a new method of conjugation”

Paul K.Nakane and Akira Kawaoi, The Journal of Histochemistry and Cytochemistry 1974

Laboratory Manual: Labeling of swine anti-human IgG with Horseradish Peroxidase

Material

- 3mg Horseradish Peroxidase (HRP) (Merck AG)
- Carbonate buffer (Merck AG) 0.1mol/l pH 8.1 to dissolve HRP, 100 μ l
- Sodium perjodate (Fluka AG) 0.1mol/l in H₂O, 25 μ l
- Ethylene glycol (Fluka AG) 2mol/l, 10 μ l
- Gel filtration column: PD 10 (Amersham-Pharmacia)
- 1mg swine anti-human IgG (Nordic SwAHu/IgG(Fc)/7S) dissolved in 100 μ l carbonate buffer (Merck AG) 0.01M pH8.1 and dialysed twice against the same buffer prior to the practical course.
- Carbonate buffer (Merck AG) 0.01mol/l pH 8.1 to dissolve swine anti-human IgG, to equilibrate the column and to elute HRP, 40ml.
- Carbonate buffer (Merck AG) 0.01mol/l pH 9.5 to make 10% solution of Sodium Borohydride.
- Sodium Borohydride (Fluka AG) 10% (in Carbonate Buffer 0.01mol/l pH 9.5), 25 μ l.
- 1 mol/l Tris-HCl pH7.2, 100 μ l.
- Tris buffered saline (TBS), 0.15% Casein, 100 μ l.
- Thimerosal (Sigma) 10mg/ml in H₂O, 30 μ l.
- Ice
- Eppendorf tubes
- 12ml tubes

Method

Labeling of swine anti-human IgG with Horseradish Peroxidase.

Preparations prior to practical Course

- **Carbonate buffer 0.1mol/l pH 8.1**
Sodium Carbonate: dissolve 5.3g in 480ml H₂O; adjust pH with 1N HCl, fill up with H₂O to 500ml.
- **Carbonate buffer 0.01mol/l pH 8.1**
Dilute carbonate buffer 0.1mol/l pH 8.1 1:10 with H₂O.
- **Carbonate buffer 0.01mol/l pH 9.5**
Sodium hydrogen Carbonate stock solution 0.1M: dissolve 4,2g in 500ml H₂O.
Sodium Carbonate stock solution 0.1M: dissolve 5.3g in 500ml H₂O.
Mix 81.6ml Sodium Hydrogen Carbonate stock solution with 18.4ml Sodium Carbonate stock solution 0.1M, dilute 1:10 with H₂O.
- **Sodium-meta- perjodate 0.1M**
Solve 2.139g in 100ml H₂O.
- **Ethylene glycol 2M**

- I Fill up 12.414g (liquid) with H₂O to 100ml.
- **Sodium Borohydrid 10%**
Dissolve 1g in 10 ml carbonate buffer 0.01mol/l pH 9.5 (Prepare freshly; Gas formation!!; make a hole in the lid).
 - **Tris-HCl 1M pH7.2**
Dissolve 12.114g Tris in 80ml H₂O, adjust pH with 1N HCl; fill up with H₂O to 100ml.
 - **Tris buffered Saline(TBS), 0.15% Casein**
TBS 10x stock solution: 30g of Tris (hydroxymethyl)-aminomethan; 2g of potassium chloride; 80g sodium chloride; add 1000ml H₂O
Dilute TBS 10x stock solution 1:10 in H₂O 1l; solve 1.5mg in 1ml TBS

In addition 3mg HRP were dissolved in 100µl carbonate puffer 0.1M pH 8.1.

Start Labeling

- Add 25 µl of 0.1M Sodium Perjodate solution to HRP (Oxidation of glycoside groups of the enzyme. Formation of aldehyde groups.)
Incubate 30-45 min
- Add 10µl ethylene glycol (Termination of oxidation)
Incubate 10-30min
- Equilibrate PD 10 column with 30ml 0.01M carbonate puffer pH 8.1.
- Add Peroxidase/Perjodate mixture to the column.
- Elute with 10 ml 0.01M carbonate puffer pH 8.1. Collect all brown drops (HRP is brown) in one tube.
- To the brown eluate add 100µl of swine anti-human IgG [10mg/ml] (The aldehyde groups react by forming Schiff bases with the amino groups of the antibodies).
Incubate 30-60 min
- Chill the antibody-HRP mixture on ice and add 25µl of Sodium Borohydride 10% solution.
- Incubate 1-2 hours on ice.
- Add 100µl of 1M Tris-HCl pH 7.2 under the hood (Attention! Formation of toxic gases!)
- Dilute antibody-HRP mixture 1:2 with casein solution.
- Add 30µl of 1% Thimerosal (antibactericide).

