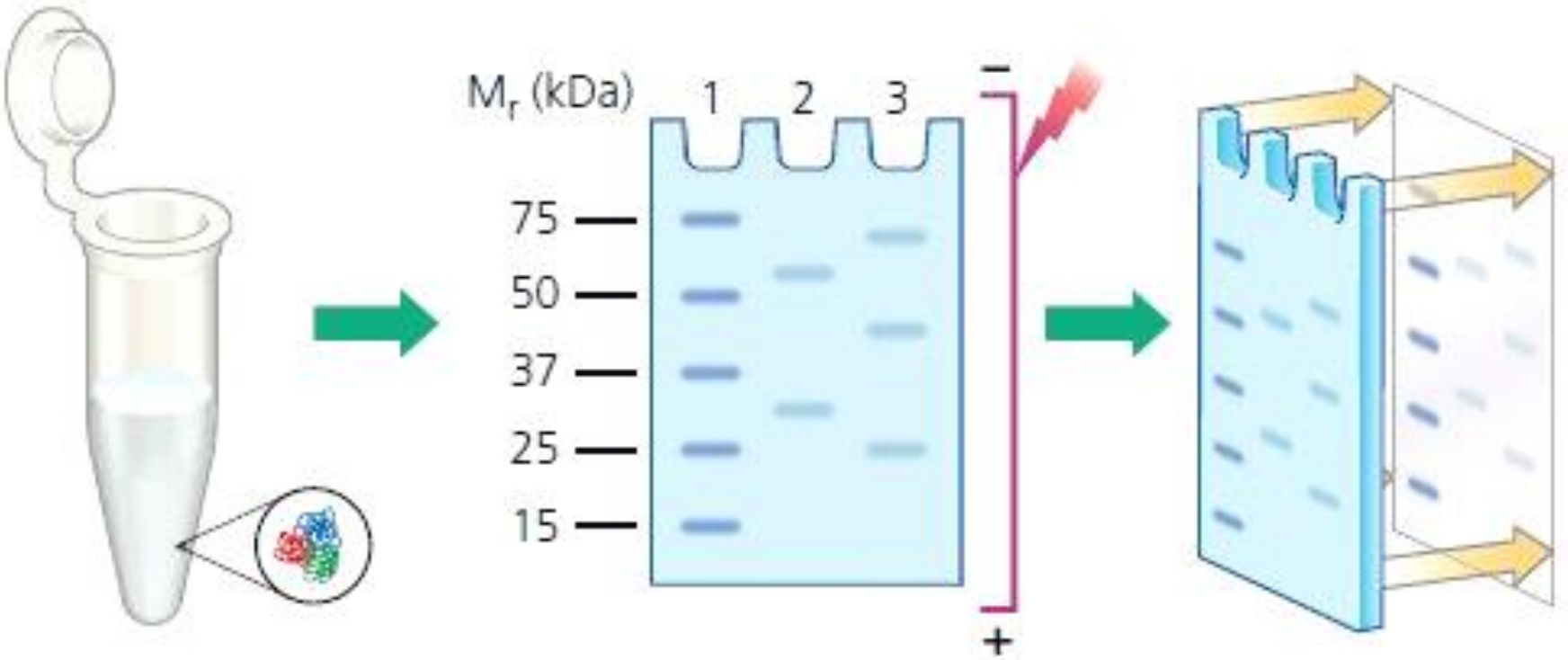

Western Blotting

Prepared by

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INTRODUCTION

- Western blotting identifies specific proteins that have been separated from one another according to their size by polyacrylamide gel electrophoresis.
- The blot is a membrane, almost always made up of nitrocellulose or PVDF (polyvinylidene fluoride).
- The gel is placed next to the membrane and application of an electrical current induces the proteins in the gel to move to the membrane where they adhere.
- The membrane is then a replica of the gel's protein band pattern, and is subsequently stained with a labelled antibody.

SAMPLE PREPARATION

- To prepare samples for running on a gel, cells and tissues need to be lysed to release the proteins of interest.
- **The first step solubilizes the proteins** so that they can migrate individually through a separating gel.
- There are many formulations for lysis buffers but a few will serve for most Western blotting experiments.
- **In brief, they differ in their ability to solubilize proteins**, with those containing sodium dodecyl sulfate and other ionic detergents considered to be the harshest and therefore most likely to give the highest yield.
- However, it is important to note though that **some antibodies will only recognize a protein in its native, non-denatured form** and will not recognize a protein that has been extracted with a denaturing detergent (SDS, deoxycholate etc.) The main consideration, then, when choosing a lysis buffer is whether the antibody chosen will recognize denatured protein samples. When this is not the case **buffers without detergent or with relatively mild non-ionic detergents (NP-40, Triton X-100) should be used.**

