# Western Blotting & ELISA

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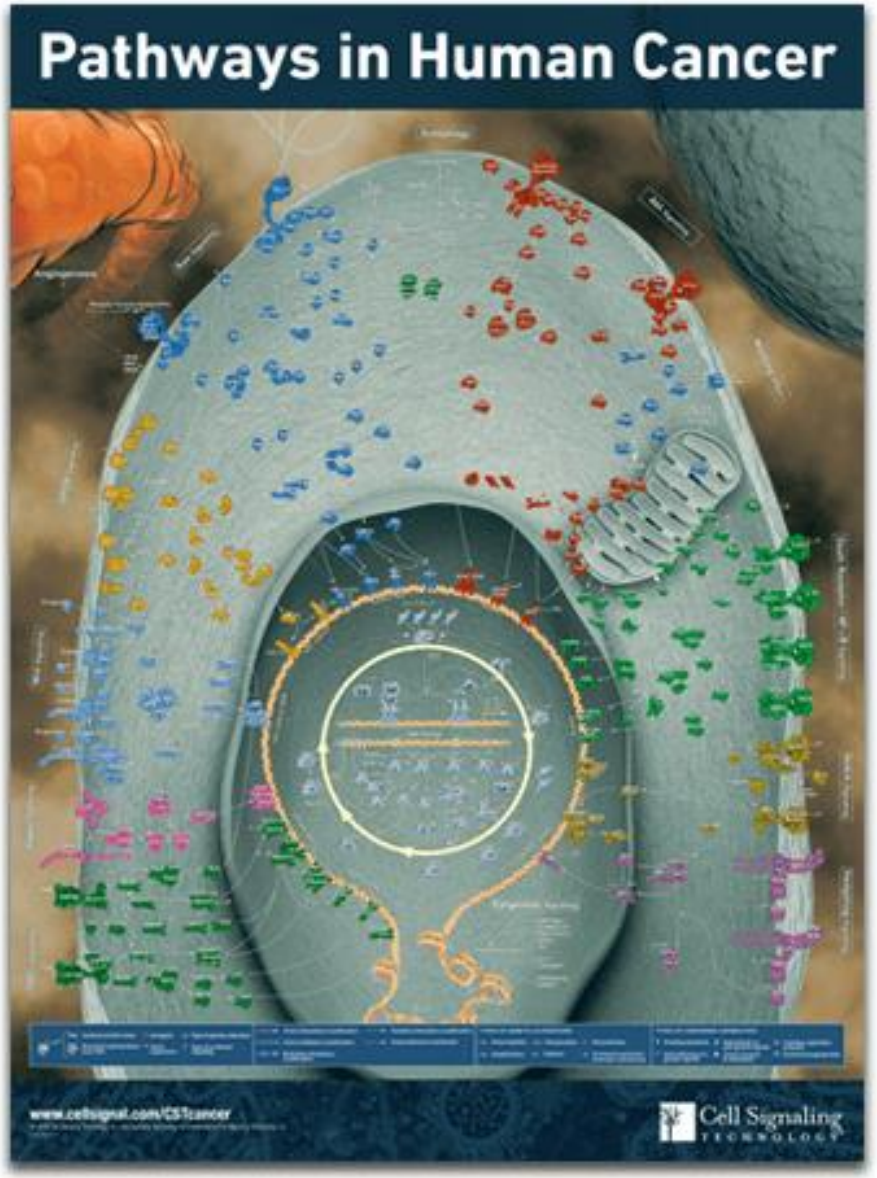
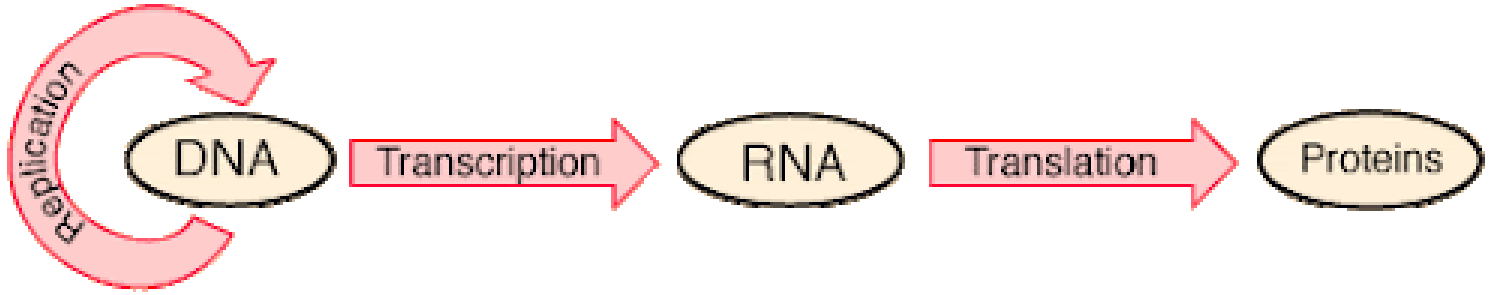
Cancer Biology Summer School 2025

# Why Detect and Quantify Proteins?

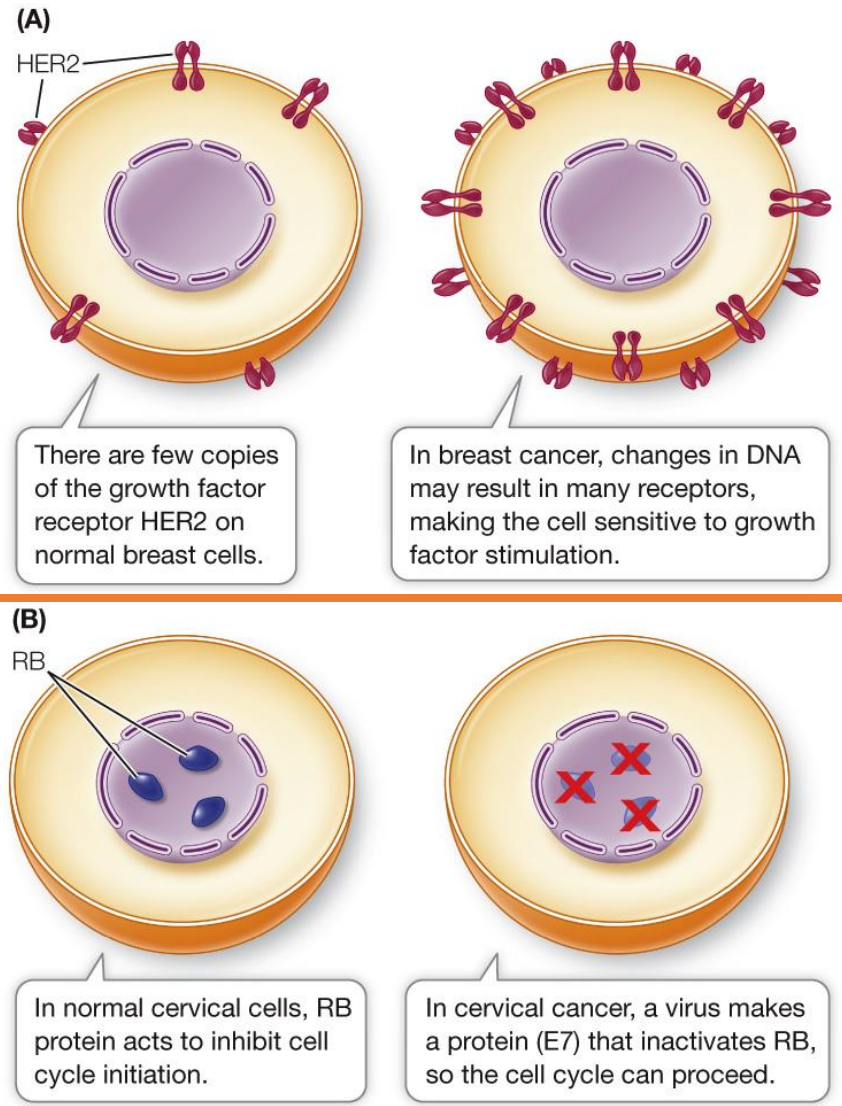



Central dogma of molecular biology

1. Diagnosis e.g. biomarkers
2. Prognosis e.g. clinical outcome, disease monitoring
3. Treatment e.g. targeting functional proteins for therapeutic purposes

# Why Detect and Quantify Proteins?



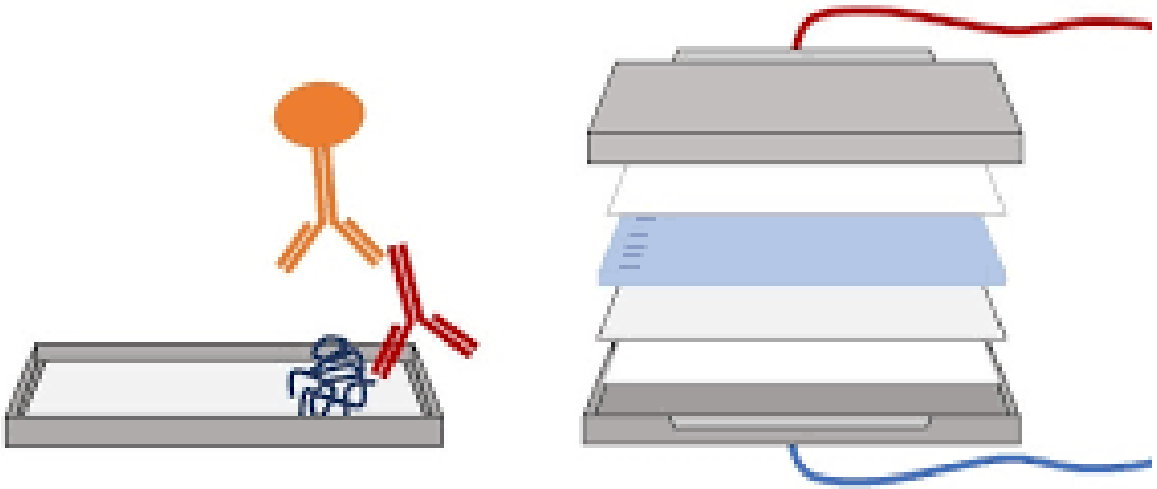

*LIFE: THE SCIENCE OF BIOLOGY 11e*, Figure 11.23


Oncogenic / Tumour Promoting
Increased in cancer vs. normal

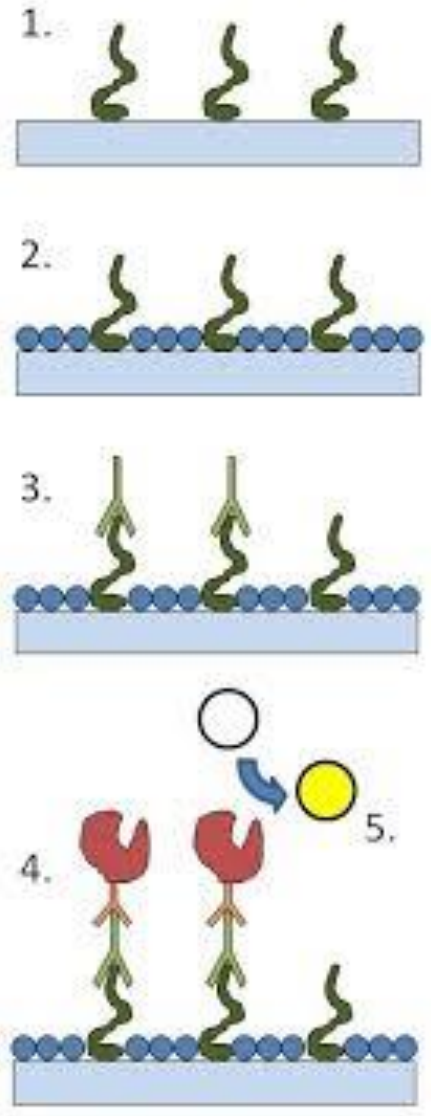
Tumour Suppressive
Decreased in cancer vs. normal

