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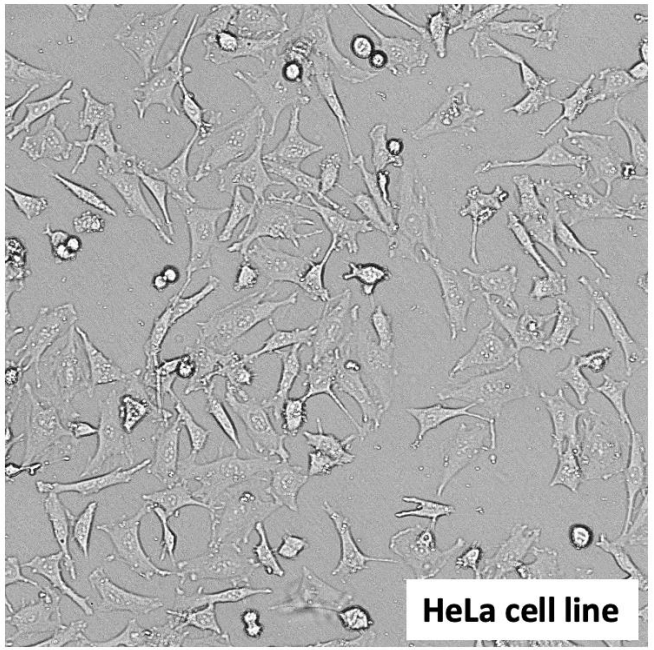
9_ Proteomics and methods

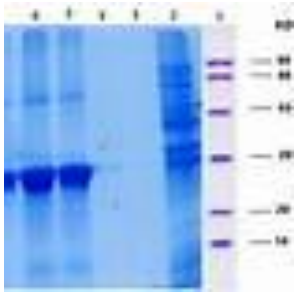
Proteomics is the large-scale study of proteins. Proteins are vital macromolecules of all living organisms, with many functions such as the formation of structural fibers of muscle tissue, enzymatic digestion of food, or synthesis and replication of DNA. In addition, other kinds of proteins include antibodies that protect an organism from infection, and hormones that send important signals throughout the body.



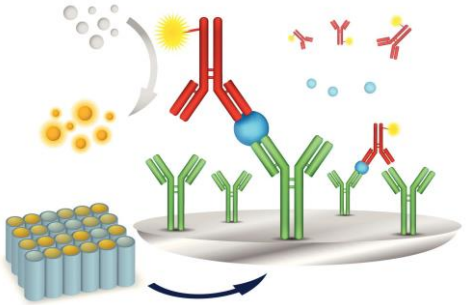
Zuzana Soldánová
Marie Brázdová

Methods - determining the physical presence of cellular proteins

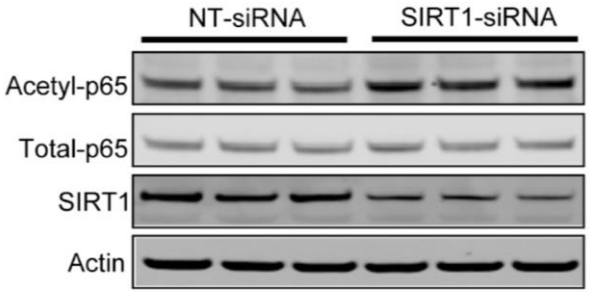
Generally, proteins may be detected by using either antibodies (immunoassays), electrophoretic separation or mass spectrometry



- Polyacrylamide gel electrophoresis (PAGE)
- SDS-PAGE
- Western blot
- Immunodetection of proteins



- ELISA (Enzyme-Linked ImmunoSorbent Assay)
- Immunoprecipitation



- Immunohistochemistry
- Isoelectric focusing
- Two-dimensional polyacrylamide gel electrophoresis

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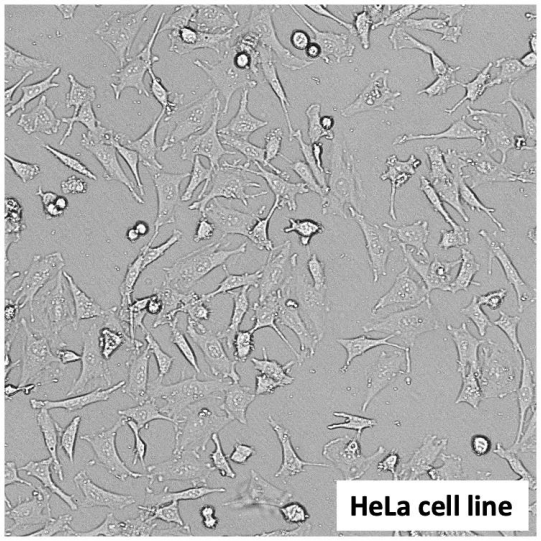
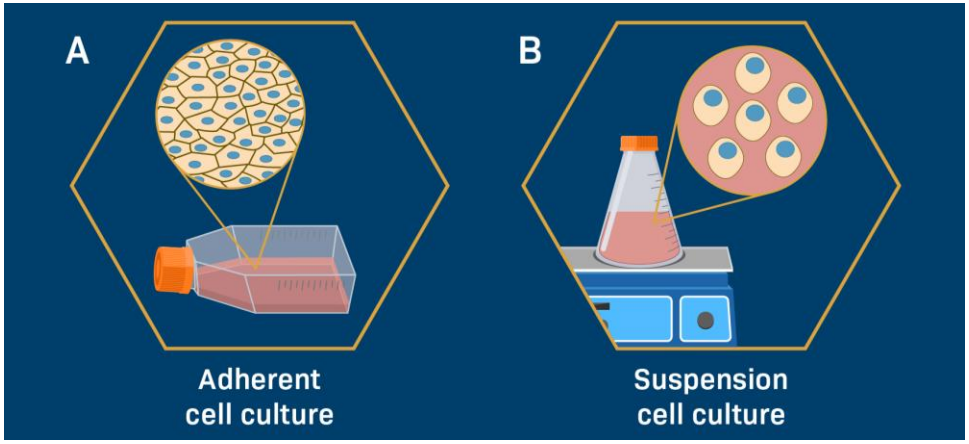
Protein sample collection

- **1st step**
- cell or tissue suspension gain
- **2nd step**
- cell or tissue breakdown
- by ultrasound
- by using a mild detergent to perforate the plasma membrane
- pushing cells through a small opening
- breaking the cells with a sealing rotary piston in a thick-walled container
- **3rd step**
- obtaining a dense homogenate or extract with larger and smaller molecules from the cytosol (enzymes, ribosomes, metabolites, membrane-enclosed organelles)
- gain of organelles in an intact state





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Homogenization of cells or tissues

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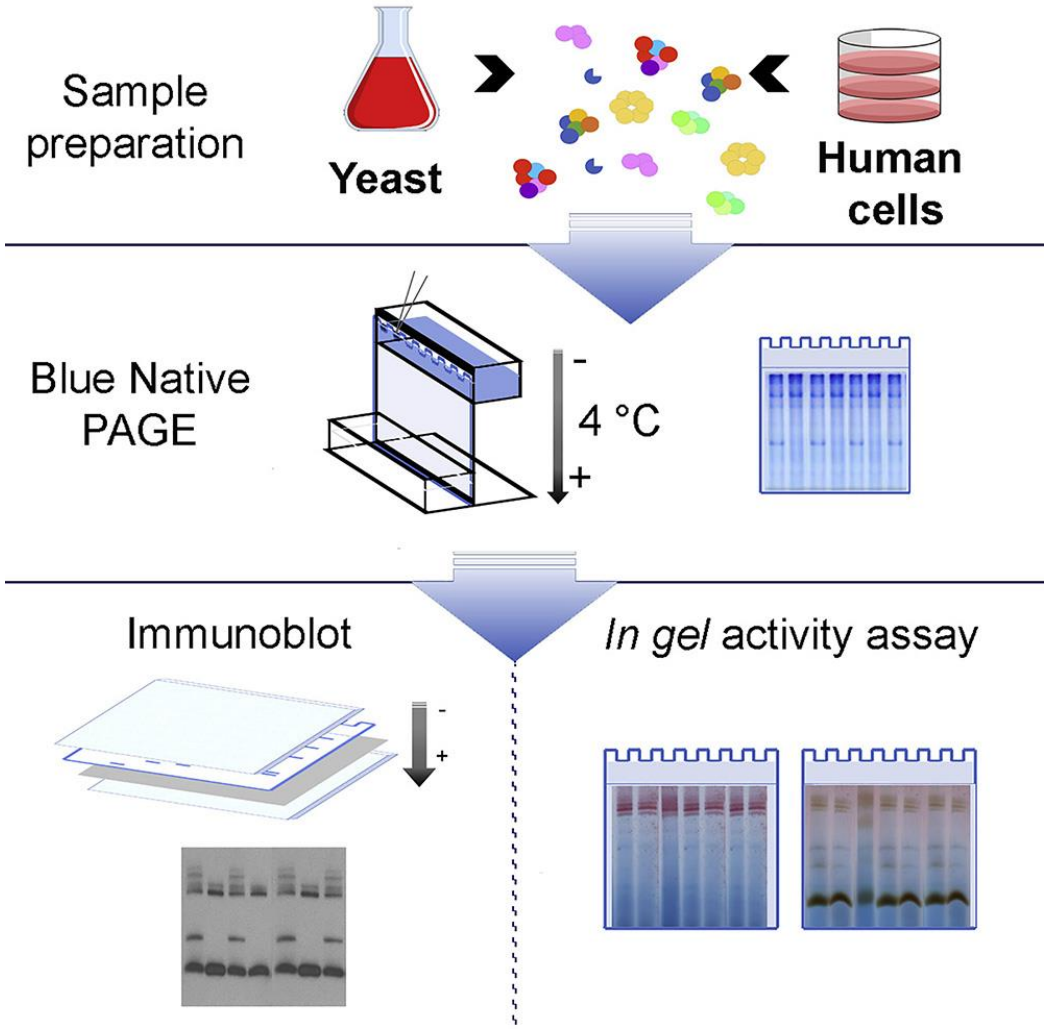
Electrophoretic techniques

the ability to move electrically charged molecules in an electric field
vertical polyacrylamide gel electrophoresis (PAGE)
alkaline buffers are used imparting a negative charge to the proteins - the proteins then move to the positive electrode (anode)
different variants
gel sandwiched between two containers filled with buffer into which the electrodes are immersed
plate variant (samples are placed in wells on the upper side of the gel)