Protein Location And Lysis Buffer Choice

Protein location	Buffer recommended
Whole Cell	NP-40 or RIPA
Cytoplasmic (soluble)	Tris-HCl
Cytoplasmic (cytoskeleton bound)	Tris-Triton
Membrane bound	NP-40 or RIPA
Nuclear	RIPA or use nuclear fraction protocol*
Mitochondria	RIPA or use mitochondrial fraction protocol*

Proteins that are found exclusively or predominantly in a sub-cellular location can be enriched in a lysate of the sub-cellular fraction compared to whole cell or tissue lysates. This can be useful when trying to obtain a signal for a weakly-expressed protein. For instance, a nuclear protein will be a larger proportion of the total protein in a nuclear lysate than it will be in a whole-cell or whole-tissue lysate, making it possible to load more of the protein per gel lane. Another advantage is the removal of potentially cross-reactive proteins present in the unused fractions.

RIPA: Radio Immuno Precipitation Assay buffer

Protein Location & Buffer Choice [cont.]

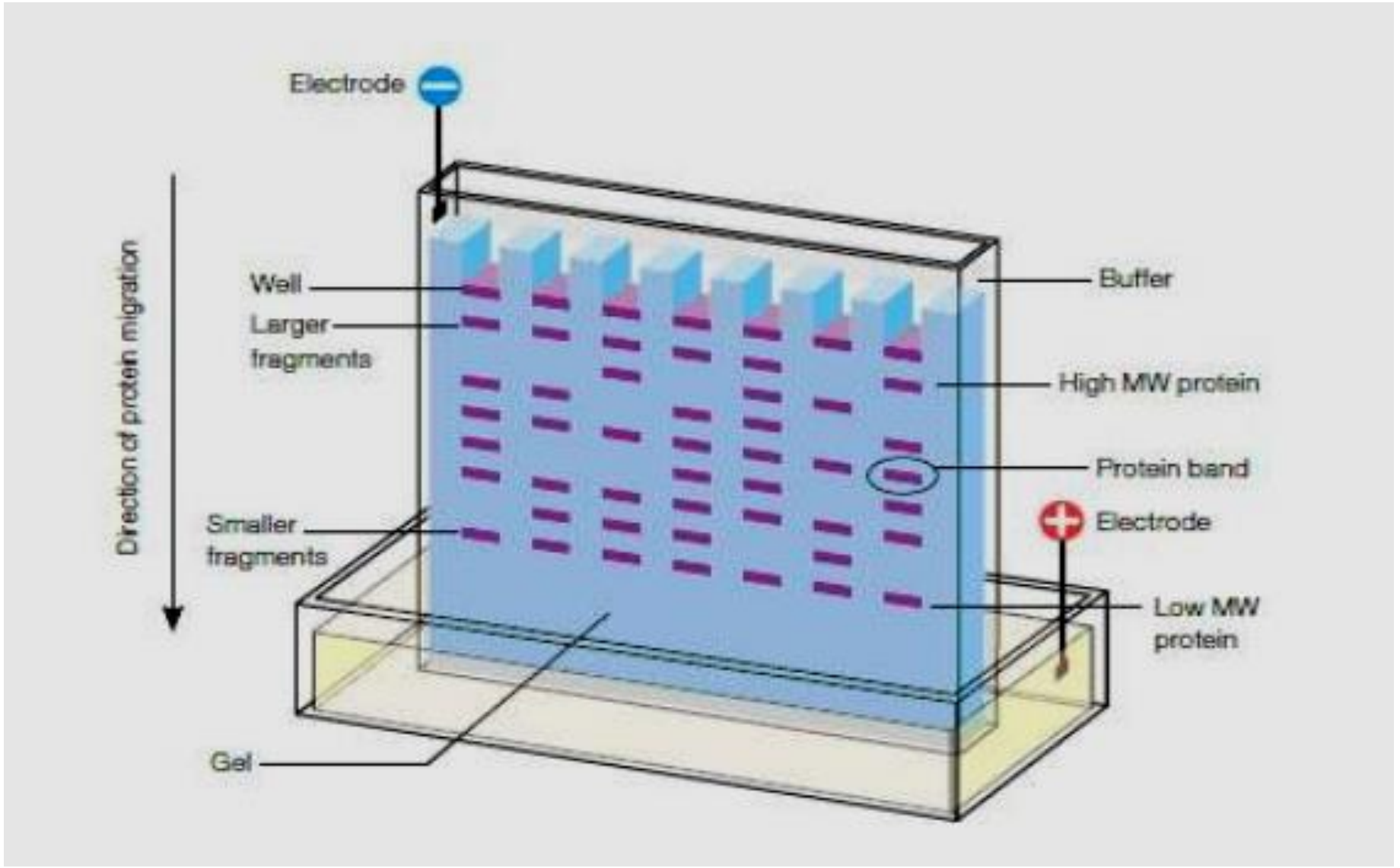
- In cases where it is important to preserve protein-protein interactions or to minimize denaturation (for example, when it is known that the antibody to be used will only recognize a non-denatured epitope), a buffer without ionic detergents (e.g. SDS) and ideally without non-ionic detergents (e.g. Triton X-100) should be used.
- Cell lysis with detergent-free buffer is achieved by mechanical shearing, often with a homogenizer.
- In these cases a simple Tris buffer will suffice, but as noted above, buffers with detergents are required to release membrane- or cytoskeleton-bound proteins.