# Methods of Protein Detection

## Western Blotting

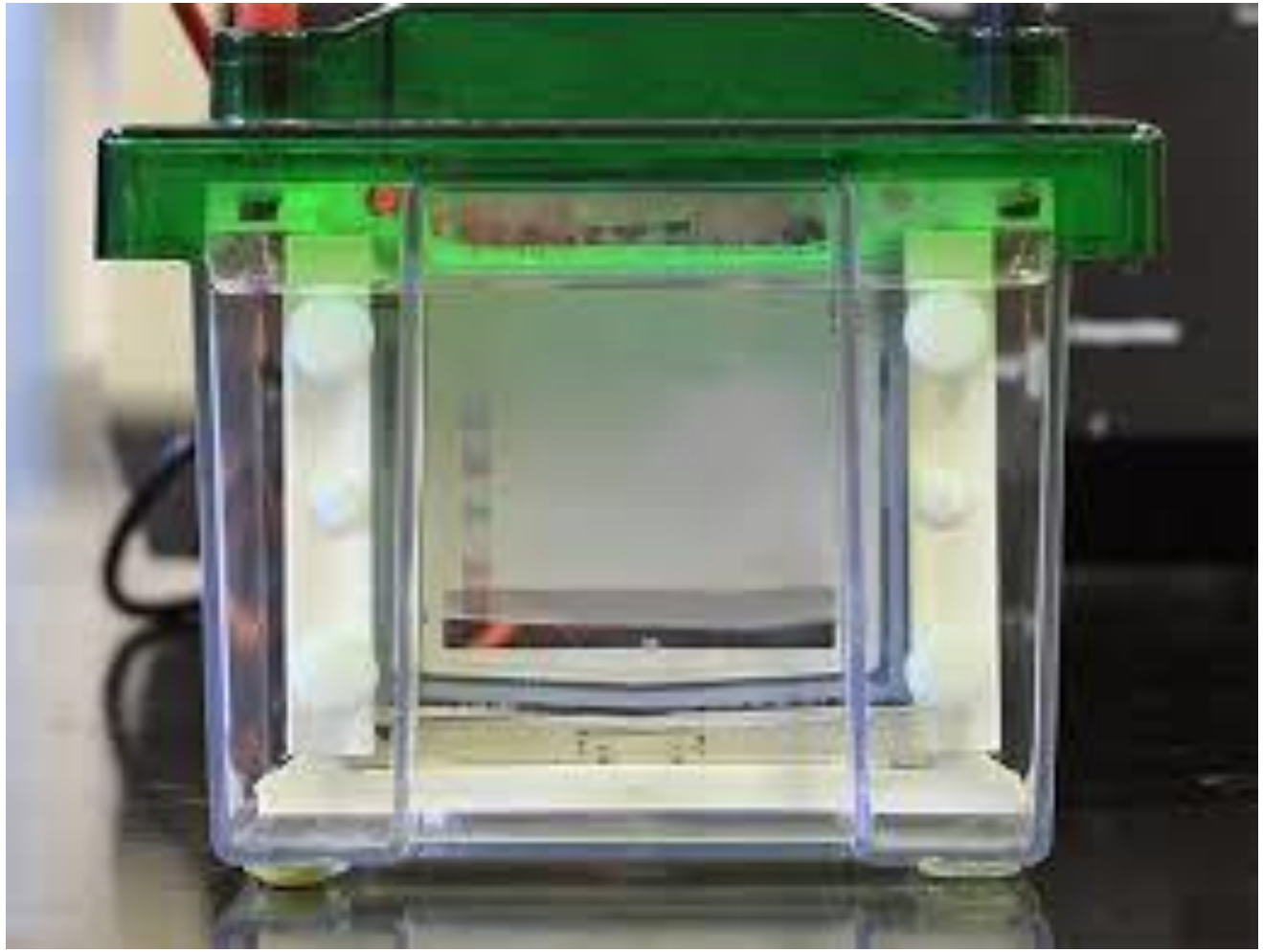


## ELISA



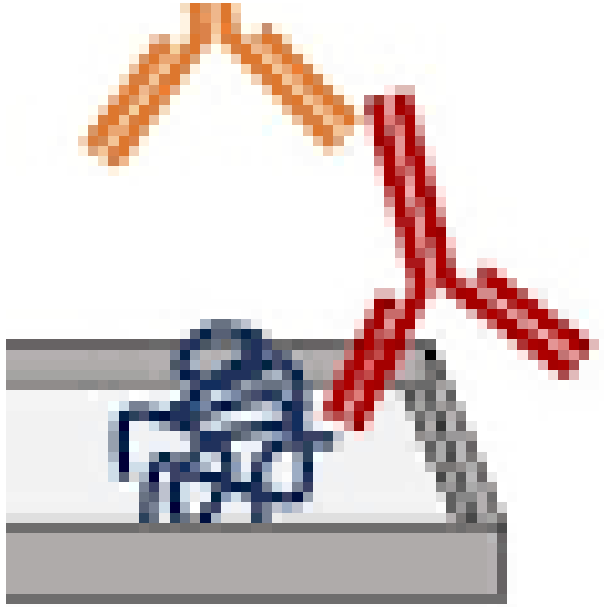
# Western Blotting: The Theory

'....identifies proteins using specific antibodies that have been separated from one another according to their size by electrophoresis'

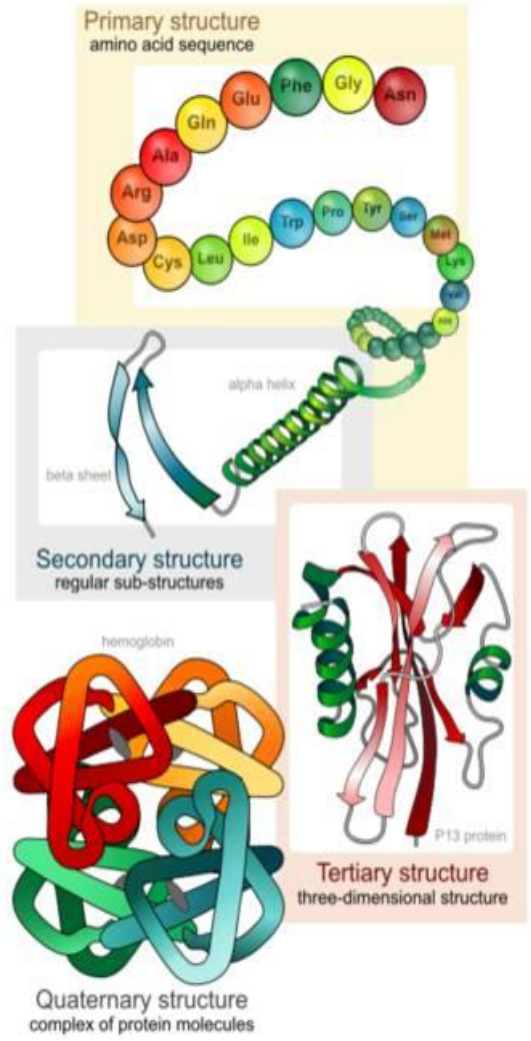


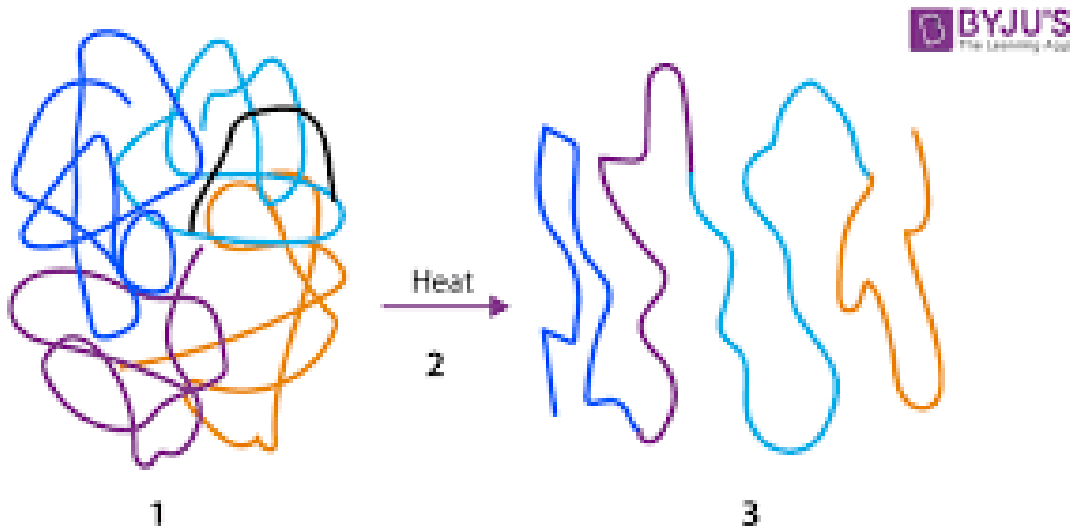
Factors usually affecting electrophoretic mobility:

- Size
- Charge



# Western Blotting: The Theory



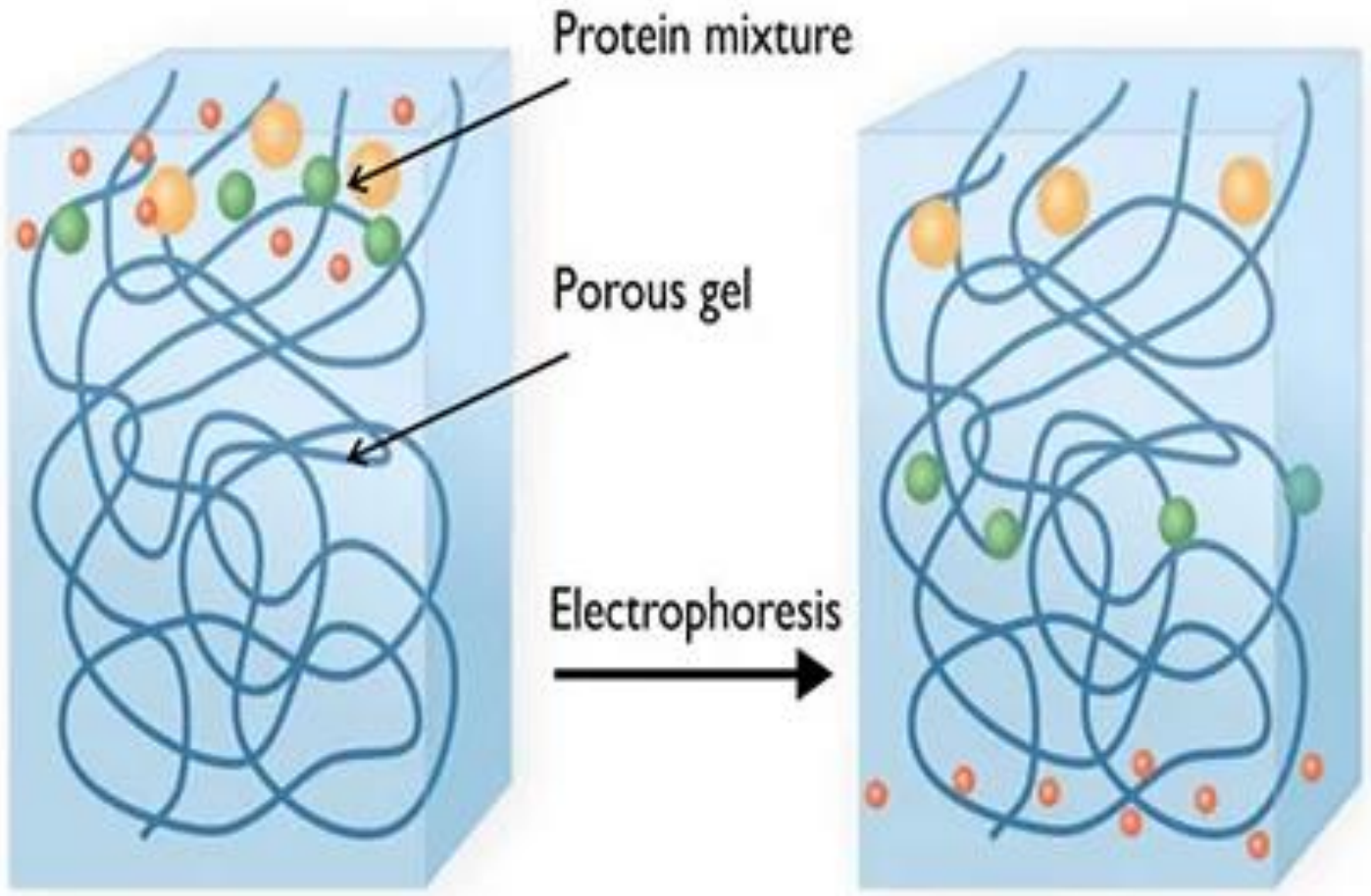




1. Anionic detergent, commonly SDS
    - Disrupts protein structure into a linear polypeptide
    - Imparts uniform negative charge
2. Reducing agent e.g. DTT, β-mercaptoethanol
    - Disrupt disulphide bonds