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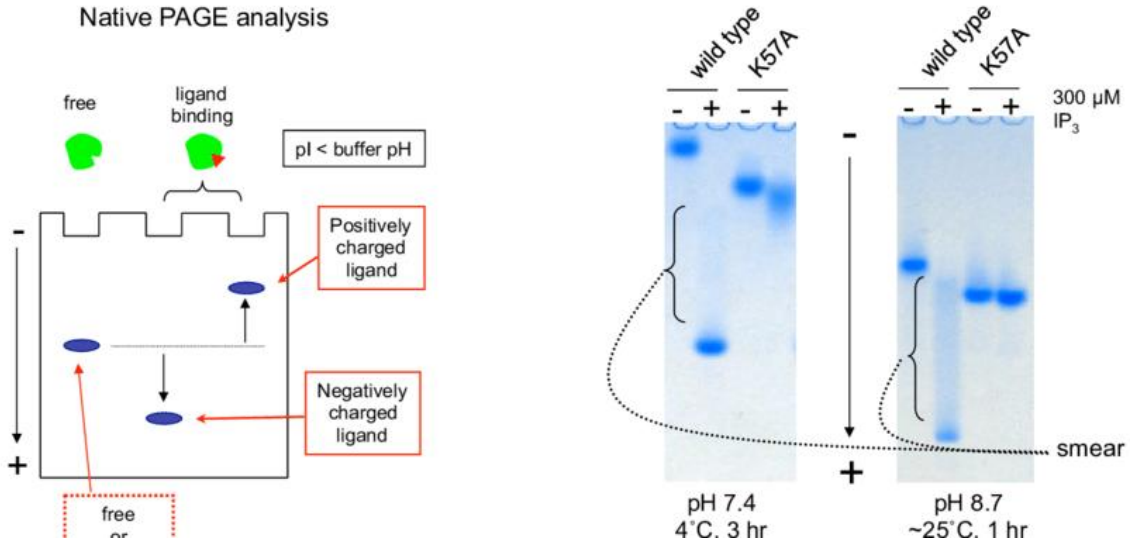
Factors influencing the mobility of proteins in a gel

- **Size**
- with increasing molecular size, the mobility of proteins in the gel decreases (molecular sieve effect)
- **Face**
- globular proteins move faster than filamentous proteins
- **Charge density**
- charge/unit mass
- the higher the charge density, the higher the mobility in the gel
- **Acrylamide concentration**
- mobility decreases with increasing concentration

listinguished by native polyacrylamide gel electrophoresis (native PAGE; Fig.2) (5). Indee
he preliminary native-PAGE experiments demonstrated that the migration patterns of th
PLC-δ1 PH domain were different between the two forms (Fig.3~5), indicating that the I
inding to the protein induces the marked migration shift.



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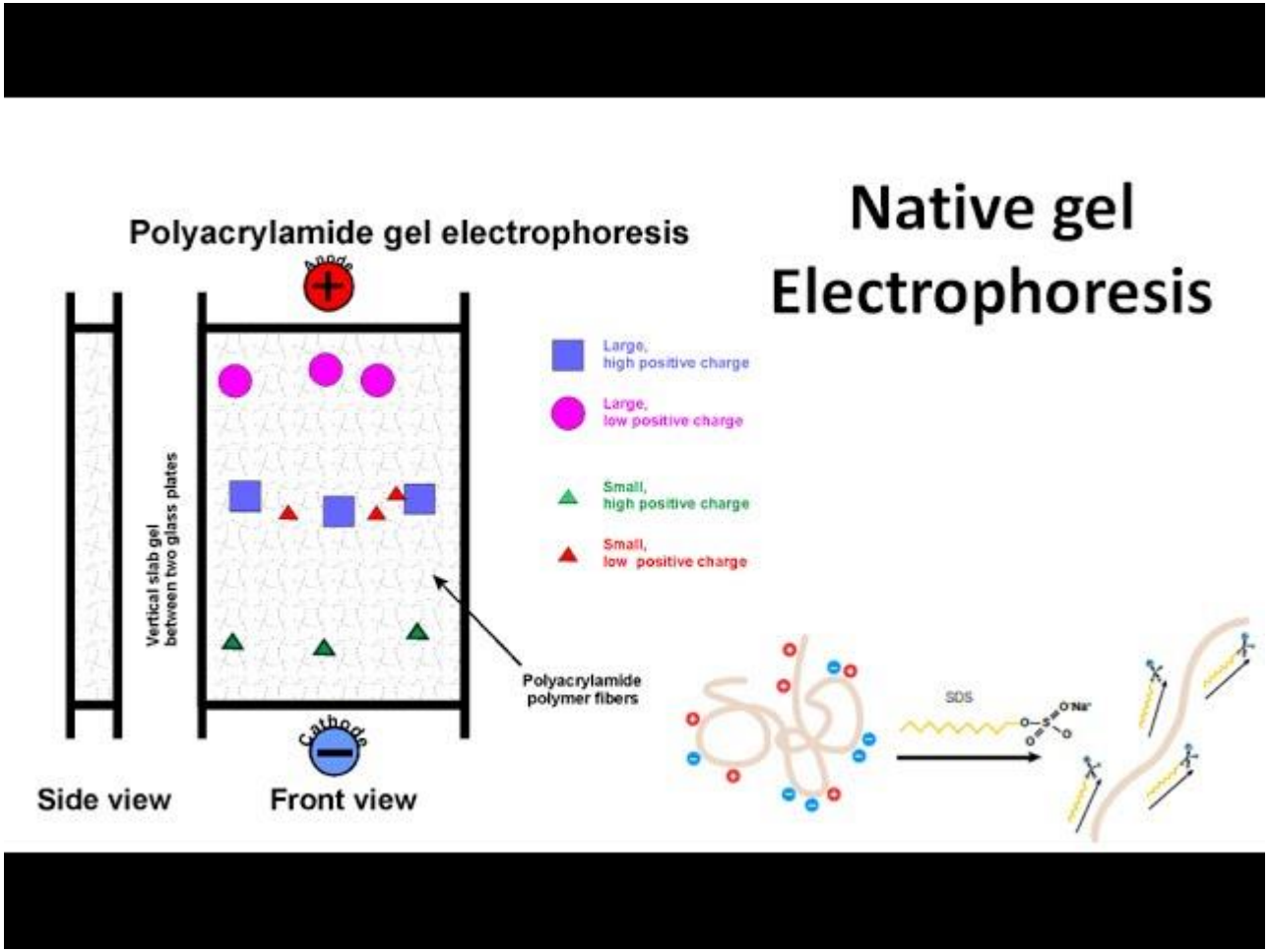
Native PAGE

- proteins
- different shapes of molecules
- they also have different charges

- DNA
- uniform in terms of shape and charge distribution

- Interpretation of the electrophoretogram in its native form is difficult.



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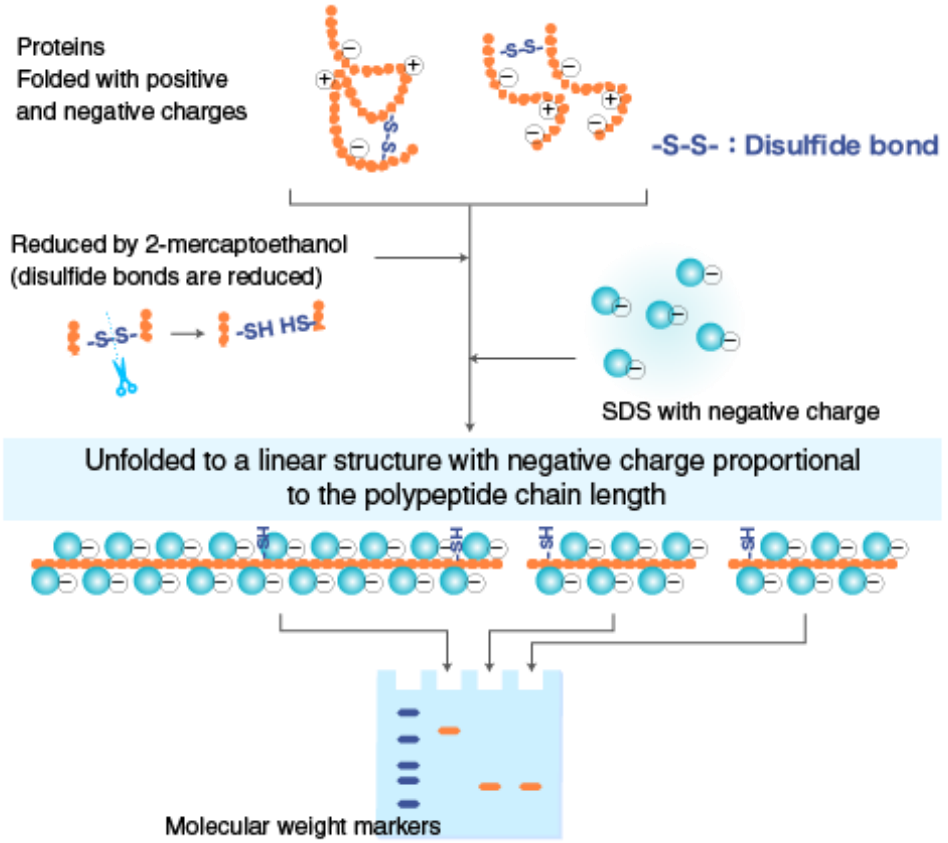
SDS-PAGE