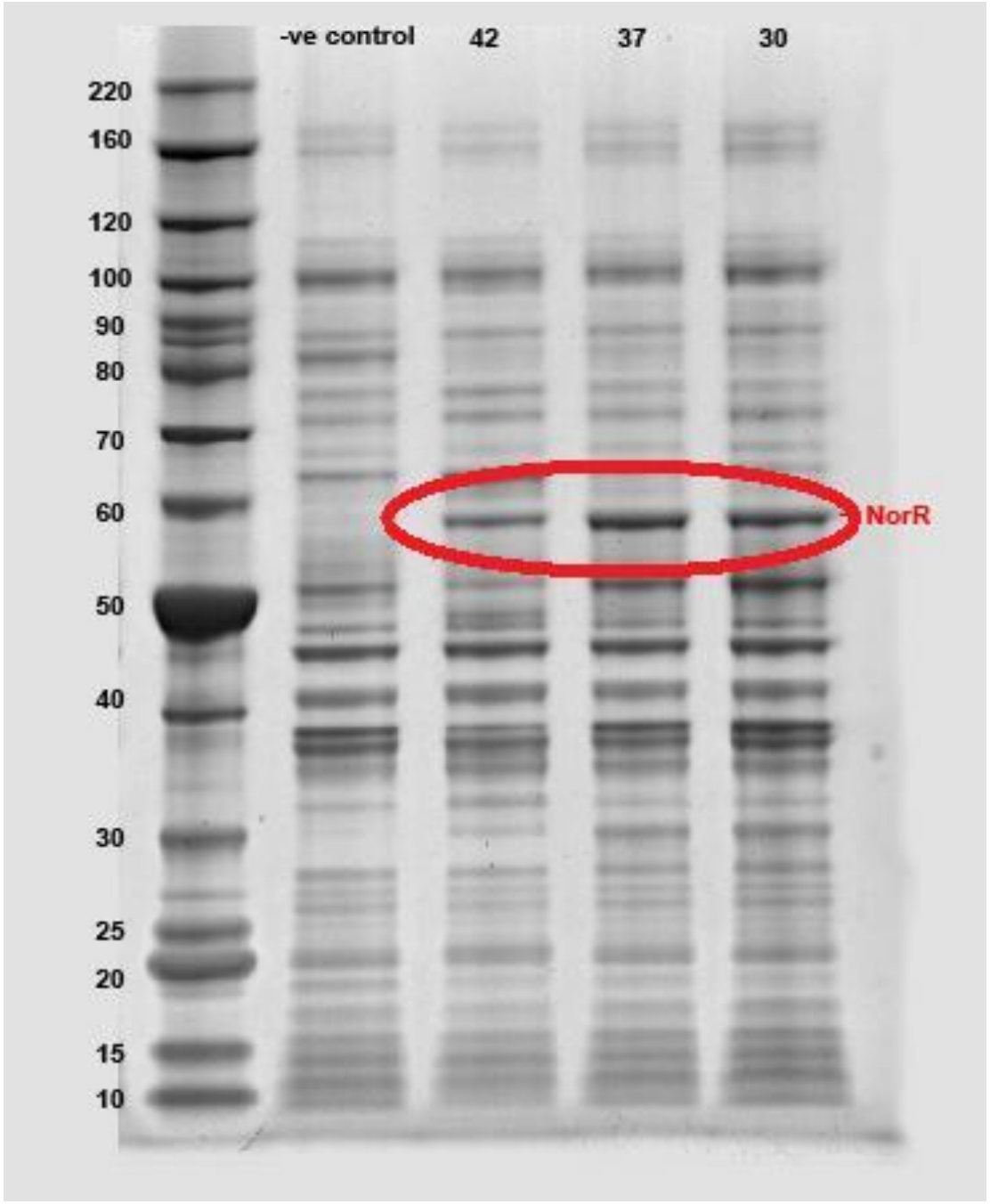
Protease and phosphatase inhibitors

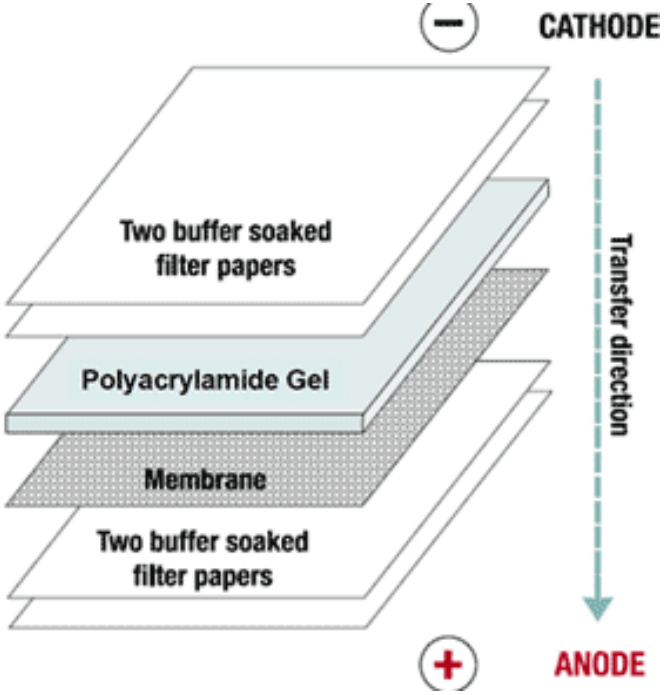
- As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin.
- These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added **fresh to the lysis** buffer.
- **Inhibitor** **Protease/phosphatase Inhibited**

Aprotinin	Trypsin, Chymotrypsin,
Leupeptin	Plasmin
Pepstatin A	Aspartic proteases
PMSF	Serine, Cysteine proteases
EDTA	Metalloproteases that require Mg^{++} & Mn^{++}
EGTA	Metalloproteases that require Ca^{++}
Na Fluoride	Serine/Threonine phosphatases
Na Orthovanadate	Tyrosine phosphatases

Separation of Proteins







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Transfer of Proteins

- Once electrophoresis is complete, the separated proteins can be transferred from within the gel onto a membrane (a western blot) made of nitrocellulose, polyvinylidene difluoride, activated paper, or activated nylon.
- **Nitrocellulose is the most commonly used membrane**.
- **Electroblotting is the most popular procedure** for transferring proteins from a gel to a membrane. Its main advantages are speed and completeness of transfer.
- This process uses an electric current to pull proteins from the gel onto the membrane. It can be achieved by immersion of a gel-membrane sandwich (**wet transfer**) or by putting the gel-membrane sandwich between absorbent paper that has been soaked in transfer buffer (**semidry transfer**).
- **The effectiveness of protein transfer is dependent on the type of gel used, the molecular mass of the protein, and the type of membrane**.
- Some limitations associated with protein transfer include a lower molecular weight limit of 10 kDa and use of specialized transfer buffers [*e.g.*, 3-(cyclohexylamino)-L-propanesulfonic acid] to facilitate transfer of proteins with a high isoelectric point.

Blocking and Antibodies

- It is important to prevent interactions between the membrane and the antibody chosen to detect the target protein.
- **To block nonspecific binding, the membrane is placed in a dilute solution of protein such as bovine serum albumin and non-fat dry milk.**
- Researchers should ensure that the **blocking buffer that is appropriate for the specific antiserum is also appropriate for the type of membrane**.
- Blocking helps mask any potential nonspecific binding sites on the membrane, thus **reducing background "noise"** in the final product of the western blot, eliminating false positives and providing a clear result.
- After blocking, the most popular method is to incubate the membrane with primary antibody, wash, re-block, and then incubate with secondary antibody and wash again.
- It is important to determine the optimal concentration of antibodies before running all the samples as optimization is a prime determinant of the sensitivity of the assay. The antibody concentration should be optimized to provide the best signal to noise ratio.
- Both monoclonal and polyclonal antibodies can be used for western analyses, with advantages and disadvantages in using either type.