<u>* Proteins separate solely based on their molecular weight *</u>

# Western Blotting: The Theory

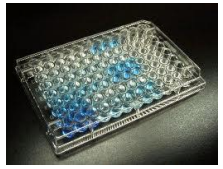
Polyacrylamide Gels



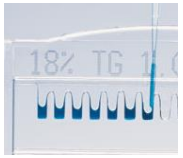
# Running a Western Blot

Sample preparation

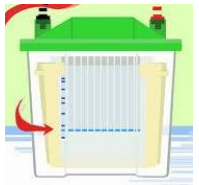
Protein quantification



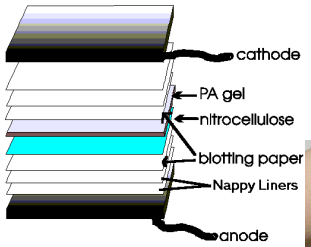
Load a gel




Run the gel




Transfer to a membrane blot




Block membrane


Immunodetection and washes

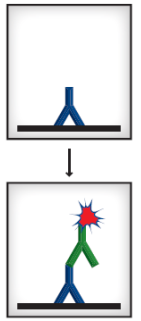
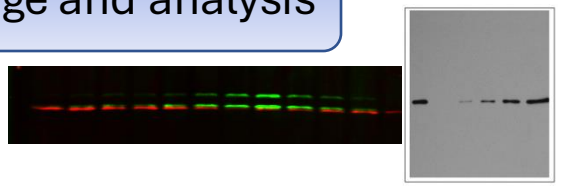
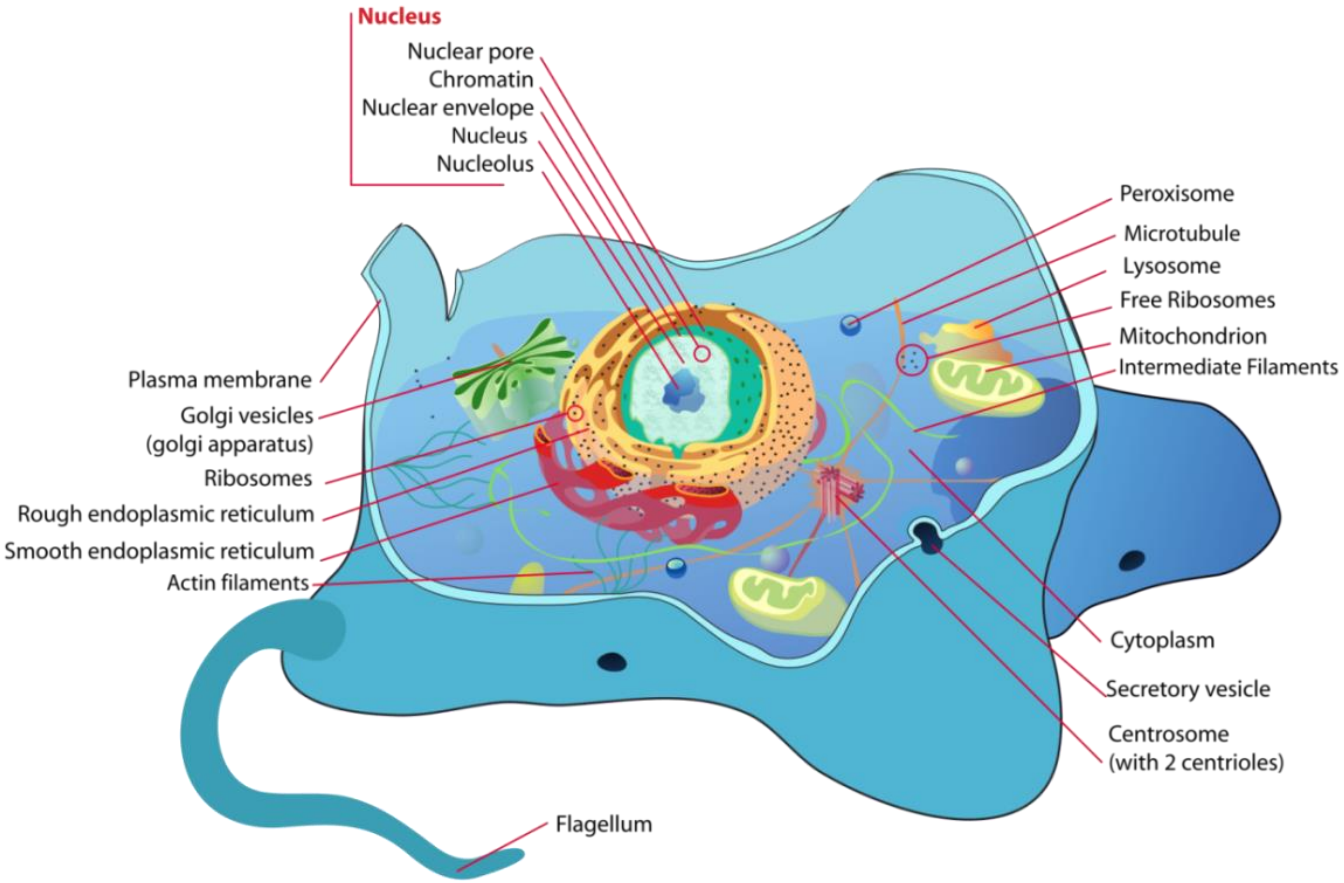