- denaturing variant of PAGE
- commonly used method
- used for protein separation
- sample preparation
- dissolution in a solution containing negatively charged molecules (SDS: sodium dodecyl sulfate)
- elimination of disulfide bonds in proteins by a reducing agent (β-mercaptoethanol)
- completion of sample preparation by boiling



https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html

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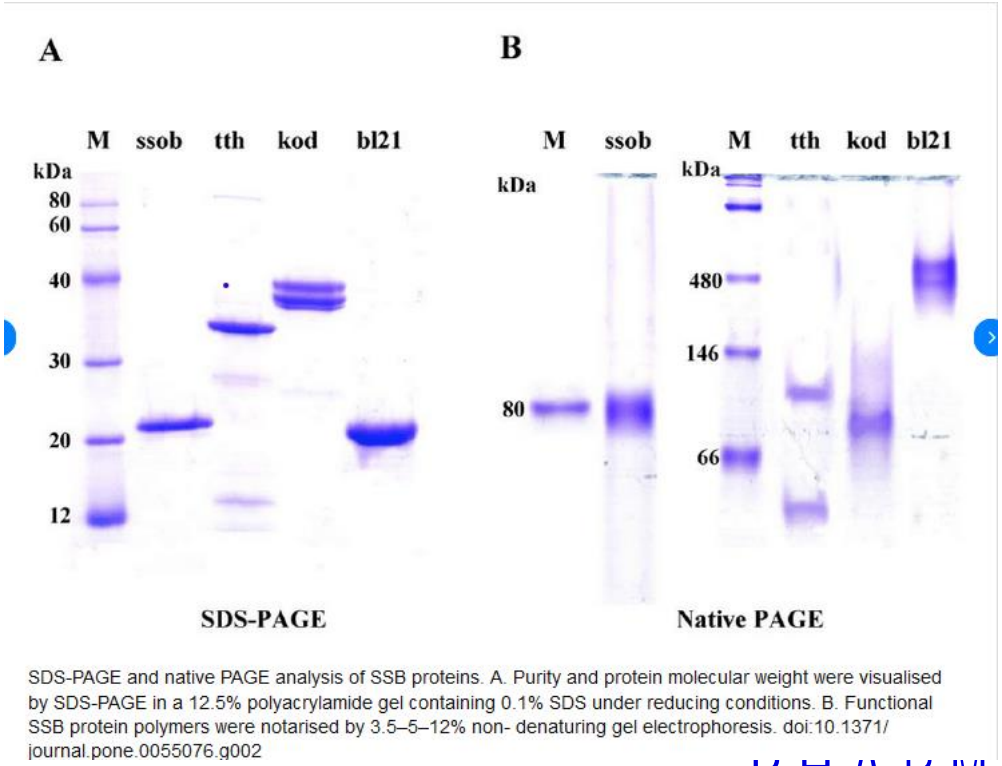
SDS-PAGE

- SDS negatively charged
- binds to proteins
- masking the protein's intrinsic charge
- protein denaturation and elimination of the effect of protein shape
- the number of SDS molecules bound to a protein is roughly proportional to its molecular weight
- equivalent charge density
- larger proteins in the gel face greater resistance
- Slower motion (molecular sieve effect)
- **Proteins are separated by molecular weight only**

SDS-PAGE vs Native PAGE



SDS-PAGE and native PAGE analysis of SSB proteins. A. Purity and protein molecular weight were visualised by SDS-PAGE in a 12.5% polyacrylamide gel containing 0.1% SDS under reducing conditions. B. Functional SSB protein polymers were notarised by 3.5–5–12% non- denaturing gel electrophoresis. doi:10.1371/journal.pone.0055076.g002

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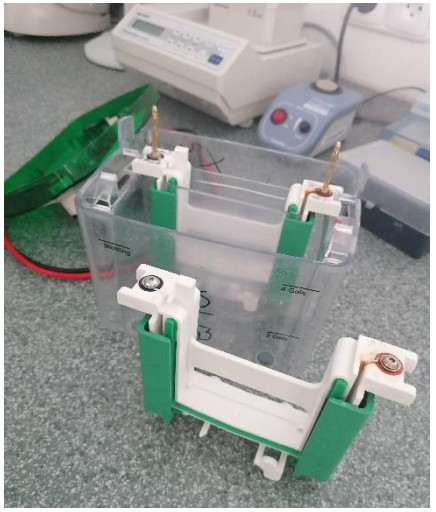
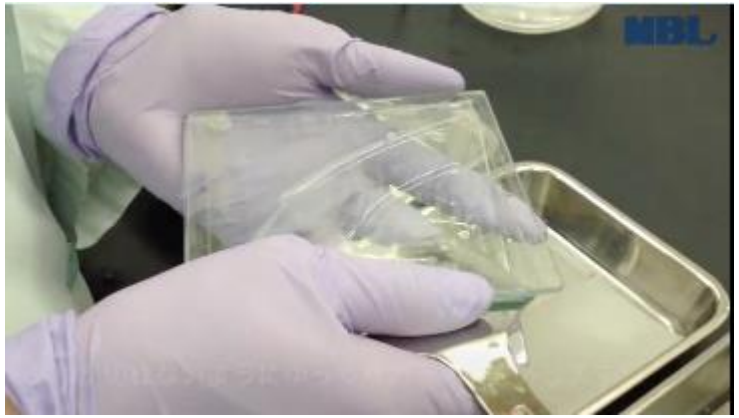
SDS-PAGE



Remove the gel assembly from the electrophoresis apparatus. Remove the gel from the glass plates using a spatula, and prepare for subsequent analysis.



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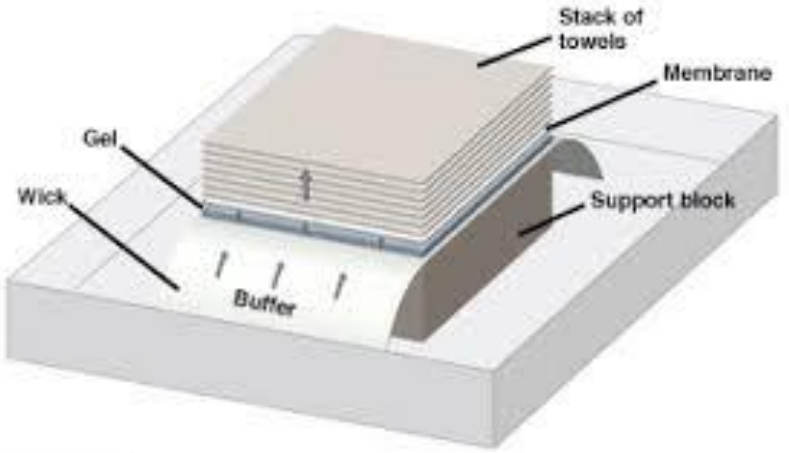
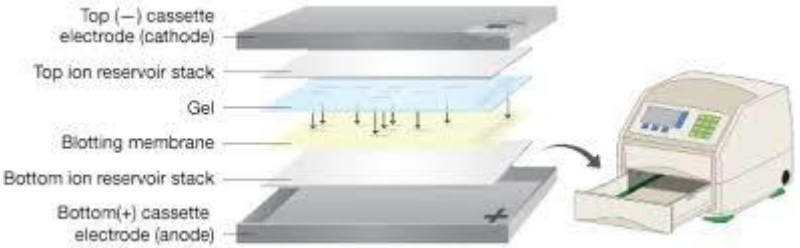
Methods of protein transmission from gel to membrane

- **capillary transfer**
- weight, glass plate, paper, filter paper, nitrocellulose filter, gel, filter paper, glass plates immersed in transfer buffer
- the dry paper absorbs the buffer by capillary forces, thus the sample is drawn from the gel to the membrane



- **electrophoretic transfer**
- Western transfer (proteins are negatively charged and travel to the positive electrode - anode)
- the driving force is the electric field strength



- **vacuum transfer**
- similar arrangement to capillary transfer, but the sample is pulled by vacuum
- faster than capillary transfer