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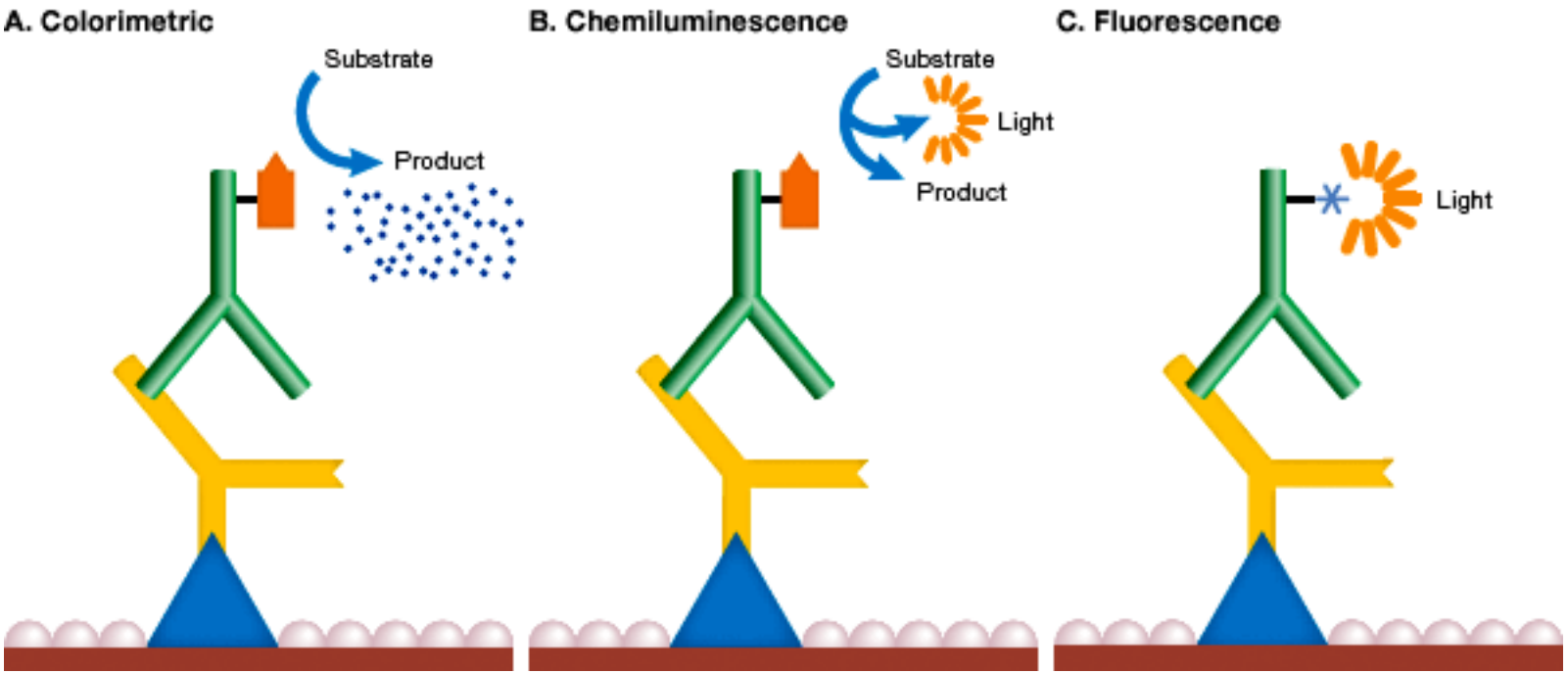
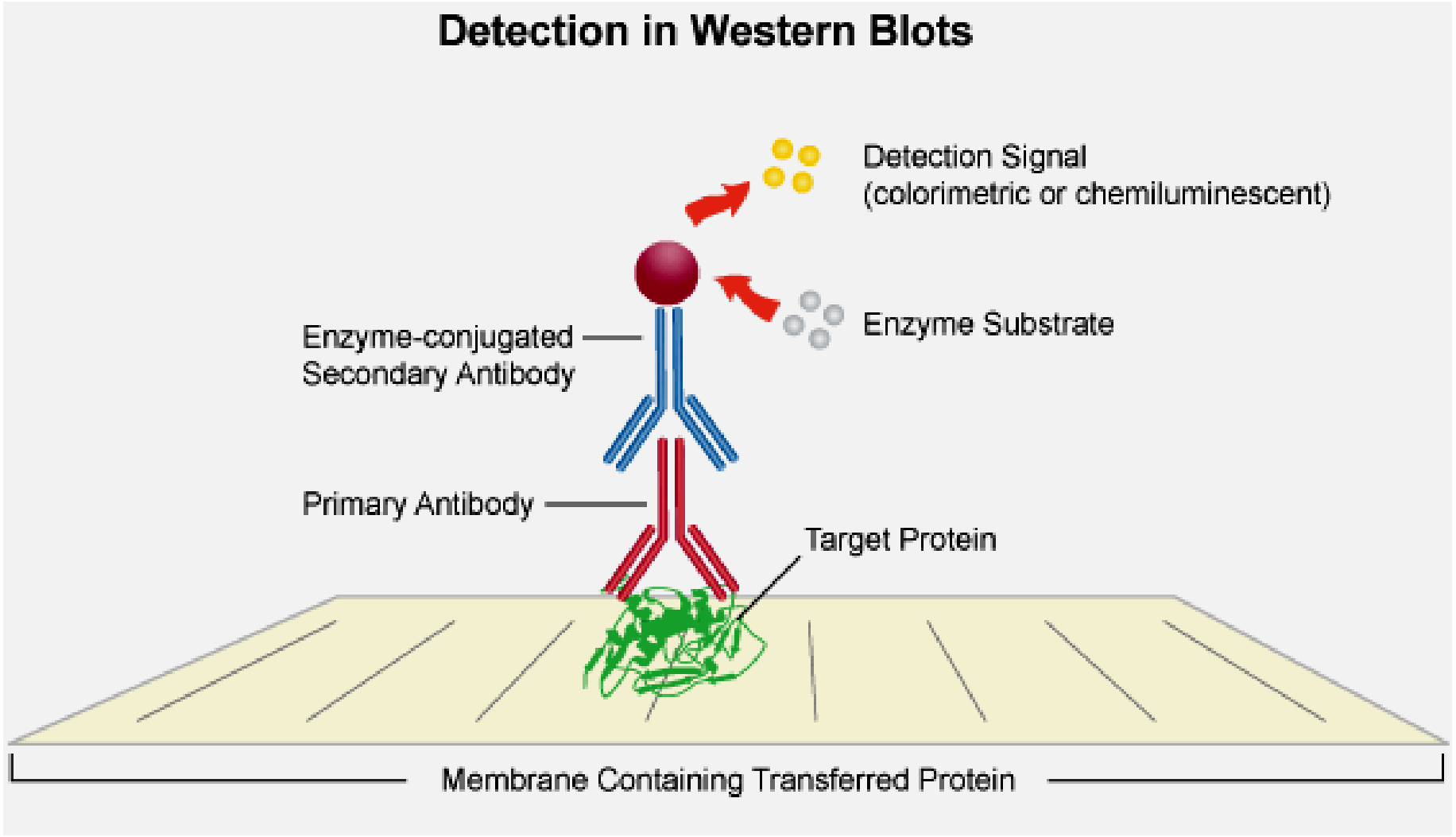
Detection