Image and analysis

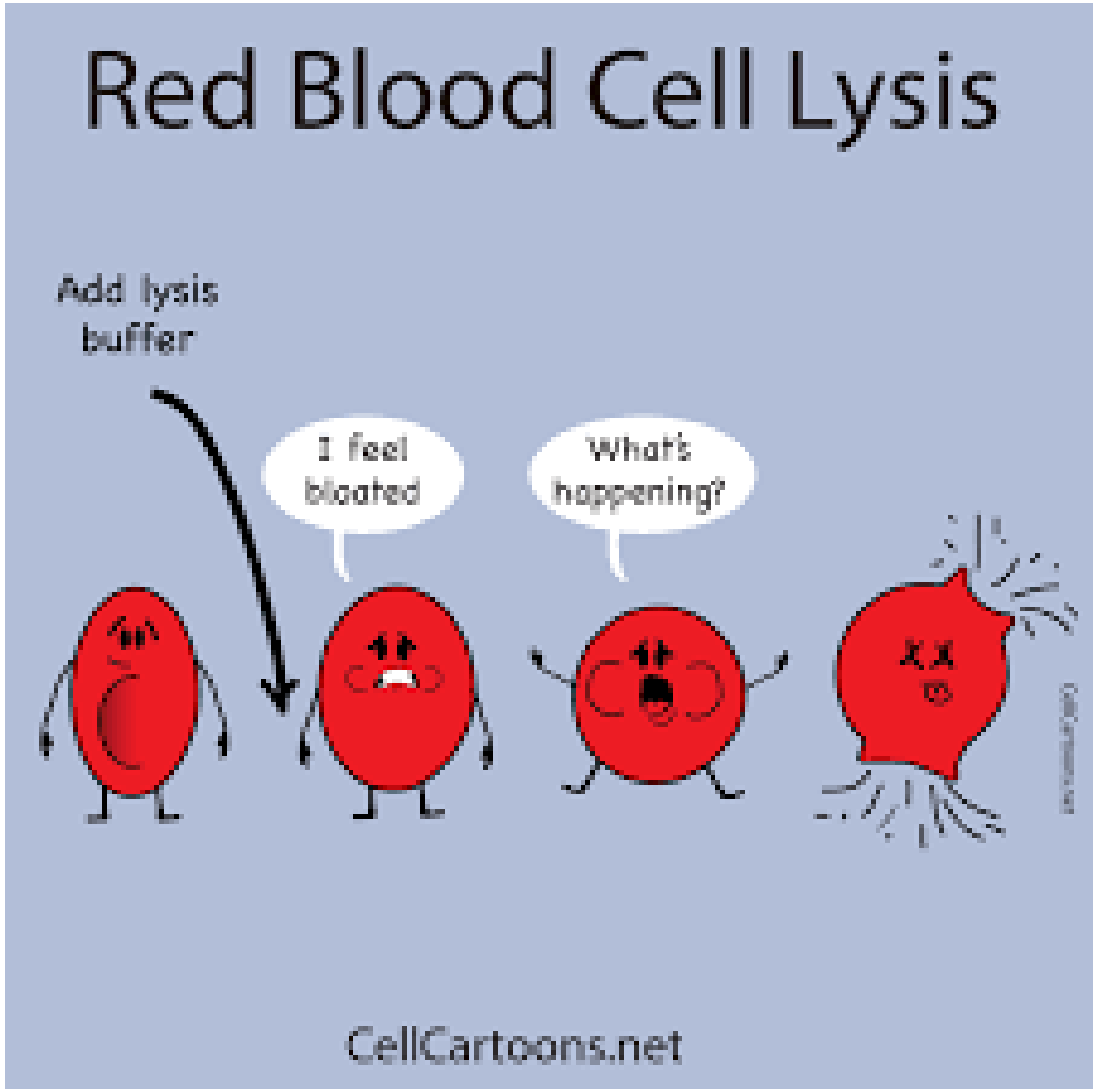


# Properties of a Protein-of-Interest




1. Size
2. Function
3. Cellular Localisation
4. Expression

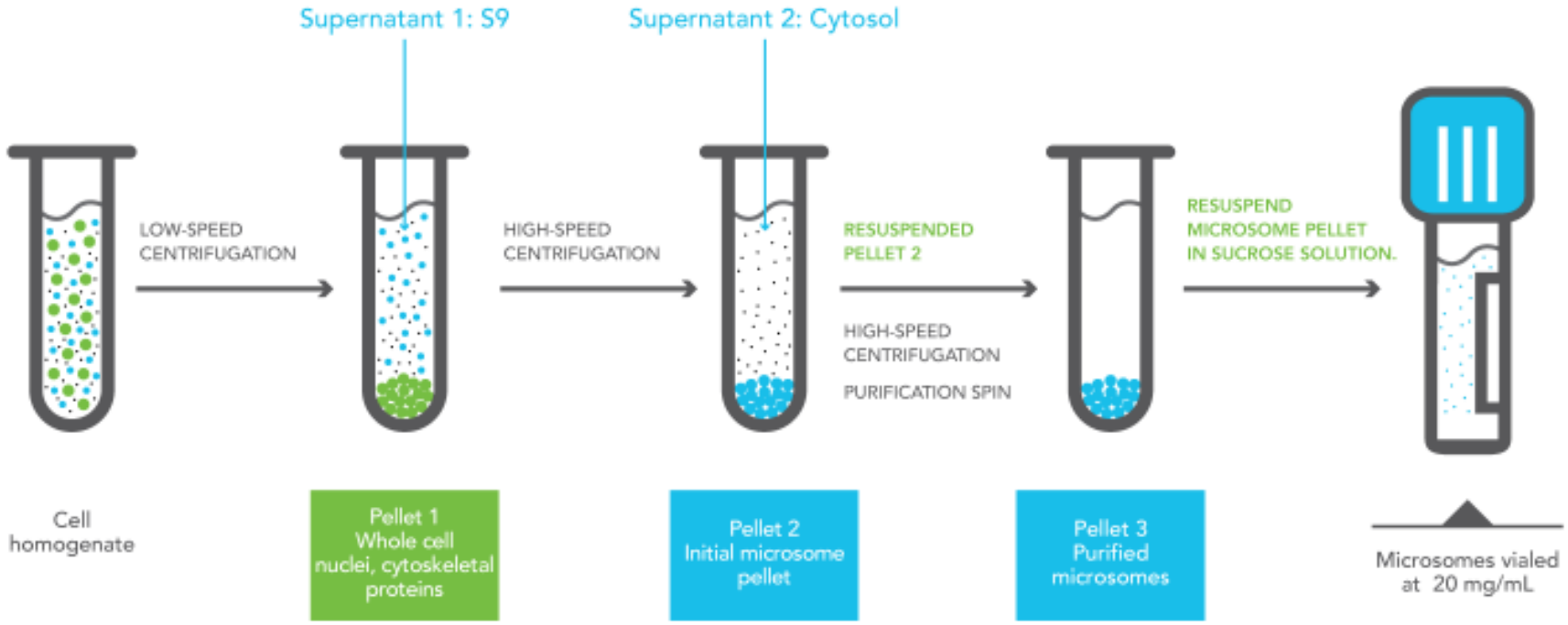
# Step 1: Cell Lysis



# Cell Lysis: Things to Think About

Objective: To break apart a cell and solubilise its constituent parts (including proteins within) to form a **cell lysate**

1. Protein Localisation




2. Required state for desired experiment

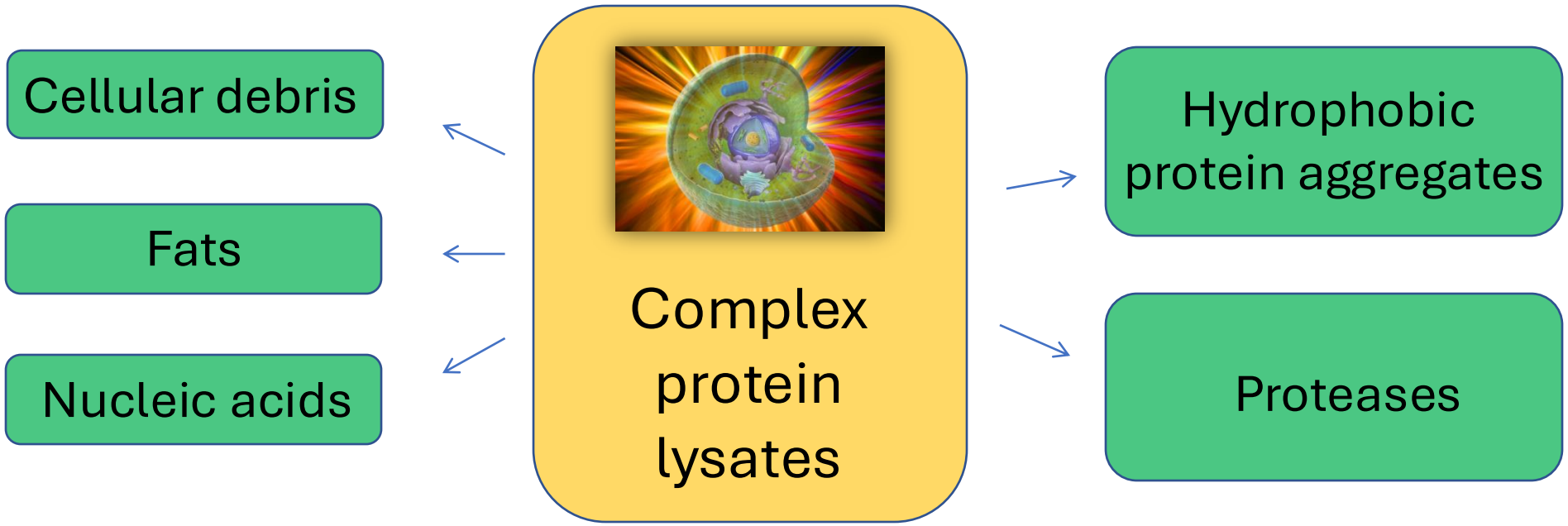
# Cell Lysis: Things to Think About

Inhibitor Cocktails: Slowing down cellular processes e.g dephosphorylation

Sonication: Using sound energy to mechanically shear cells and increase yield

# Cell Lysis: Things to Think About

Contaminants negatively affecting Western results




The more care you take, at every step, the nicer your Western blot will look.

*Masashi Narita, 2015 and 2016 and 2017 and 2018....*

# Typical Sample Preparation from Cultured Cells

~6 x $10^6$ cells

Wash with ice-cold PBS

Scrape cells into ice-cold PBS (~1 ml)

Transfer to pre-cooled eppendorf tube

Spin to pellet cells at 4°C

Remove supernatant and resuspend pellet in ~500µl ice-cold lysis buffer + inhibitors

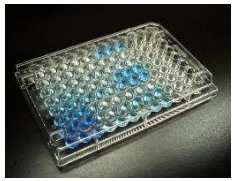
Sonicate sample on ice (3x10s)

Agitate at 4°C for 30 min

Spin max speed at 4°C for 20 mins to pellet cell debris

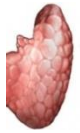
Carefully aspirate supernatant into a pre-cooled clean tube (discard any pellet)

Freeze rest of sample (-20°C or -80°C)



Keep aliquot for protein assay (typical ~5mg/ml)

# Typical Sample Preparation from Frozen Tissue



Frozen Tissue

Add pre-cooled lysis buffer + inhibitors (~300µl / 5 mg)

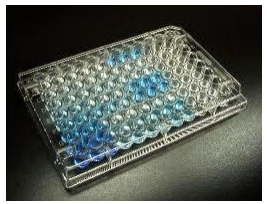
Homogenise (e.g. using Precellys)

Agitate at 4°C for 2 hours

Spin max speed for 20 mins at 4°C to pellet debris

Carefully aspirate supernatant into a pre-cooled clean tube (discard any pellet)

Freeze rest of sample (-20°C or -80°C)

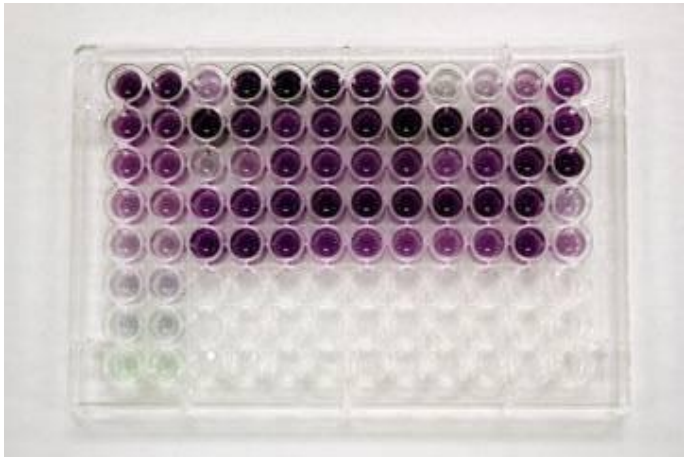


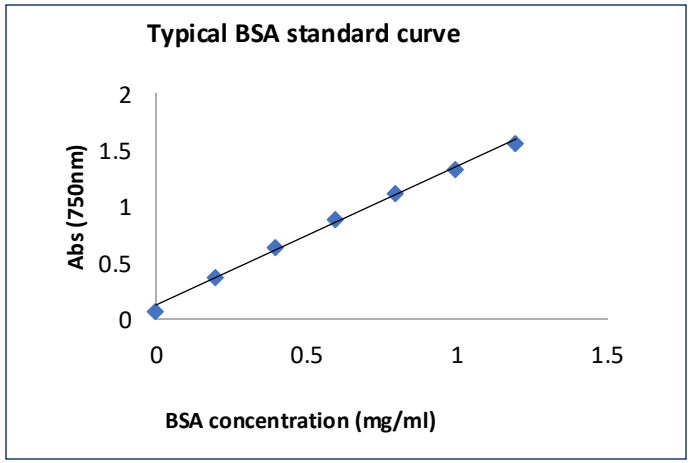
Keep aliquot for protein assay (~150mg wet brain tissue = ~15 mg protein)