Questions

- Why was casein solution added to the antibody-HRP mixture?
- Why is 3mg HRP and 1mg antibody a feasible ratio for labeling?
- What other possibilities for antibody labeling do exist?

Second Course Day

Enzyme-Linked Immunosorbent Assay (ELISA)

What is an ELISA?

There are a number of ways to determine whether an antibody has bound to its target antigen. One method is an ELISA. It is frequently used for diagnostic detection of specific molecules or organisms. Generally an ELISA has the following steps (**Figure 8**):

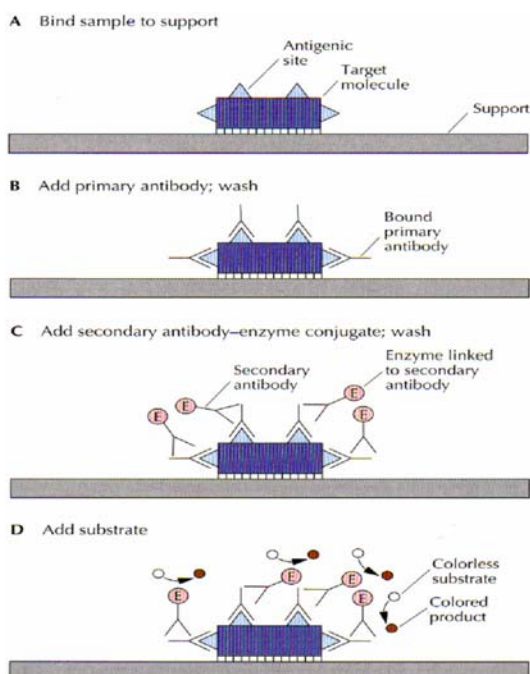


Figure 8: Generalized ELISA protocol for detecting a target antigen. The enzyme (E) is conjugated to the secondary antibody. (Glick & Pasternak - *Molecular biotechnology: principles and applications of recombinant DNA*, 1998).

After the solid support is initially coated (**Figure 8A**) with an appropriate amount of the sample, the whole surface must be blocked (coated with an inert protein, usually albumin) to ensure biospecific binding of the subsequent components. If the target site is present in the sample, the primary antibody binds to it (**Figure 8B**), the secondary antibody binds to the primary antibody (**Figure 8C**), and the attached enzyme catalyzes the reaction, after adding of a substrate (**Figure 8D**), yielding a colored product. The color intensity reflects the amount of the target and can be measured. Conversely, if the added primary antibody does not bind to the sample, the first washing step removes it. Consequently, the secondary antibody-enzyme conjugate has nothing to bind to and is removed during the second washing step, and the final mixture is colorless.

The principal feature of ELISA is the specific binding of the primary antibody to the target site. If the target molecule is, for example, a protein, then a purified preparation of this protein is generally used to raise polyclonal antibodies (usually in a rabbit) to detect the protein by ELISA. For certain diagnostic assays polyclonal antibodies have drawbacks: (1) the composition of antibodies may vary between batches; (2) polyclonal antibodies cannot distinguish between similar targets (e.g. when the difference between a target pathogen and a nontarget nonpathogen is a single determinant). These problems can be overcome by using monoclonal antibodies.

How is an ELISA done in the Laboratory?

ELISA reactions are performed in transparent wells of normalized micro titer plates usually containing 96 wells and made of polystyrene or poly vinyl chloride. Adsorption of the tested sample is based mostly on hydrophobic interactions. Various proteins bind to the plastic with different attraction – particularly coating-efficient are immunoglobulins and serum albumin. The enzymes commonly used as conjugates are: peroxidase from horseradish (abbreviated HRP, POD, POX), alkaline phosphatase (AP), or less often urease, β -galactosidase or glucose oxidase. HRP is the smallest of the enzymes normally used for conjugation to antibodies and is an enzyme of choice when steric hindrance may be of importance. The chromogenic reactions are quenched for example by acidifying the reaction mixture and quantified by measuring optical density (absorbance) on an ELISA plate reader. The analytical beam of light of defined wavelength projects vertical to the plate along the axis of a well. In older type of apparatus the beam jumps from well to well. In modern readers the plate is illuminated from above with a matrix of beams and the intensity of transmitted light is measured with a matrix of fotodetection elements. This solution allows for simultaneous quantification in all the wells, which is particularly important for kinetic measurements. Results can be compared when the OD values are in the linear range 0 - 1.0.

Different Applications of an ELISA

The assay may be performed according to several basic schemes. The abbreviations used below are: **I** – plastic support; **Ag** – antigen; **Ab** – antibody against the antigen; **AB** – antibody of another animal species against the same antigen; **anti-Ab** – antibody of another species against the antibody Ab; **anti-AB** – antibody of another species against the antibody AB; (W) – washing step; ***E** – marker enzyme; S – chromogenic substrate for the enzyme.