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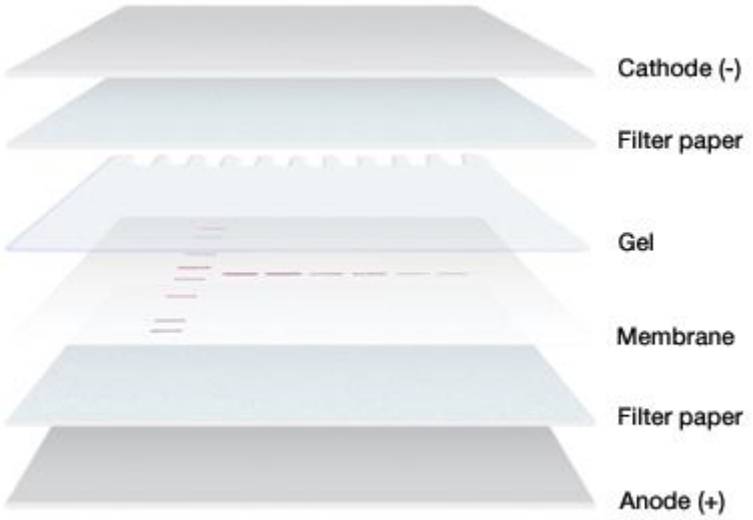
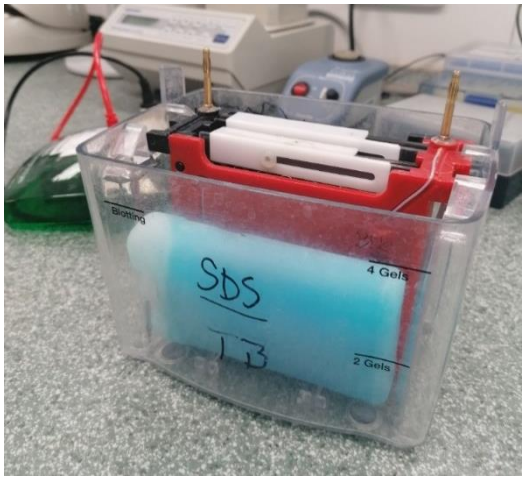
Western transfer

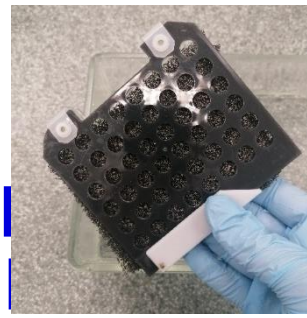
electrophoretic transfer
Western transfer (proteins are negatively charged and travel to the positive electrode - anode)
the driving force is the electric field strength

- **transfer of proteins separated by electrophoresis from a gel to a solid filter (membrane)**
- **composition: sponge, filter paper, gel, membrane, filter paper, sponge**



Mini Trans-Blot cell.

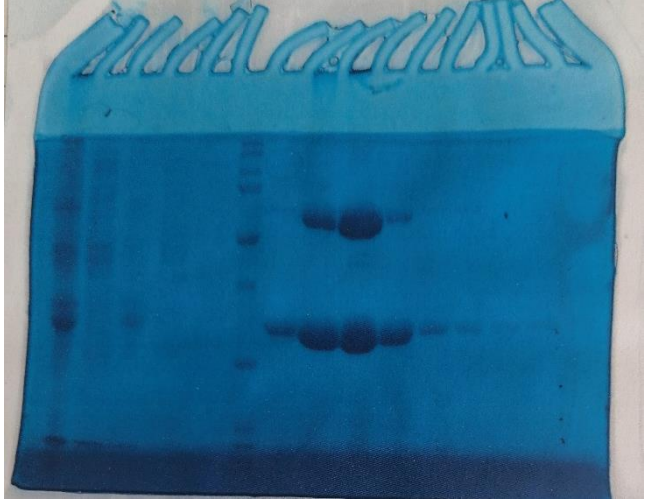




Protein visualisation - staining

- **Non-specifically**
- **staining of all proteins in the gel**
- **Coomassie Brilliant Blue**
- **Silver**

Coomassie Brilliant blue



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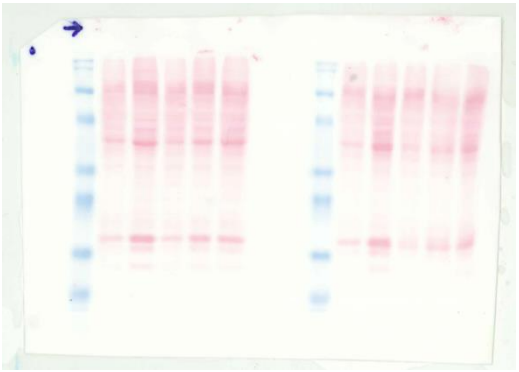
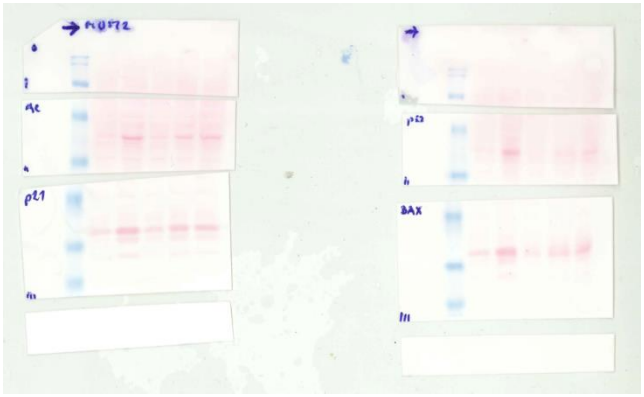
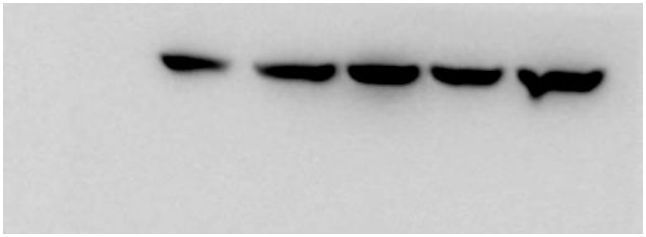


- **Specific-Immunodetection**
- **staining of only the selected protein (membrane)**
- **antibodies**





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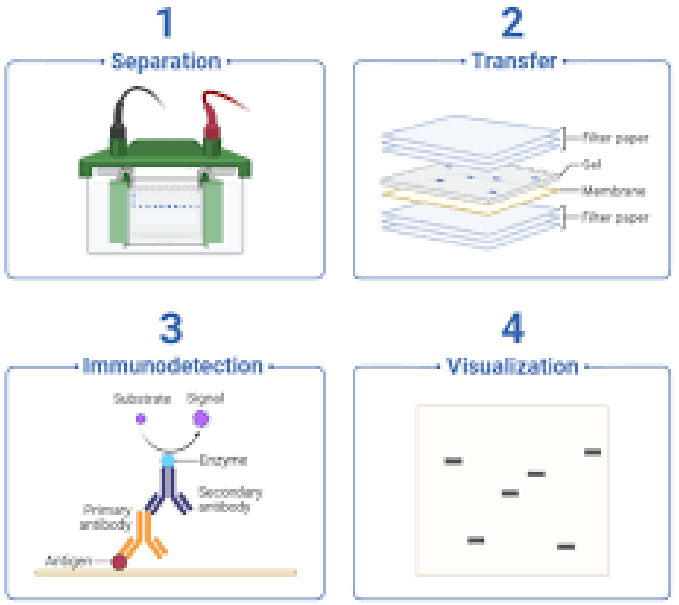
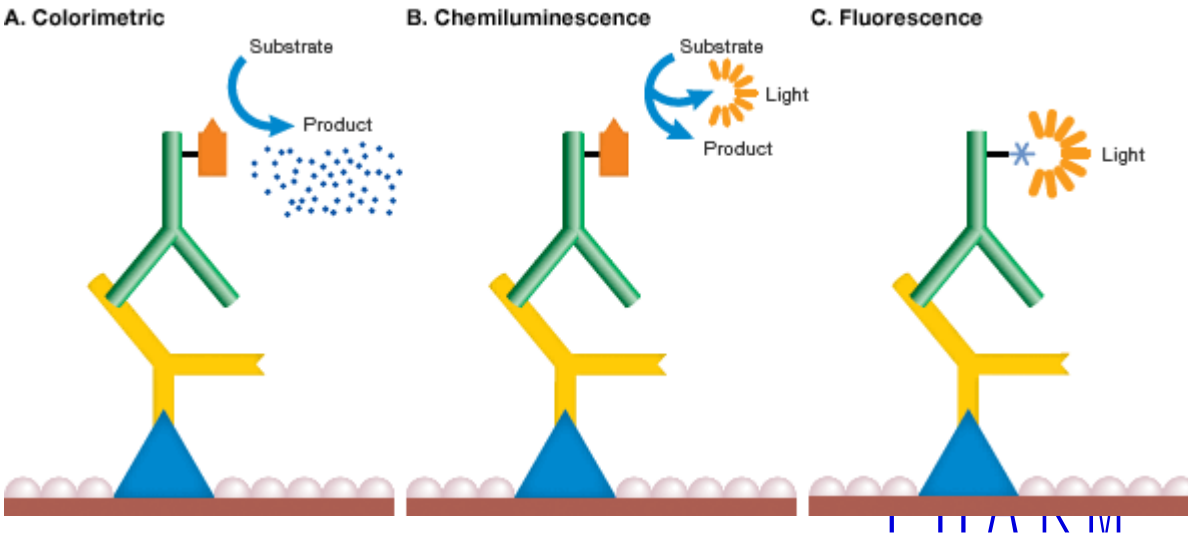
Immunodetection of proteins