- The probes that are labelled and bound to the protein of interest need to be detected on the western blot.
- For detection colorimetric, radioactive, and fluorescent methods can be used. However, chemiluminescent detection is used most often these days.





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Detection by Chemiluminescence

- Enhanced chemiluminescence (ECL) is a sensitive method and can be used for relative quantitation of the protein of interest.
- The primary antibody binds to the protein of interest and the secondary antibody, usually linked to horseradish peroxidase, is used to cleave a chemiluminescent agent.
- The reaction product produces luminescence, which is related to the amount of protein.
- Only a single light detector is required, and the light is detected by photographic film.

Chemiluminescence

- Chemiluminescence (CL) makes use of molecules that emit light in the visible or UV range (sometimes also in the IR range) below their glowing temperature. Several hundreds of organic and anorganic compounds are capable of chemiluminescence.
- The molecules are not excited physically (laser, light, heat, electricity), but reach the excited state due to an enzyme-catalyzed chemical reaction.
- The emittance of energy occurs by light quanta, not by heat. Therefore, chemiluminescence is called “cold luminescence“.
- Chemiluminescence appears at oxidation processes, the reaction product is transferred into an energetically excited level and stabilizes itself by energy emittance in the form of light.
- The reaction product is chemically different from the initial components.
- If chemiluminescence processes take place in living organisms, it is called **bioluminescence**, *e.g.* the luciferin/luciferase system of the firefly which is applied variously in biochemistry and molecular biology.