Direct ELISA

$I\text{-Ag}(W) + \text{Ab}^*\text{E}(W) + S \rightarrow \text{readout}$

or

$I\text{-Ab}(W) + \text{Ag}^*\text{E}(W) + S \rightarrow \text{readout}$

Indirect ELISA

$I\text{-Ag}(W) + \text{Ab}(W) + \text{anti-Ab}^*\text{E}(W) + S \rightarrow \text{readout}$

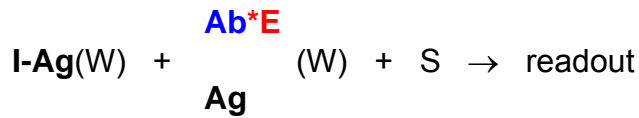
Sandwich direct ELISA

$I\text{-Ab}(W) + \text{Ag}(W) + \text{AB}^*\text{E}(W) + S \rightarrow \text{readout}$

Sandwich indirect ELISA

$I\text{-Ab}(W) + \text{Ag}(W) + \text{AB}(W) + \text{anti-AB}^*\text{E}(W) + S \rightarrow \text{readout}$

Competitive direct ELISA



Competitive indirect ELISA



or



Indirect ELISA allows for stronger signal amplification, has extremely high sensitivity and is used more often. ELISA is named competitive when the two competing agents are introduced to the well simultaneously. In an alternative procedure referred to as inhibition assay the two competing agents are added subsequently with a washing step in between. It is always necessary to control every single step of ELISA, for example confirm that antibodies do not bind to plastic or to the protein used for blocking but are specific.

Recommended further reading

Coligan, John E., et. al., editors. *Current Protocols in Immunology*. New York: John Wiley and Sons, 1999.

Laboratory Manual: ELISA

Material

- Costar EIA/RIA Half Well Plate
- Commercial Swine anti-Human IgG (SwAHu/IgG (H+L)/7S, 10mg, Nordic Immunological Laboratories, Tilburg, Netherlands) diluted to a concentration of 5µg/ml with Carbonate Buffer 0.1M pH 9.6, 4ml.
- BSW17 diluted to a concentration of 5µg/ml with Carbonate Buffer 0.1M pH 9.6, 700µl.
- TBS, 0.15% Casein (ELISA Blocking and Antibody Incubation buffer), 15ml for Blocking, 30ml for dilutions.
- Human IgG Preparation [10mg/ml] from at least 20000 individuals (Sandoglobulin, Ivlg), 12µl.
- Human Serum, isolated Human IgG (Eluate), and Flow through isolated on the first course day.
- Human IgE [2µg/ml], 150µl
- HRP conjugated anti-Human IgG from the first course day.
- Commercial HRP conjugated goat anti-Human IgG (Fc) (GAHu/IgG (Fc) Pox, Nordic Immunological Laboratories, Tilburg, Netherlands) 1:1000 in PBS, 0.15% Casein, 2.5ml.
- HRP conjugated anti-Human IgE (BSW17Pox) 1:2000 in PBS, 0.15% Casein, 800µl.
- TBS, 0.1% Tween 20 (Wash Buffer), 100ml.
- TBS (Wash Buffer), 100ml.
- 3,3',5,5'-Tetramethylbenzidine (TMB, chromogen), 5ml.
- H₂SO₄ (Stopping Solution), 5ml.
- Eppendorf pipettes and tips

Method

Enzyme-linked Immunosorbent Assay (ELISA) to test Material from First Course Day.

Preparations prior to practical Course

Prior to the practical course a Costar EIA/RIA Half Well Plate was coated and blocked (see scheme below):

- Commercial Swine anti-Human IgG and Mouse anti-Human IgE (BSW17) were diluted with 0.1M Carbonate Buffer pH 9.6 to a final concentration of 5µg/ml.
- Wells were incubated with 50µl of diluted protein and incubated for 2h at 37°.
- Protein solution was removed from the wells and the plate was blocked for 2h at 37° with 150µl/well TBS, 0.15% Casein.