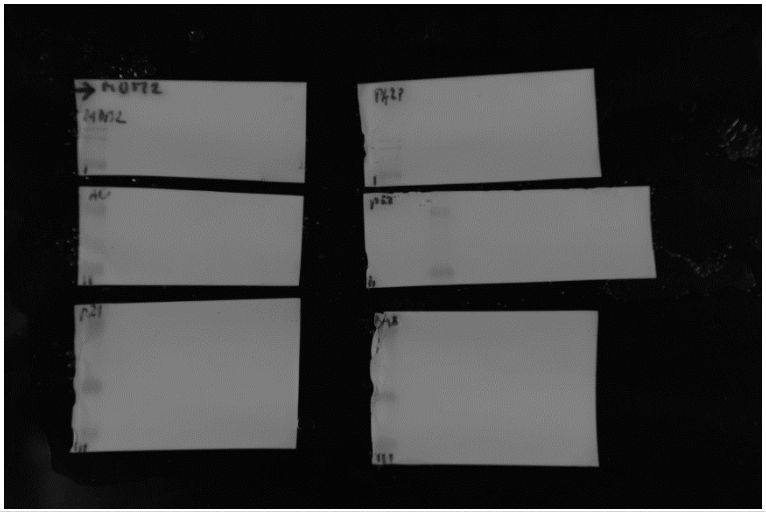
1. saturation of free binding sites on the membrane
2. cheap protein solutions (milk, BSA,...)
3. binding of the primary antibody to the respective antigen while washing the filter with a specific antibody solution
4. washing
5. binding of the secondary antibody containing the conjugated enzyme
6. washing
7. providing the appropriate substrate for the conjugated enzyme
8. signal detection
9. chemiluminescence
10. color detection
11. for example, radioactive probes or the aforementioned secondary antibodies are used to make the protein visible on the membrane

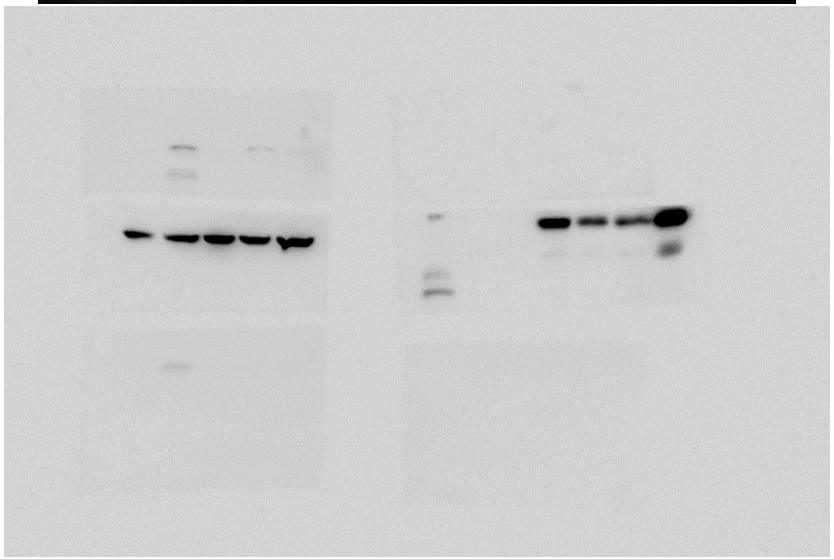




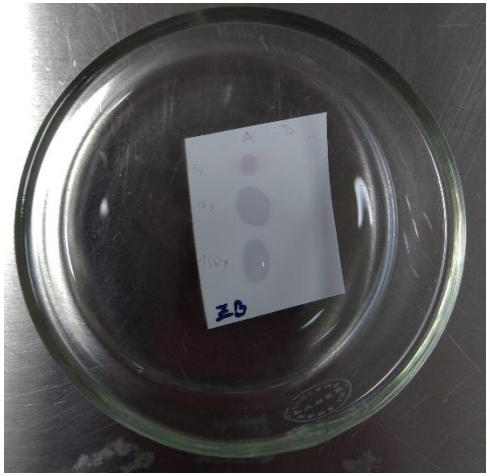
Mechanism of detection chemistries. In each method of western blot detection, a detectable signal is generated following binding of an antibody specific for the protein of interest. In colorimetric detection (**A**), the signal is a colored precipitate. In chemiluminescence (**B**), the reaction itself emits light. In fluorescence detection (**C**), the antibody is labeled with a fluorophore.

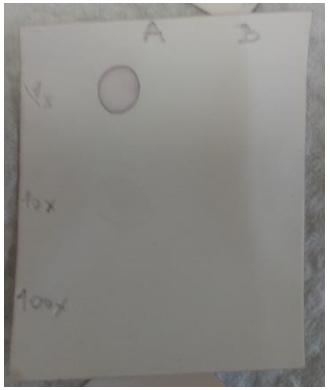
Immunodetection of proteins





Patobiochemistry





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Antibodies

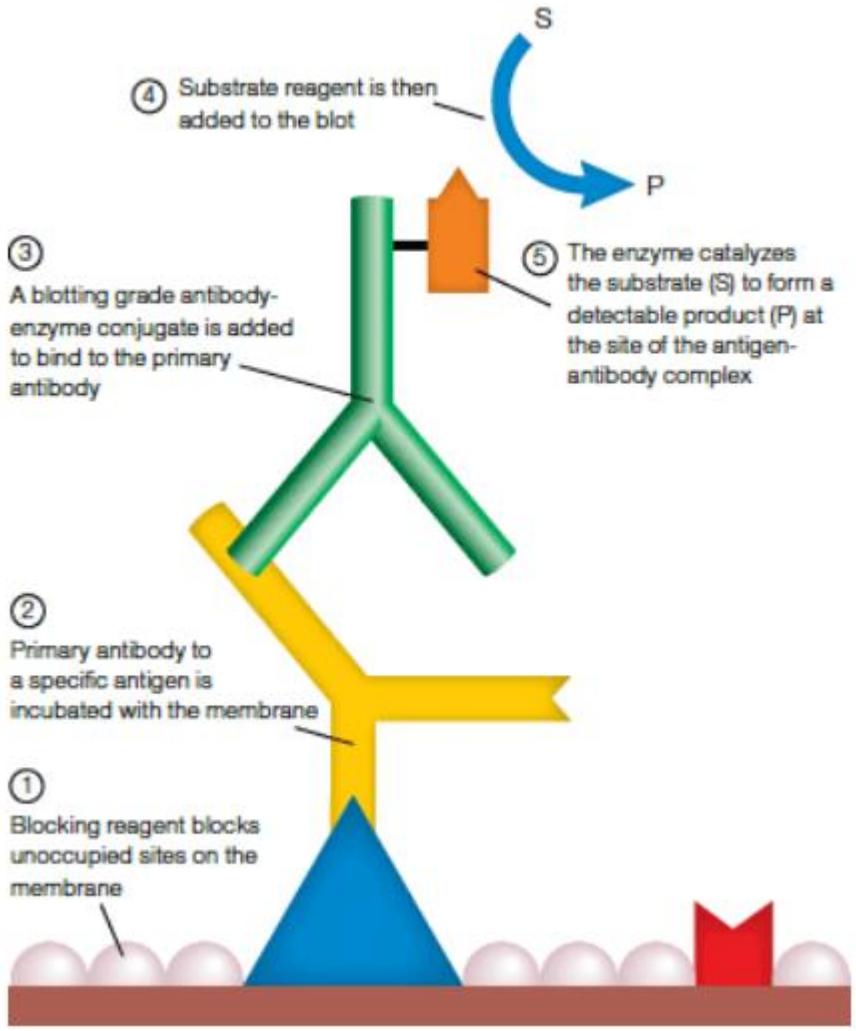
Immunodetection (immunological detection) is used to identify specific proteins blotted to membranes. This section provides an overview of immunodetection methods, workflow, protocol, and troubleshooting tips.

- **they are able to recognize specific protein epitopes**
- serve as probes
- distribution

- **monoclonal**
- they are obtained from hybridomas
- **polyclonal**
- they are obtained from the blood of immuniz animals

•**The primary antibody,** which is directed against the target antigen; the antigen may be a ligand on a protein, the protein itself, a specific epitope on a protein, or a carbohydrate group

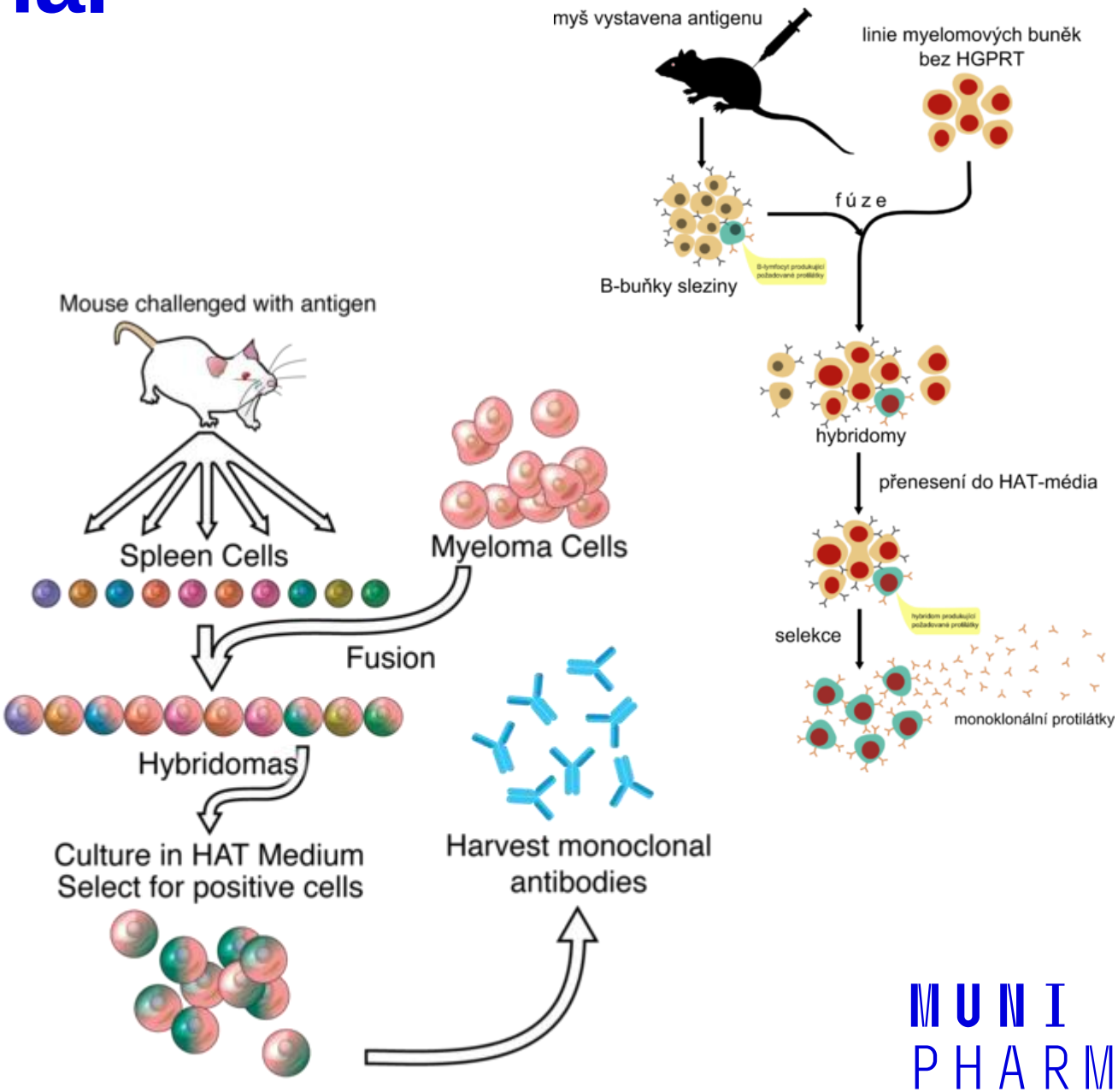
•**The secondary antibody,** which recognizes and binds to the primary antibody; it is usually conjugated to an enzyme such as AP or HRP, and an enzyme-substrate reaction is part of the detection process (see figure below)