# Step 2: Protein Quantification

# Methods for Protein Quantification

## 1. Colorimetric Assays

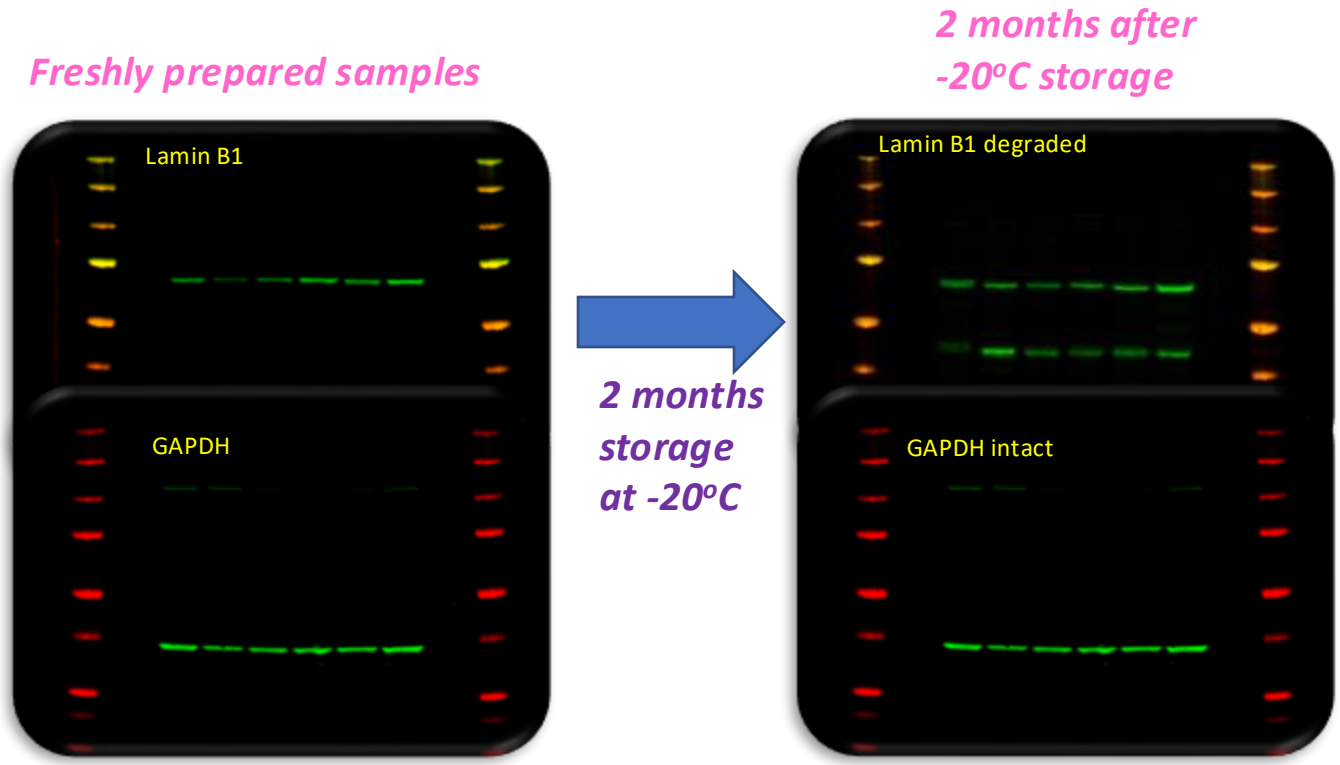




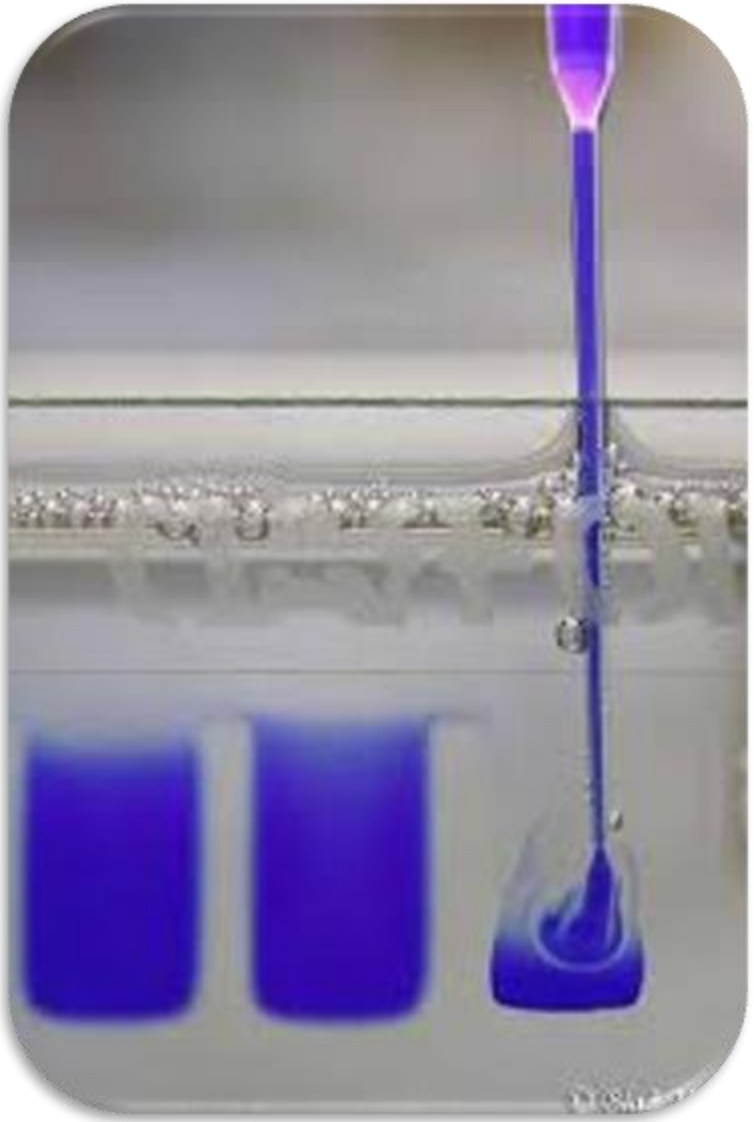

## 2. Fluorescent Qubit

# Protein Quantification: Things to Think About

Protein degradation with long-term storage



# Step 3: Loading a Gel



# Preparing Samples for Loading

1. Add loading buffer to samples

| 2 x Laemmli buffer component | Function |
|---|---|
| 4% SDS | Denaturing agent (disrupts 3D structure) |
| 10% Beta-mercaptoethanol or dithiothreitol (100mM) | Reducing agent (breaks disulphide bonds) |
| 20% glycerol | Increases the density of the sample to maintain the sample at the bottom of the well |
| 0.0004% bromophenol blue | To visualise protein migration (dye is anionic and small so it migrates the fastest to provide a ‘dye front’ |
| 0.125M Tris-HCl | To provide a pH buffer |

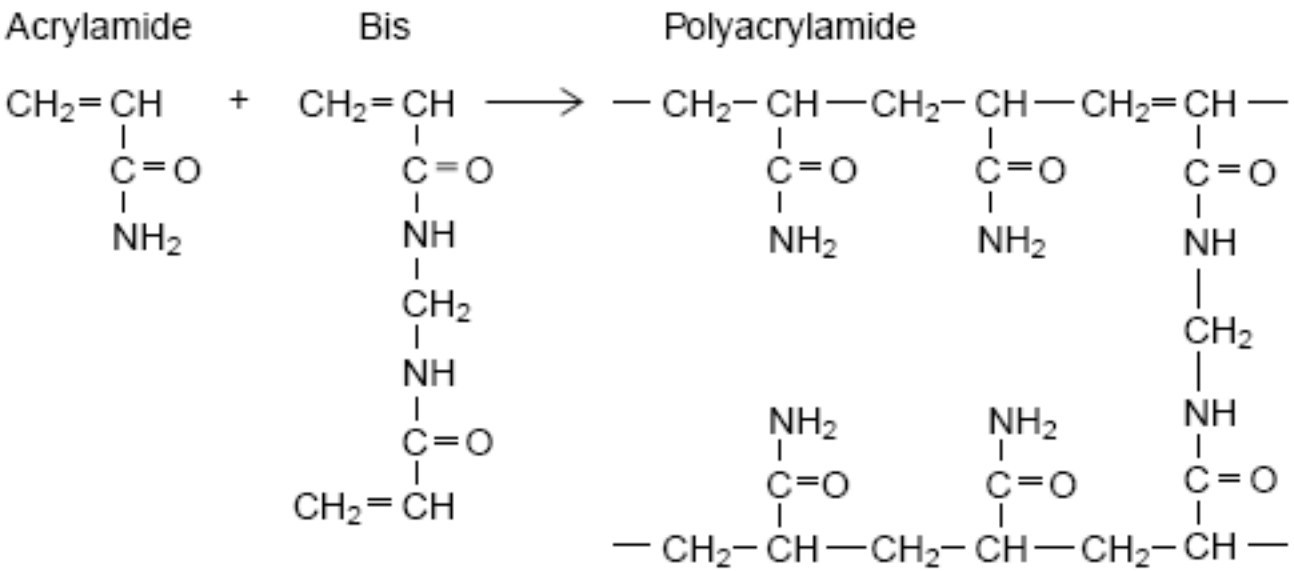
# Preparing Samples for Loading

2. Heating


3. Vortex

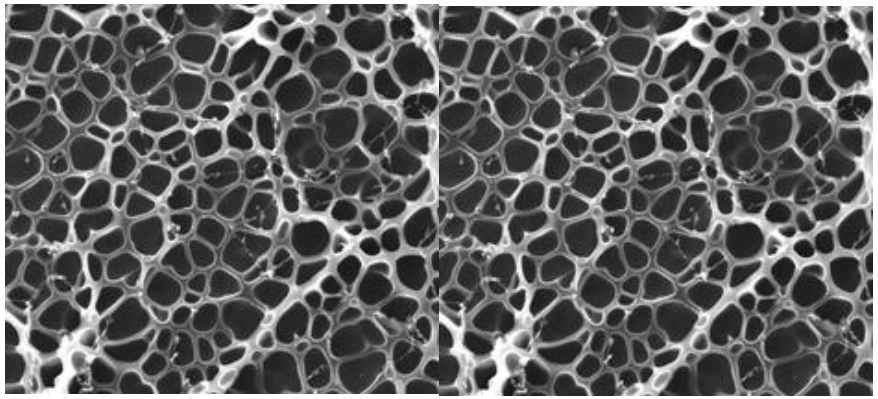
# Preparing Polyacrylamide Gel

Cross-linker




+ Ammonium Persulphate (APS, initiator)

+ TEMED (catalyst)



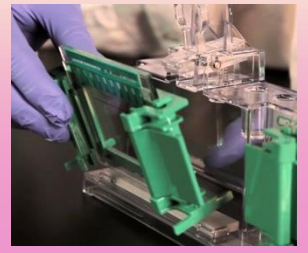
Pore size determined by

1. Total amount of acylamide present (%T)
2. Total amount of cross-linker (%C)

# Gel Options

| Gel types | When to use them |
|---|---|
| Tris-Glycine | Separation of medium to high MW proteins |
| Tris-Hepes | Separation of medium to high MW proteins |
| Bis-Tris | Separation of small to medium proteins (1-200KDa) |
| Tris-Tricine | Separation of small proteins (<20KDa) |
| Tris-Acetate gels | Separation of large proteins (up to 400KDa) |
| Native gels | Separation of proteins in their native state |

*All require different running buffers*



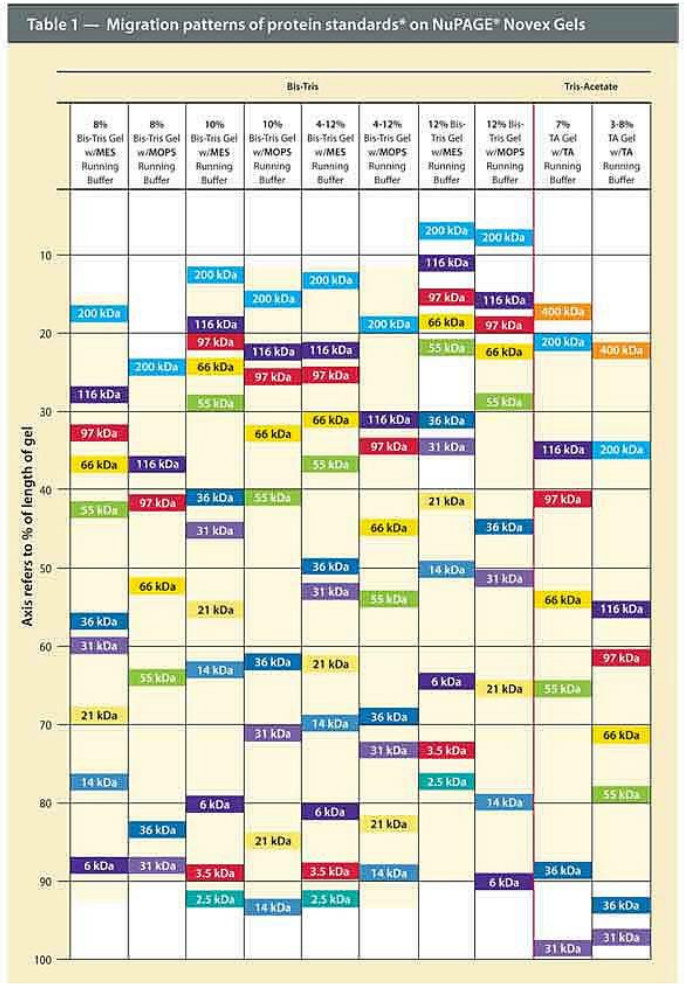
Manually poured

Pre-cast

Sizes (mini, midi, large-format)

- Single percentage (e.g. 10%)
- Gradient gels (e.g. 4-12%)