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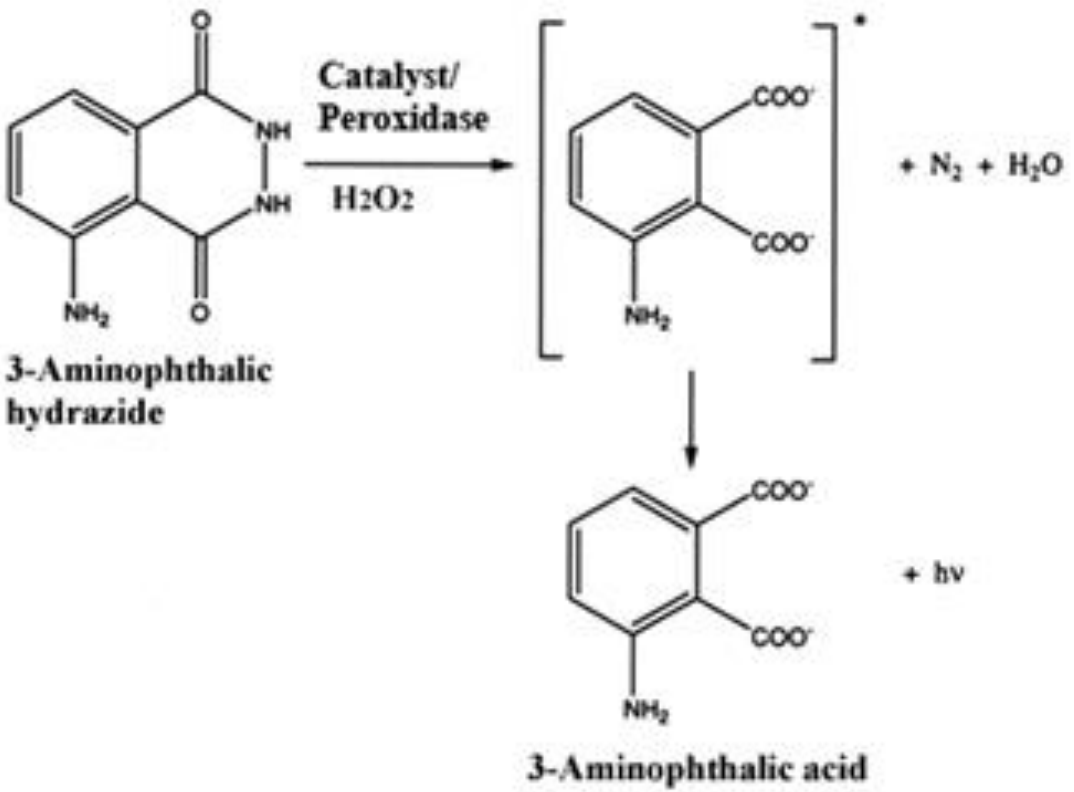

Reaction course of luminol with hydrogen peroxide (*indicates the excited transfer state of the 3-aminophthalic acid resulting by oxidation, which changes to normal state emitting light quanta).

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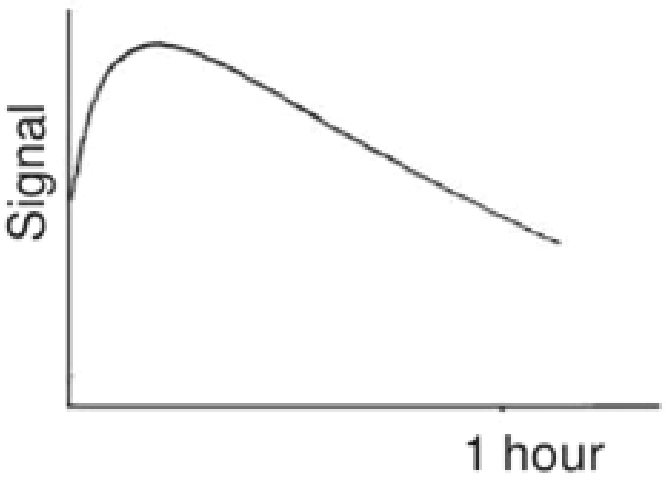
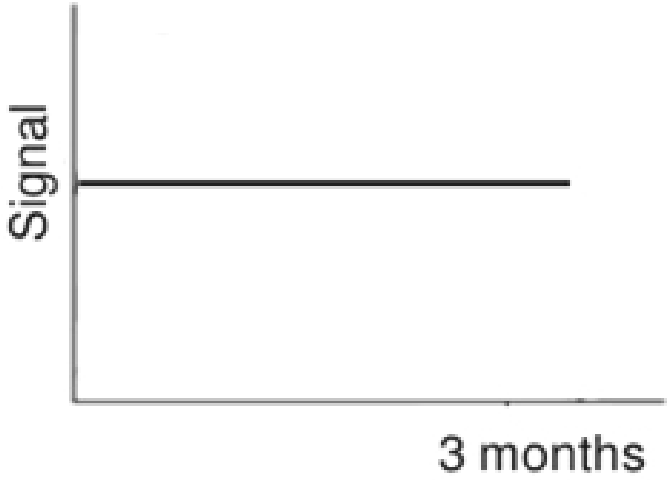
Chemiluminescence [cont.]

- The chemiluminescence of luminol and of other luminophores is the basis of many analytic proof methods and systems in biochemistry and is used for biosensors, immunoassays and microarrays (*e.g.* ELISA, Western Blot).
- In most cases the enzyme horseradish peroxidase (HRP) is used as catalyst for chemiluminescence reaction. This enzyme is linked to a secondary antibody in immunoreactions in a solid or fluid phase in a specific biochemical or biological reaction.