Monoclonal antibody

- they are produced using hybridomas
- the latter arises from the fusion of a tumor leukemia cell (immortality) and B-lymphocytes of an immunized animal (antibody production)
- expensive preparation, but in the end an almost unlimited source of antibody
- specific to only one epitope

the fusion of a tumor leukemia cell (immortality) and B-lymphocytes of an immunized animal (antibody production)



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Polyclonal antibodies

- they are obtained from the serum of immunized laboratory animals
- relatively cheap and quick preparation
- **Polyclonal vs. monoclonal antibodies**
- This summary table highlights the five main differences between the two types of antibodies.
- react with multiple epitopes

Polyclonal antibodies	Monoclonal antibodies
Refer to a mixture of immunoglobulin molecules that are secreted against a particular antigen.	Refer to a homogenous population of antibodies that are produced by a single clone of plasma B cells.
Produced by different clones of plasma B cells.	Produced by the same clone of plasma B cells.
Production does not require hybridoma cell lines.	Production requires hybridoma cell lines.
A heterogeneous antibody population.	A homogenous antibody population.
Interact with different epitopes on the same antigen.	Interact with a particular epitope on the antigen.

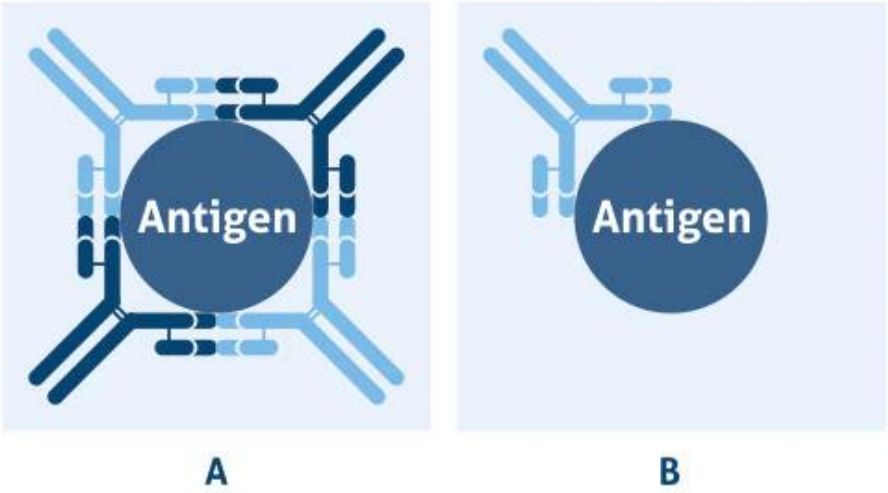


Figure 1. A) Polyclonal antibodies bind to the same antigen, but different epitopes; and B) monoclonal antibodies bind to the same epitope on a target antigen.

•Inexpensive and relatively quick to produce (+/- 3 months).
•Higher overall antibody affinity against the antigen due to the recognition of multiple epitopes.
•Have a high sensitivity for detecting low-quantity proteins.
•High ability to capture the target protein (recommended as the capture antibody in a sandwich ELISA).
•Antibody affinity results in quicker binding to the target antigen (recommended for assays that require quick capture of the protein; e.g., IP or ChIP).
•Superior for use in detecting a native protein.
•Easy to couple with antibody labels and rather unlikely to affect binding capability.

Monoclonal

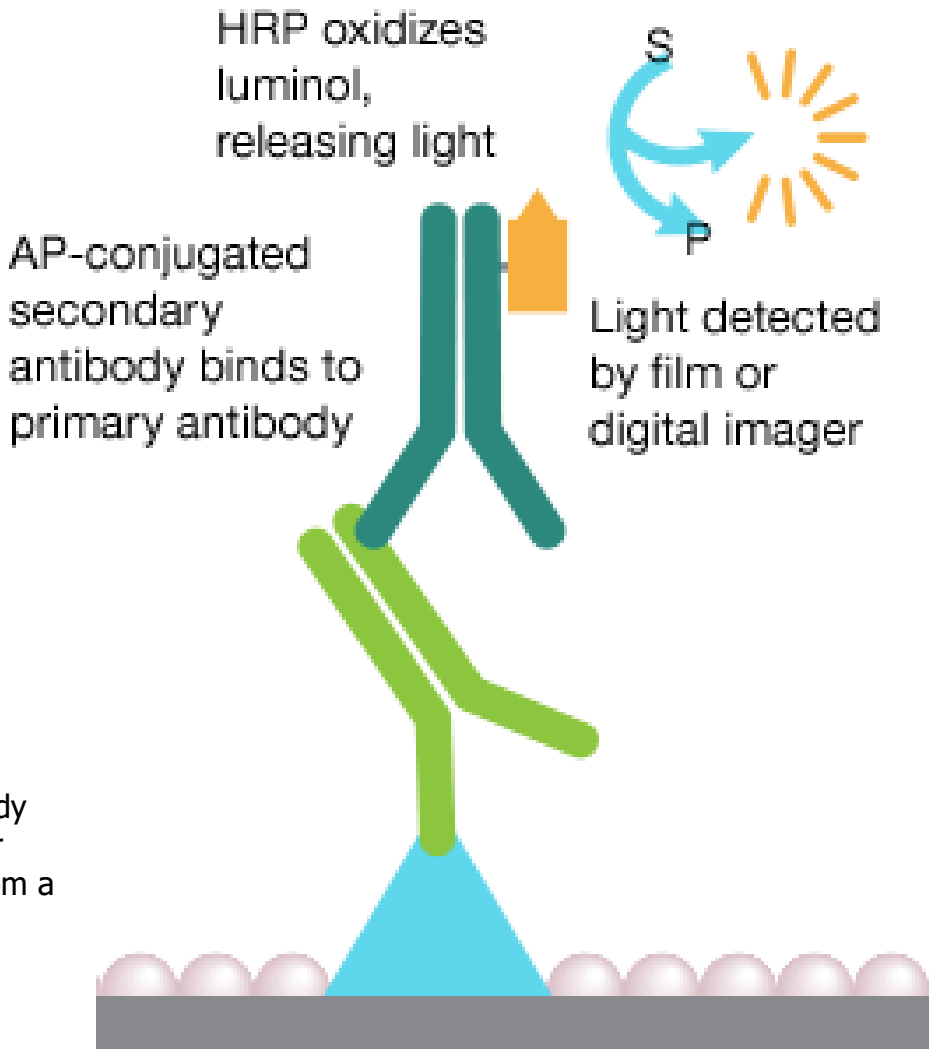
Polyclonal

Specificity	Specificity for a single epitope.	Varying specificities to multiple epitopes
Identification	Identifies whether a particular region of a protein is present	Identifies the entire target protein via binding at multiple sites. Since multiple epitopes are targeted, there is a higher likelihood of detection of the target
Cross-Reactivity	May cross-react with other proteins that share this epitope, such as isomers or common motifs	Higher background and cross-reactivity possible due to detection of multiple epitopes, any of which may be shared by related proteins
Sensitivity	Usually less sensitive since only a single antibody molecule binds to each target	More sensitive because signal is amplified through the binding of several antibodies per target
Cost	More expensive to produce initially, but available in an unlimited supply	Less expensive to produce initially, but supply is limited to immunized animal(s). Greater variability between preparations

https://www.bio-rad.com/en-cz/applications-technologies/western-blotting-immunodetection-techniques?ID=PQEEPOBWLN4A

Primary antibody

- binds to a specific antigen epitope
- a labeled secondary antibody binds to it



Primary Antibody Incubation
After blocking, the membrane is incubated in a solution containing the primary antibody, usually diluted in blocking buffer. The time and temperature of incubation depends on the binding affinity of the antibody to the target protein and should be determined for each antibody individually. One hour at room temperature with gentle agitation is a good starting point. In order to reduce the background staining, the amount of Tween 20 used in the buffers is also important.