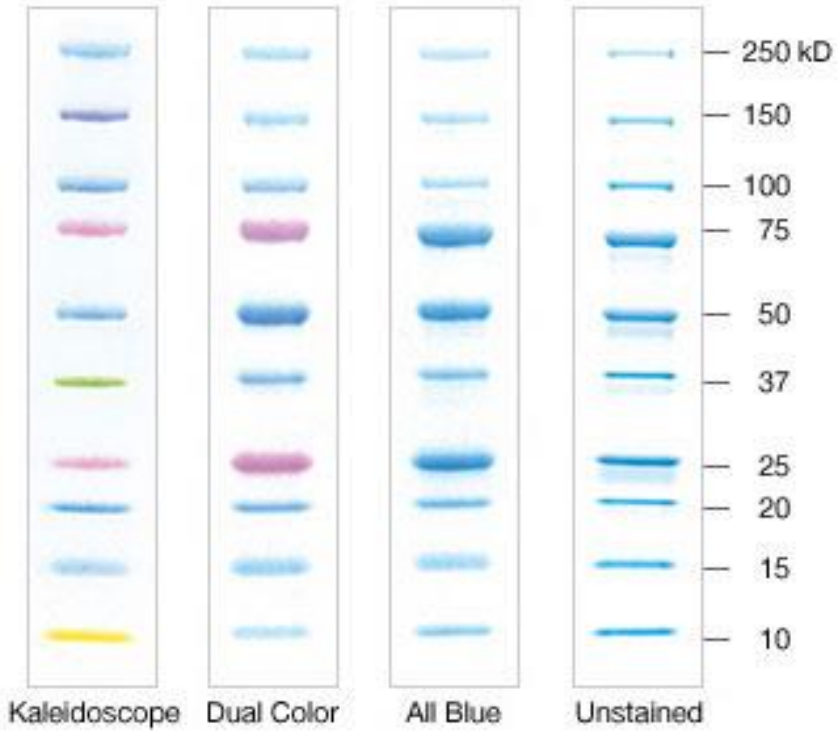
Typical migration patterns




*The smaller the size of protein, the higher the percentage of acrylamide you will need to slow it for sufficient resolution*

# Standards for Comparison

## 1. Molecular weight marker

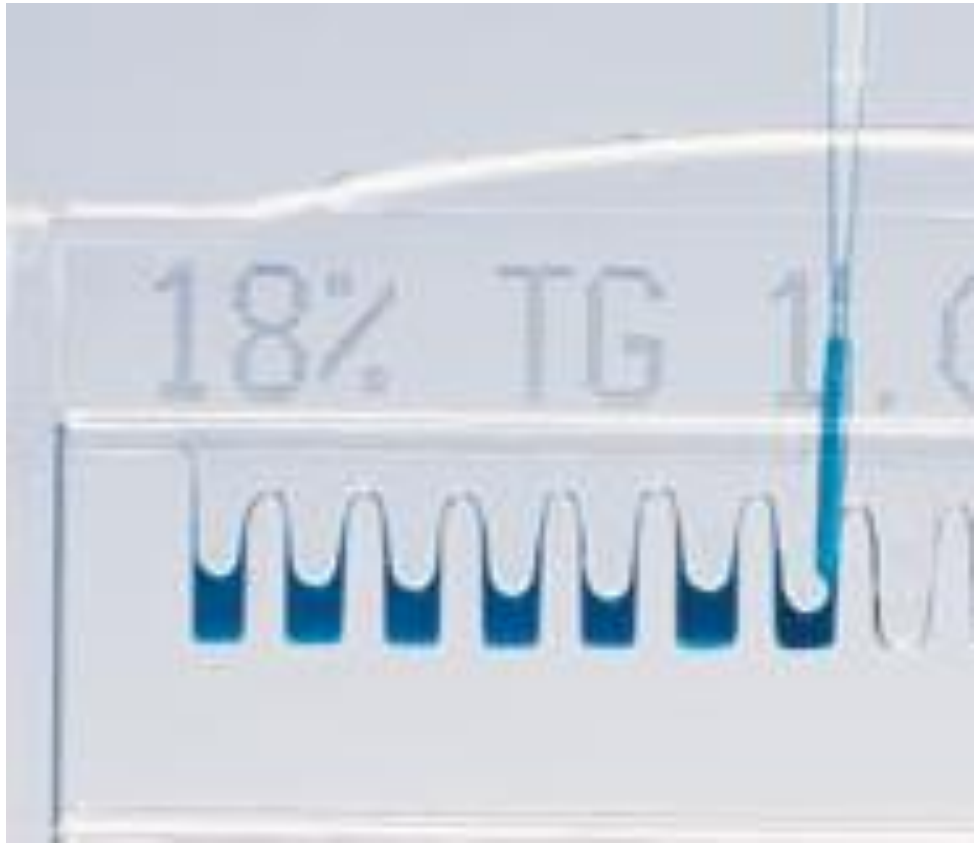



## 2. Loading controls

| Loading Control | Sample type | Molecular weight (KDa) |
|---|---|---|
| Beta actin | Whole cell/cytoplasmic | 43 |
| GAPDH | Whole cell/cytoplasmic | 35 |
| Tubulin | Whole cell/cytoplasmic | 55 |
| VDAC | Whole cell/Mitochondrial | 31 |
| COXIV | Whole cell/Mitochondrial | 16 |
| Lamin B1 | Nuclear | 38 |

# Actually Loading the Gel

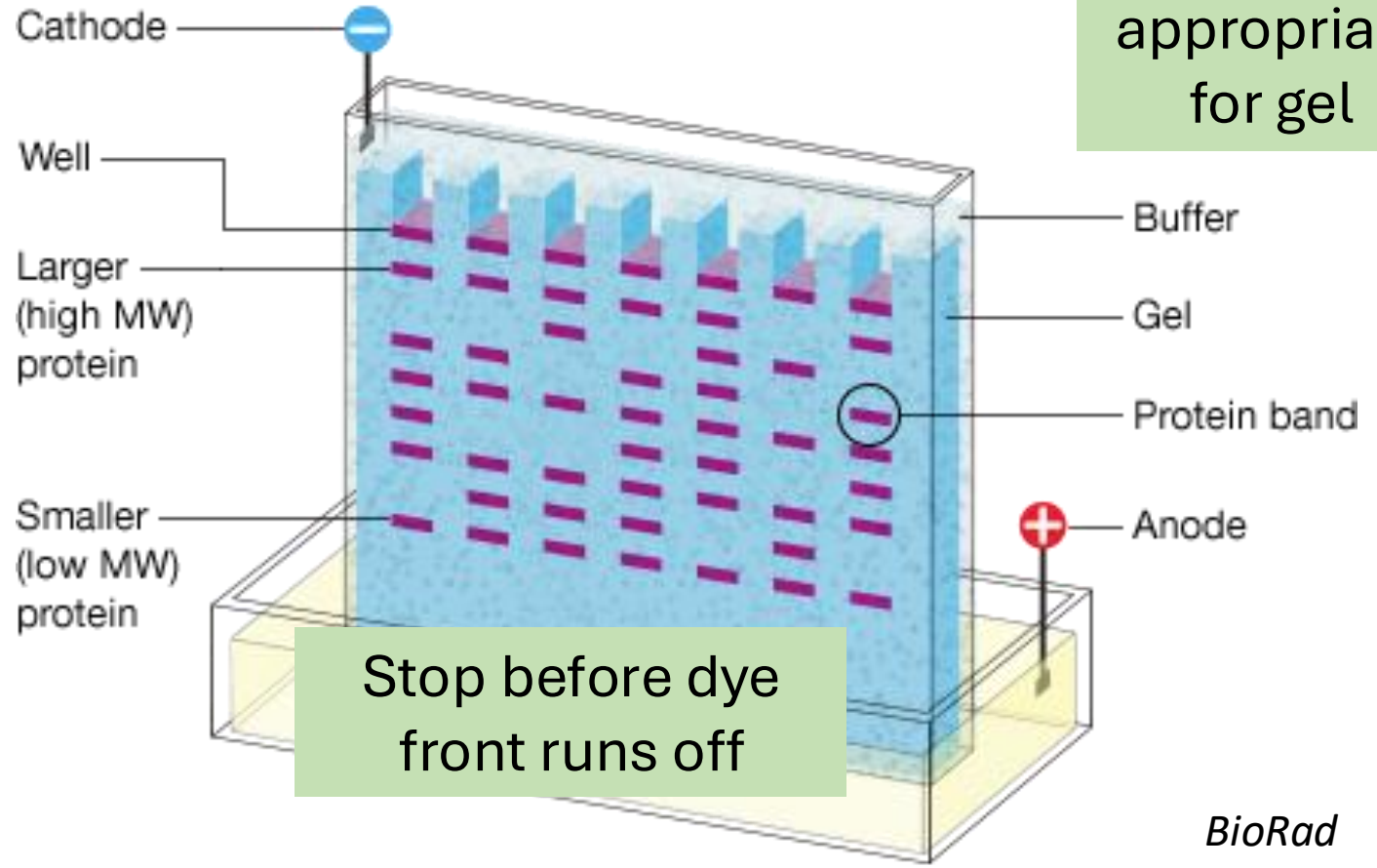
| Well Types | Maximum Load Volume |
|---|---|
| 1 well 1.0 mm | 700 µL |
| 2D well 1.0 mm<br>1.5 mm | 400 µL<br>600 µL |
| IPG well 1.0 mm | 7 cm IPG Strip |
| 9 well 1.0 mm | 28 µL |
| 10 well 1.0 mm<br>1.5 mm | 25 µL<br>37 µL |
| 12 well 1.0 mm | 20 µL |
| 15 well 1.0 mm<br>1.5 mm | 15 µL<br>25 µL |
| 17 well 1.0 mm | 15 µL |



# Running the Gel

Appropriate voltage (generally voltage limit)

Buffer appropriate for gel



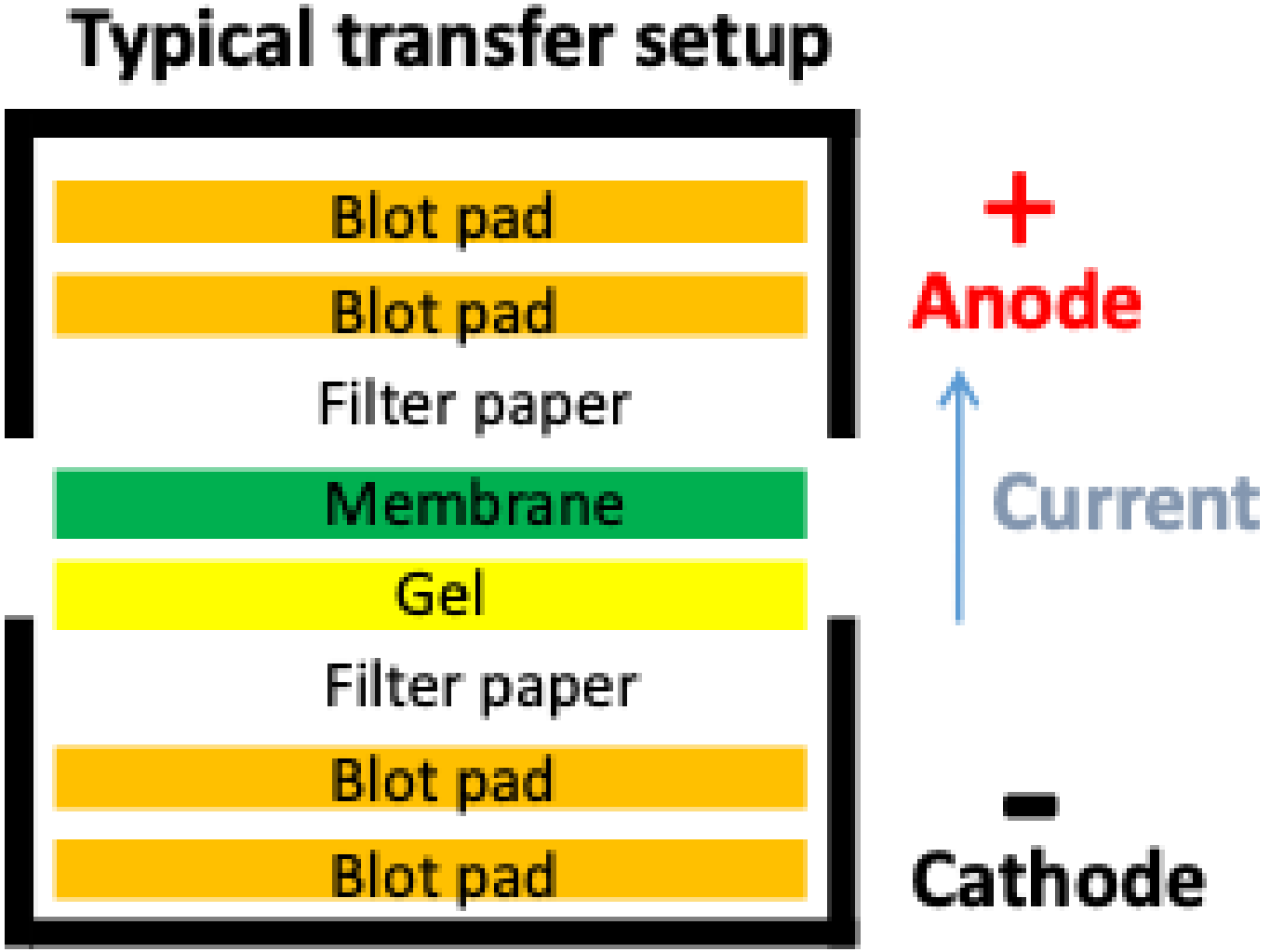

Stop before dye front runs off

*BioRad*

Transfer immediately to avoid diffusion!