Incubation of Antibodies

- Make a 1:5 dilution series, in TBS, 0.15% Casein, starting with 1:50 using the non purified Human serum and the isolated IgG (HulgG) from the first course day as well as Ivlg (Sandoglobulin) as a control:

A	B	C	D	E	F
1:50	1:250	1:1250	1:6250	1:31250	1:156250

Table 4: Serial Dilutions

- To have enough volume for all wells you finally need for each dilution at least 350µl.
- As controls use the Flow through from the first course day as well as the provided Human IgE [2µg/ml].
- Distribute the diluted antigens 50µl/well as indicated in the scheme below (2nd line).
- Incubate the ELISA plate for 2 hours at 37°C.
- Wash the ELISA plate 2x shortly with TBS-0.1%Tween20
2x 5min with TBS

Incubation of HRP labeled Antibodies

- Dilute the HRP labeled anti-hu IgG 1:5 – 1:10 by filling up to 5ml with TBS, 0.15% Casein. The commercial HRP conjugated anti-human IgG and HRP conjugated BSW17 are already diluted 1:2000 in TBS, 0.15% Casein.
- Distribute the diluted antibodies 50µl/well as indicated in the scheme below (3rd line)
- Incubate the ELISA Plate for 1 hour at 37°C.

Developing the ELISA

- Wash the ELISA plate 2x shortly with TBS-0.1%Tween20
2x 5min with TBS
- Develop the ELISA with 50µl/well TMB (positive wells become blue)
- Stop the reaction by adding 50µl/well 1M H₂SO₄.

Questions

- Comparing the titer of Ivlg to your isolated HulgG: How much IgG could you isolate from Human serum? How efficient was the purification (compare your values to **Table 1** on page 5)? Do you still have IgG in the washing solution? What might be the cause for this phenomenon?
- Do you still have IgE in your purified IgG fraction? If yes, what might be the cause for this “background”?
- How efficient was the labeling of the anti-human IgG with Horseradish Peroxidase (HRP), in comparison to the commercial available HRP labeled antibodies?

ELISA scheme:

	1	2	3	4	5	6	7	8	9	10	11	12
A	α -hu IgG Ivlg A Comm. α -hu IgG HRP	α -hu IgG Ivlg B Comm. α -hu IgG HRP	α -hu IgG Ivlg C Comm. α -hu IgG HRP	α -hu IgG Ivlg D Comm. α -hu IgG HRP	α -hu IgG Ivlg E Comm. α -hu IgG HRP	α -hu IgG Ivlg F Comm. α -hu IgG HRP						
B	α -hu IgG HulgG A Comm. α -hu IgG HRP	α -hu IgG HulgG B Comm. α -hu IgG HRP	α -hu IgG HulgG C Comm. α -hu IgG HRP	α -hu IgG HulgG D Comm. α -hu IgG HRP	α -hu IgG HulgG E Comm. α -hu IgG HRP	α -hu IgG HulgG F Comm. α -hu IgG HRP						
C	α -hu IgG Hu Serum A Comm. α -hu IgG HRP	α -hu IgG Hu Serum B Comm. α -hu IgG HRP	α -hu IgG Hu Serum C Comm. α -hu IgG HRP	α -hu IgG Hu Serum D Comm. α -hu IgG HRP	α -hu IgG Hu Serum E Comm. α -hu IgG HRP	α -hu IgG Hu Serum F Comm. α -hu IgG HRP						
D	α -hu IgG Ivlg A Self made α -hu IgG HRP	α -hu IgG Ivlg B Self made α -hu IgG HRP	α -hu IgG Ivlg C Self made α -hu IgG HRP	α -hu IgG Ivlg D Self made α -hu IgG HRP	α -hu IgG Ivlg E Self made α -hu IgG HRP	α -hu IgG Ivlg F Self made α -hu IgG HRP						
E	α -hu IgG HulgG A Self made α -hu IgG HRP	α -hu IgG HulgG B Self made α -hu IgG HRP	α -hu IgG HulgG C Self made α -hu IgG HRP	α -hu IgG HulgG D Self made α -hu IgG HRP	α -hu IgG HulgG E Self made α -hu IgG HRP	α -hu IgG HulgG F Self made α -hu IgG HRP						
F	BSW17 Hu Serum A BSW17 HRP	BSW17 Flow through BSW17 HRP	BSW17 HulgG A BSW17 HRP	BSW17 Ivlg A BSW17 HRP								
G	BSW17 Hu IgE BSW17 HRP	BSW17 - BSW17 HRP	α -hu IgG - Comm. α -hu IgG HRP	α -hu IgG - Self made α -hu IgG HRP								
H	Controls											

Figure 9: ELISA scheme (Dilutions are indicated in the letter code shown in **Table 4** above (e.g. A means 1:50).

More general Questions

- What are the limitations of the ELISA test? Compare ELISA to other protein-quantification methods.
- Which component must be additionally added to the substrate solution when the conjugated enzyme is peroxidase?
- List several applications of an ELISA.
- Did you know that:
 - sodium azide is an inhibitor of peroxidase
 - ELISA can be used to detect DNA (PCR-ELISA)
 - ELISA is the basis of pregnancy tests
 - ELISA was first described by Engvall and Perlman in 1971
 - an allergy diagnostics test based on ELISA (CAST-ELISA) was invented in the Institute of Immunology, Inselspital, Bern?