Antibody Concentration
The optimum antibody concentration is the dilution of antibody that still yields a strong positive signal without background or nonspecific reactions. Instructions for antibodies obtained from a manufacturer typically suggest a starting dilution range. For custom antibodies or for those where a dilution range is not suggested, good starting points are:
•1:100–1:1,000 dilution when serum or tissue culture supernatants are the source of the primary antibody
•1:500–1:10,000 dilution of chromatographically purified monospecific antibodies
•1:1,000–1:100,000 dilution may be used when ascites fluid is the source of antibody

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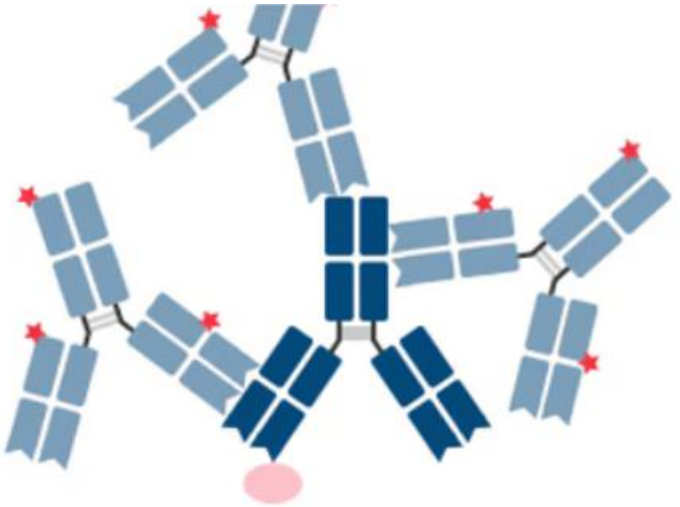
Secondary antibody

- they are specific against only a small number of primary antibodies
- anti-mouse IgG
- anti-rabbit IgG

Secondary antibodies are specific for the isotype and species of the primary antibody.
For example, a goat anti-rabbit secondary is an antibody raised in goats against a primary antibody raised in rabbits.
Secondary antibodies bind to a number of different conserved regions on the primary antibody, and act to amplify the signal, increasing detection sensitivity. Secondary antibodies are labelled with either an enzyme for colorimetric or chemiluminescent detection or with a fluorescent dye for fluorescent detection of the protein of interest.

- **<u>marked in different ways</u>**
- radioactively
- horseradish peroxidase (HRP)
- alkaline phosphatase
- biotin
- fluorescent marker



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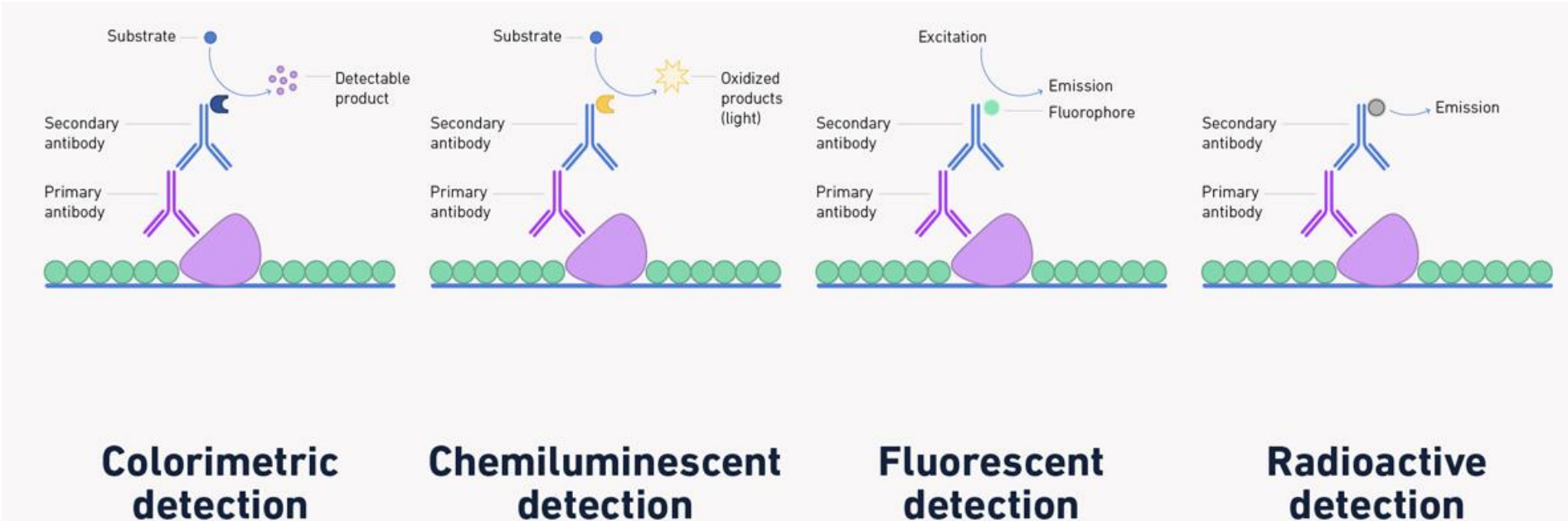
Detection Methods

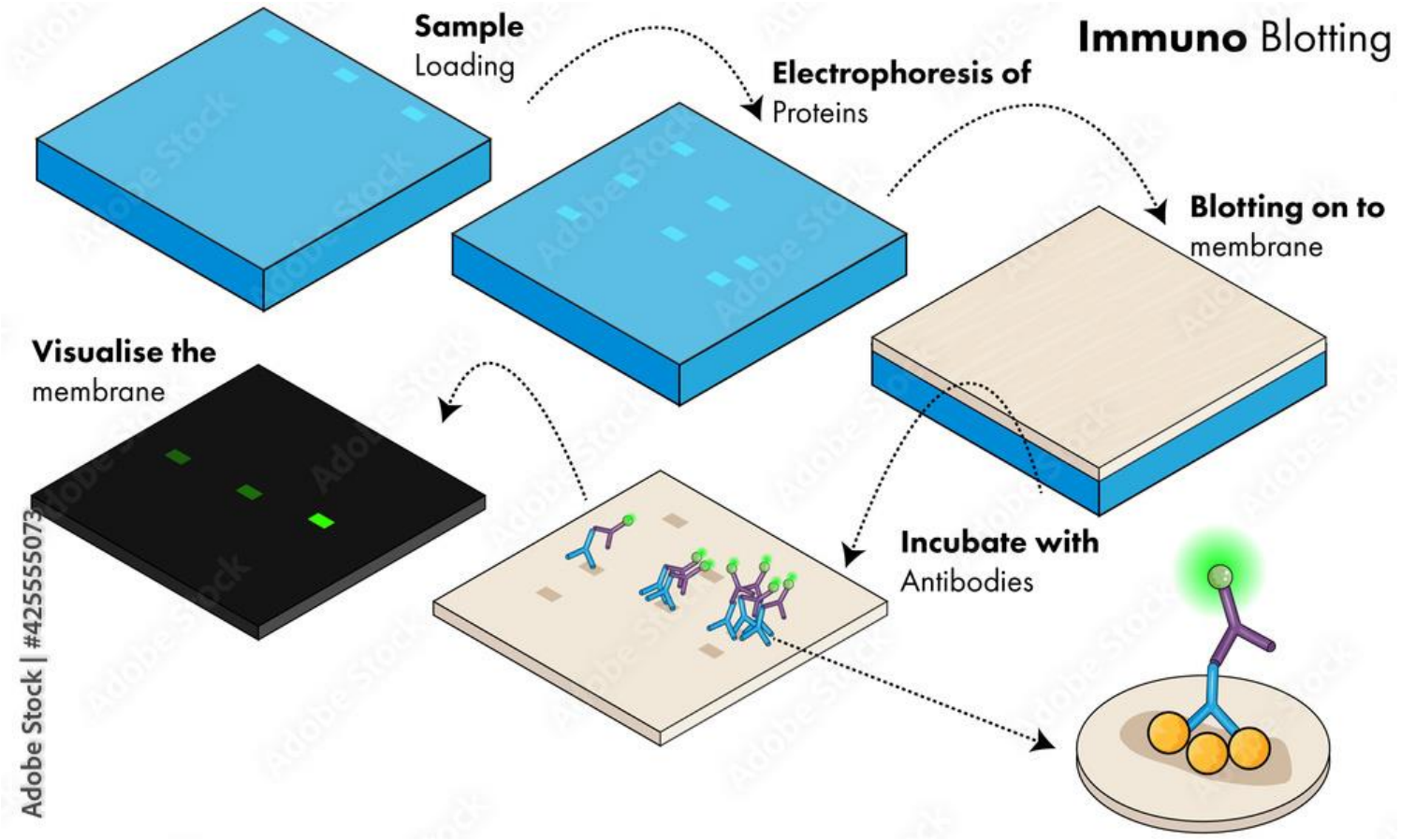
- **colorimetric methods**
- **chemiluminescence**
- **bioluminescence**
- **chemifluorescence**
- **fluorescence/autoradiography**
- **gold-labeled antibodies**

In the past, many different methods were used for western blot detection, but now the vast majority employ enzymatic chemiluminescence or fluorescent detection. Thus, most secondary antibodies are conjugated to an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) for use with a chemiluminescent substrate or labeled with a fluorescent compound for imaging.