Chemiluminescence vs. Fluoresecence



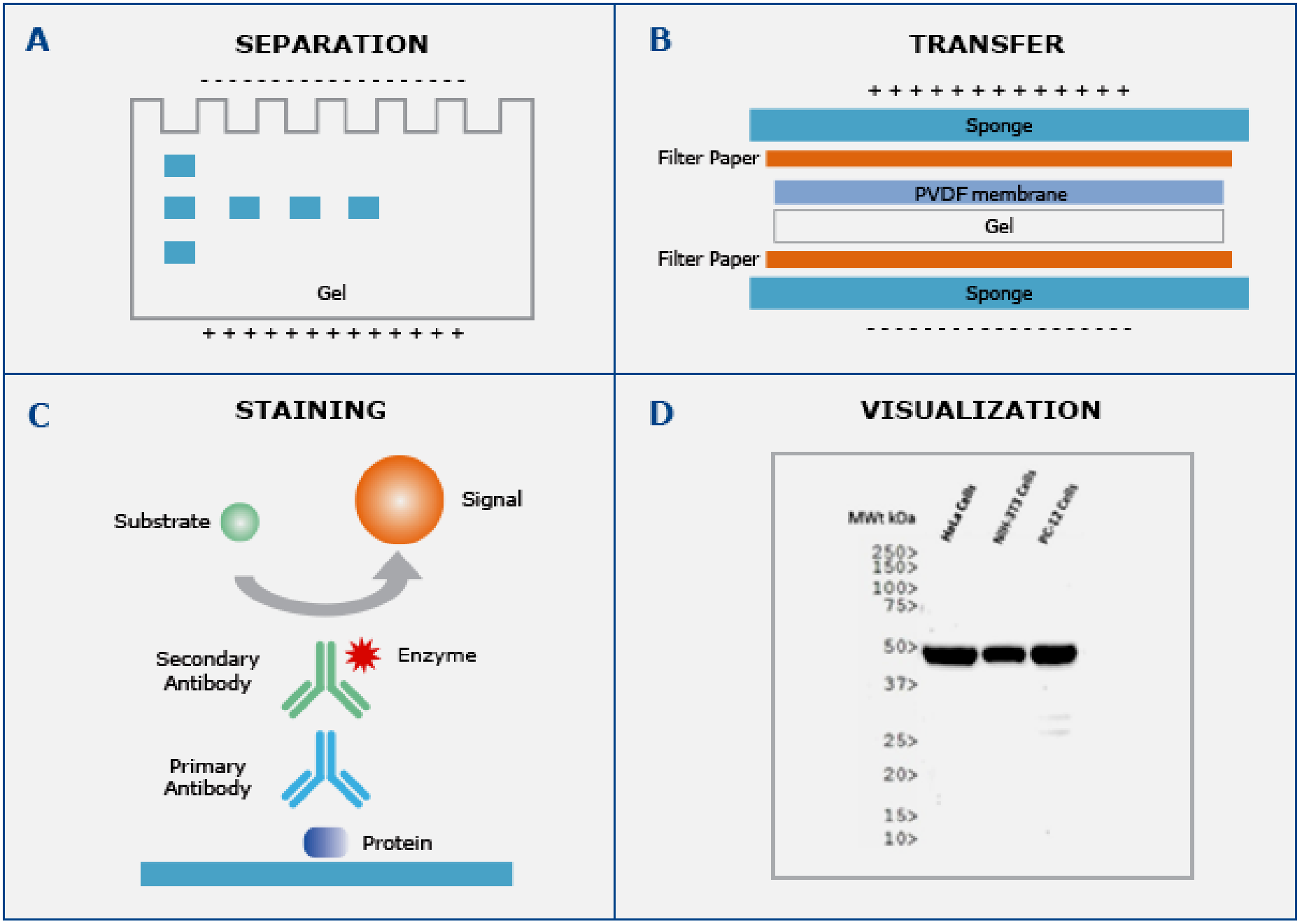


Chemiluminescence

- Unstable signal declining within minutes
- High variation between blots
- Skills and controlled handling needed for accurate quantitation
- *Good choice for confirmatory Westerns*

Fluorescence

- Stable signal for months
- High reproducibility
- Accurate quantitation
- *First choice for quantitative Westerns*


A
SEPARATION
Gel
B
TRANSFER
Sponge
Filter Paper
PVDF membrane
Gel
Filter Paper
Sponge
C
STAINING
Substrate
Signal
Secondary Antibody
Enzyme
Primary Antibody
Protein
D
VISUALIZATION
MWt kDa
250>
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