# Step 4: Transferring

# Setting up the ”Transfer Sandwich”

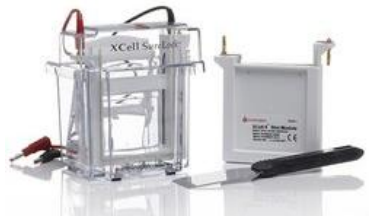



Remember membranes bind proteins!

# Options for Transferring

### *Full Wet*

- Method of choice for transferring large proteins (>150kDa)
- Transfer in approx. 1.5L of cooled transfer buffer either for 1.5 hours (with ice pack) or overnight in a cold room.



### *Semi-Wet*

- Uses less buffer than full wet and is good for transfer of proteins of all molecular weights (except very heavy)
- Transfer in approx. 200ml of cooled transfer buffer for 1.25 hours.

### *Semi-Dry*

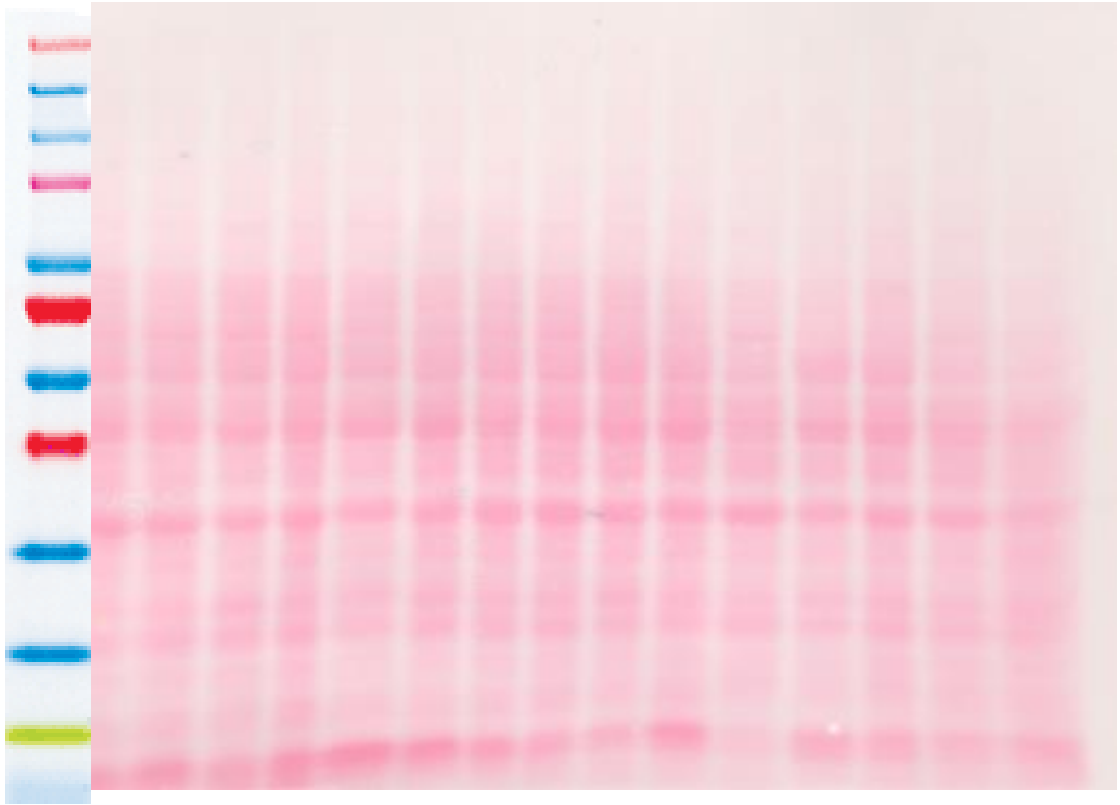
- Faster transfer (~15-60mins) but not as efficient and cannot transfer large proteins (>150kDa).
- Low buffering capacity means its no good for prolonged transfers.
- Prone to current leakage.

### *Dry*

- Very quick (~7mins) and efficient transfer of proteins under 150kDa, but loses efficiency for proteins larger than this.
- Produces well resolved bands.
- No need for transfer buffer.

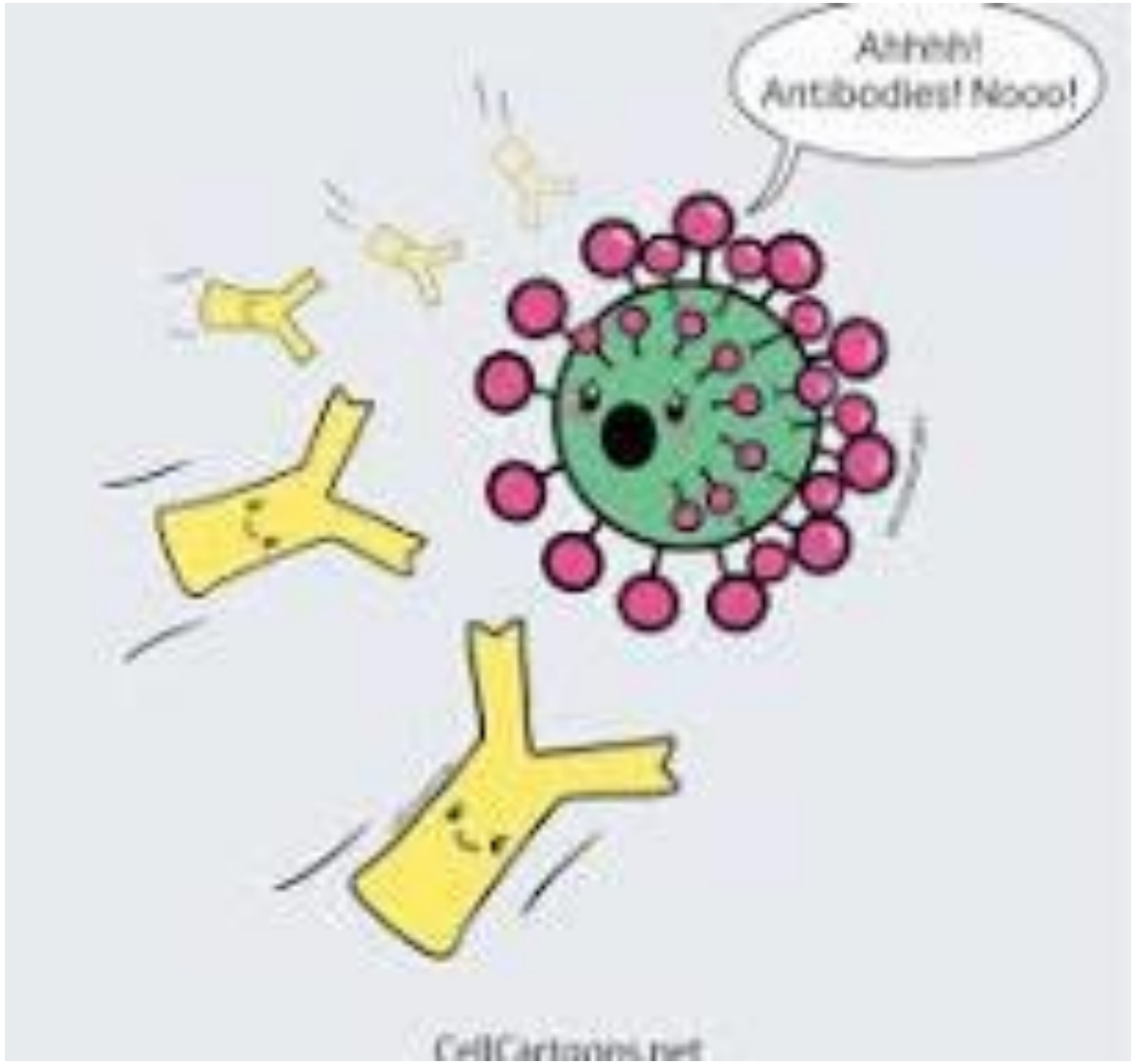
# Staining Proteins on Membrane

Ponseau staining of membrane



Membrane can be PVDF or nitrocellulose – personal choice, both work, various options available for both e.g. pore size