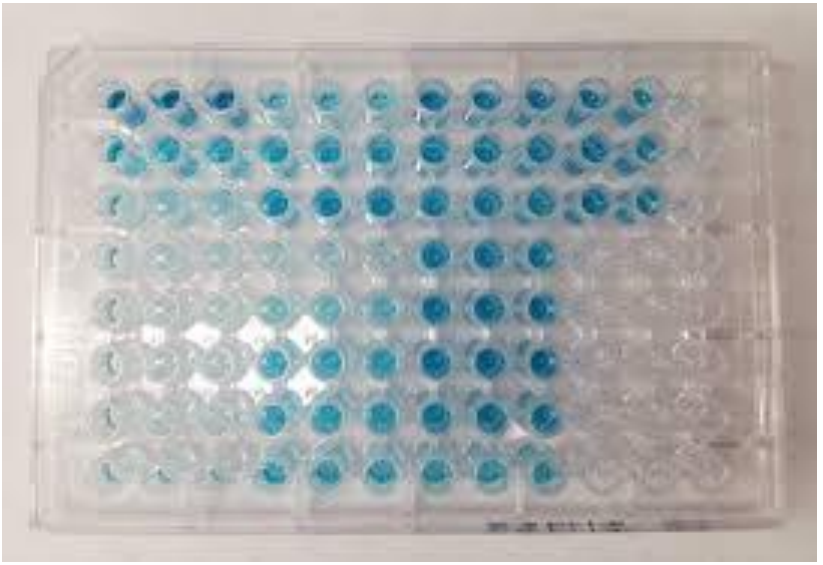
Imunobloting

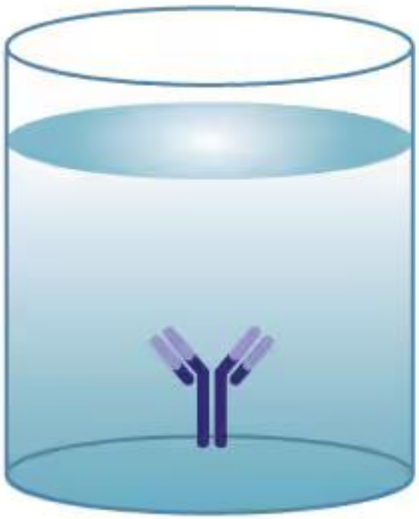
1. separation of the proteins of the given sample by gel electrophoresis
2. transfer of proteins to the membrane (western blotting)
3. incubation of the membrane with a specific antibody
4. incubating the membrane with a secondary labeled antibody
5. detection of bound antibody

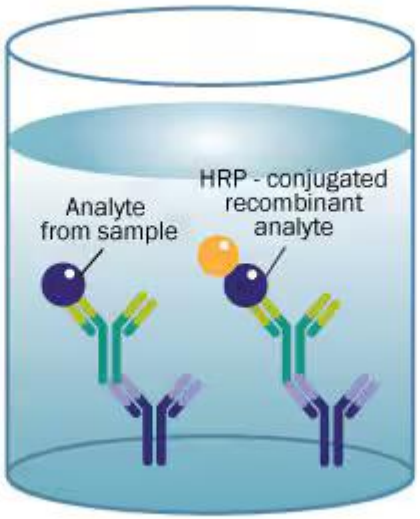


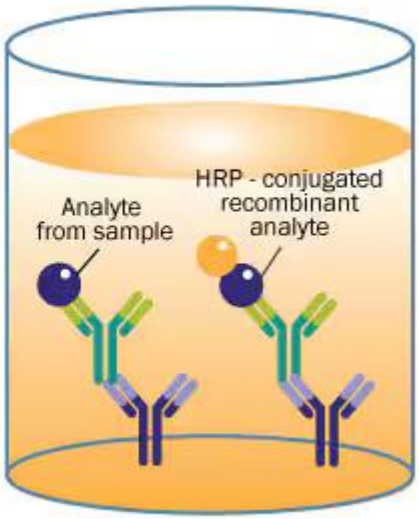
ELISA

- **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay
- uses two antibodies against one protein
- 2 different epitopes
- one antibody is bound to a carrier
- most often on the wall of the reaction vessel



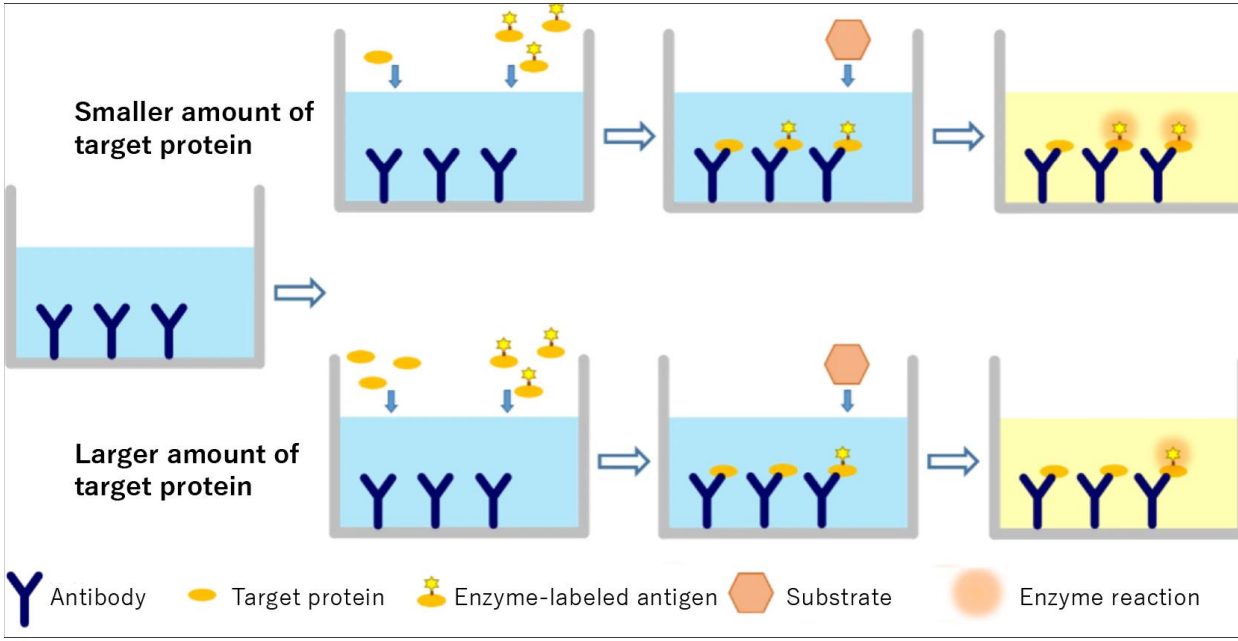






ELISA

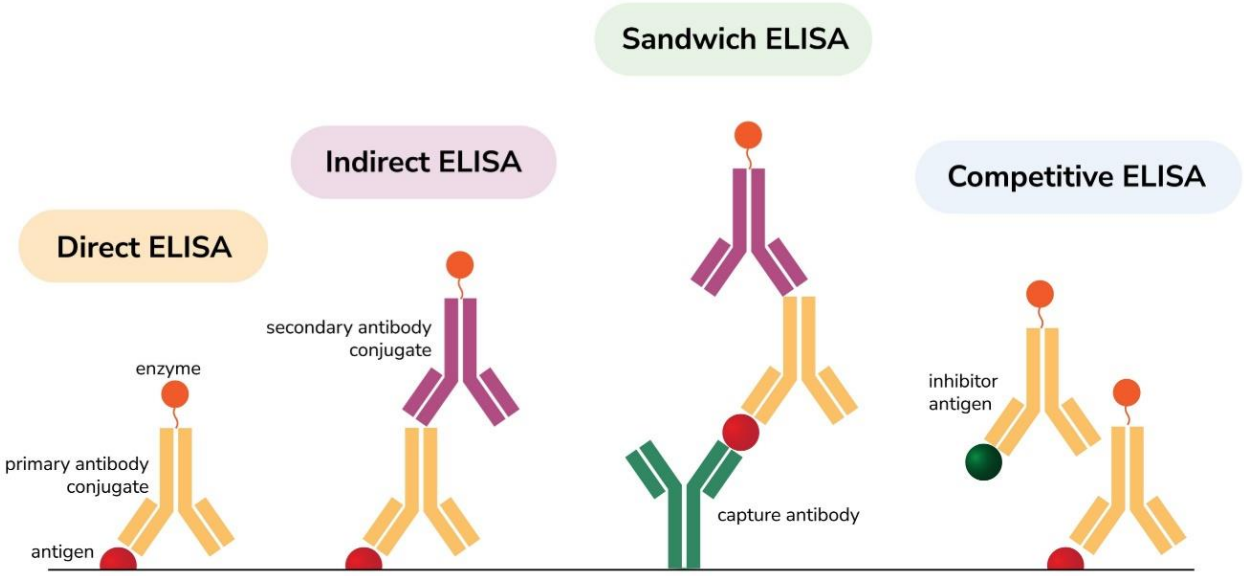
- benefits
- high sensitivity
- pg/ml
- small amount of sample needed
- the possibility of using semi-automatic systems



MUNI PHARM

ELISA

Types of ELISA



– **design:**

1. binding of the protein to the first antibody
2. washing
3. binding of the second antibody to the protein
4. washing
5. detection of a second clot

MUNI PHARM

AlphaLISA

- modification of the ELISA method
- "ELISA in solution"
- no washing
- both the donor and the acceptor are bound to the target molecule
- the donor produces singlet oxygen
- the result is signal amplification
- a wide range of uses