# Step 5: Immunodetection



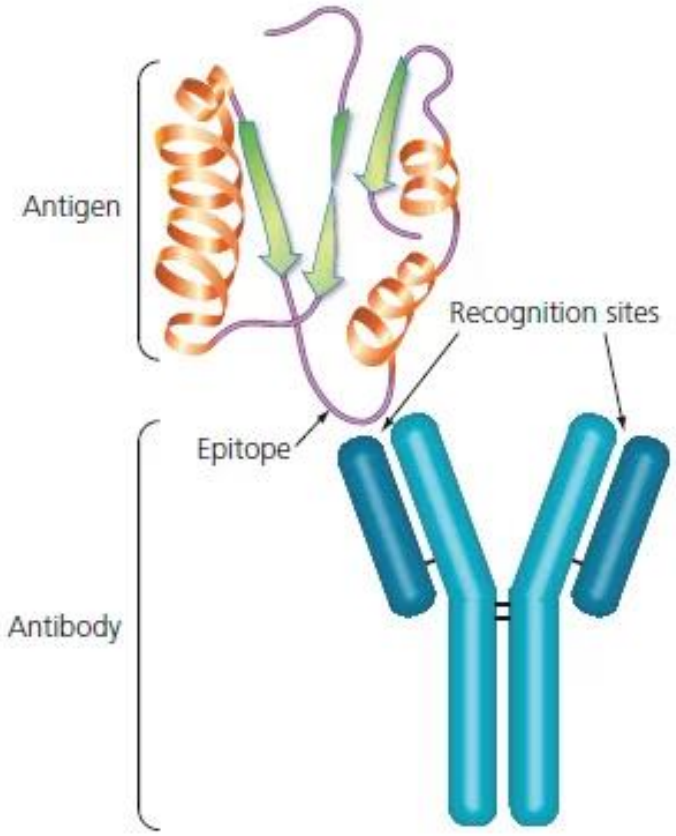
# Blocking the Membrane

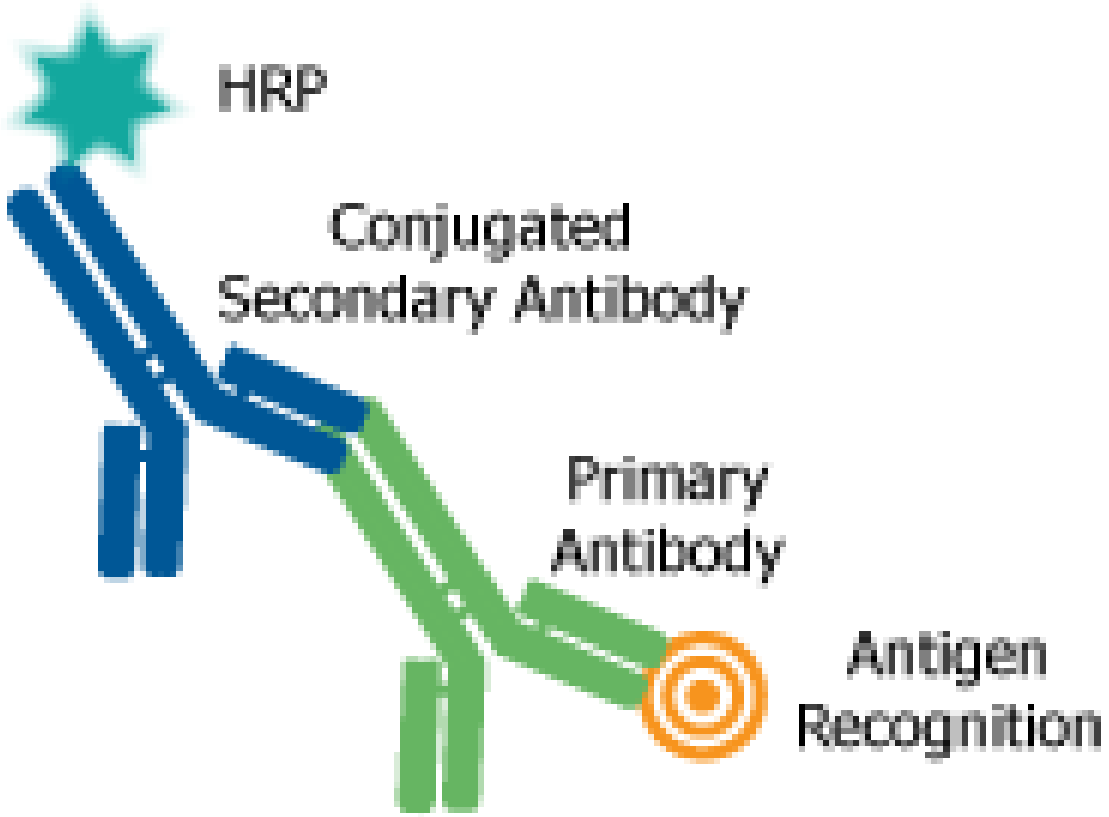

Typical block buffers:

- 5% non-fat milk
- 1% Casein
- 2-3% BSA
- Non-mammalian block buffer

Made up in the same buffer as antibody

# Probing with Antibody



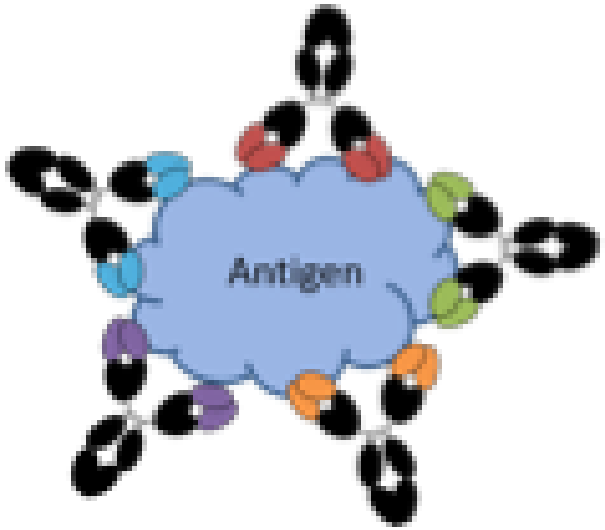




Typically use a primary-secondary combination:

- Primary for antigen detection
- Secondary for visualization e.g. HRP

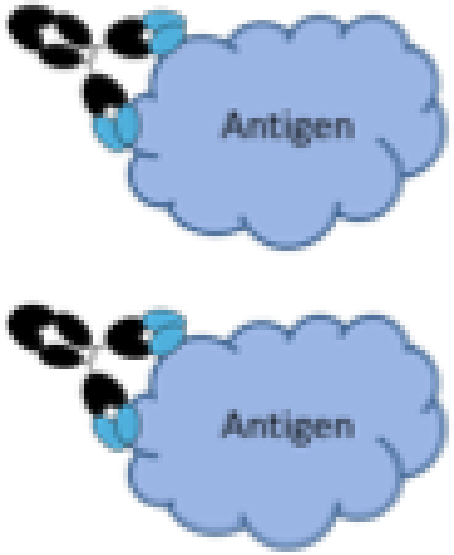
# Antibodies: Polyclonal vs. Monoclonal

## Polyclonal




- Several different antibodies recognizing different epitopes
- Usually more sensitive

## Monoclonal




- A single clone of an antibody
- Recognises only one specific epitope on the protein-of-interest
- Usually more specific

# Grand Master Tips

- Your Western will only be as good as your primary antibodies!
- Check for pictures, references, recommendations etc.
- Try to use antibodies that have been tried and tested for Western blotting (some antibodies only work in IHC where epitope is in its native form)
- Typically dilute antibodies in block buffer + 0.1% Tween for incubation overnight at 4$^{o}$C or 1 hr at room temperature
- Use a rocker to ensure homogenous covering and even binding over the membrane.
- Time required will be dependent on the binding affinity of the antibody for the protein and the abundance of the protein
- If possible use a lower concentration of antibody for longer periods to improve specificity.
- Important to wash after antibody incubations (PBS + 0.1% Tween or TBS + 0.1% Tween), 4 x 5min

# Grand Master Tips

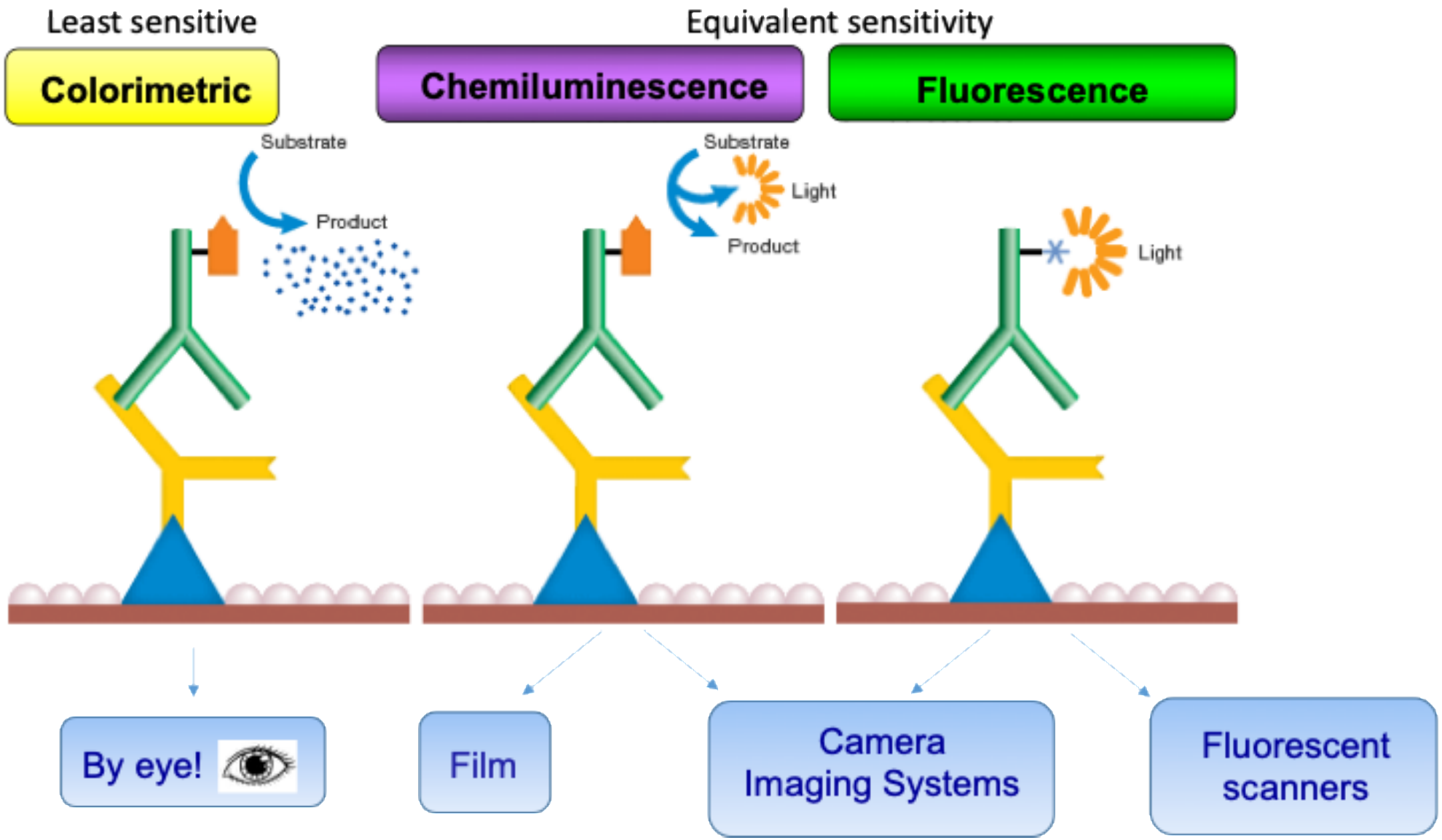
**Concentration of primary to use**

- Typically around 1:1000 (Can vary from 1:100 – 1:100,000!)
- Use application sheets for a recommended dilution to start with, but empirically determining concentration may be necessary to optimise a blot.
- Ideally do a dilution series to find optimal dilution
- Too little antibody will lead to a lack of signal.
- Too much antibody will lead to the appearance of non-specific bands

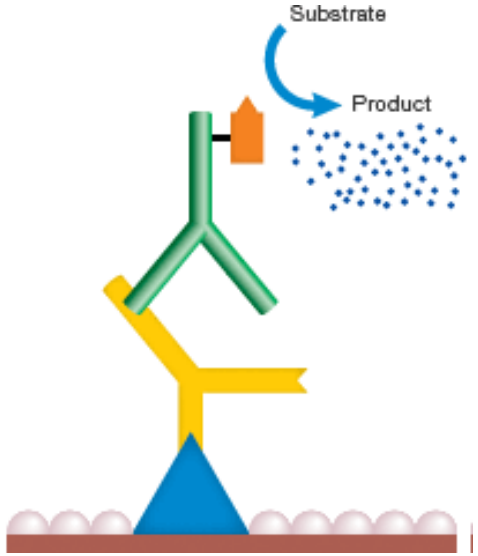
For multiplexing, use primary antibodies derived from different species

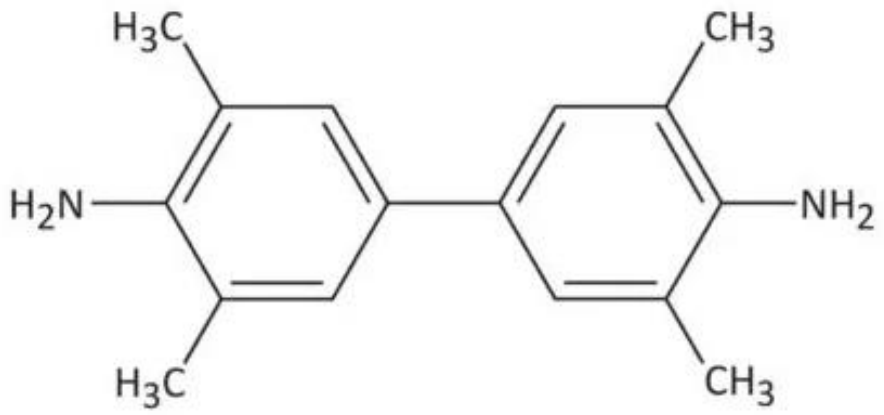
# Step 6: Imaging

# Options for Imaging

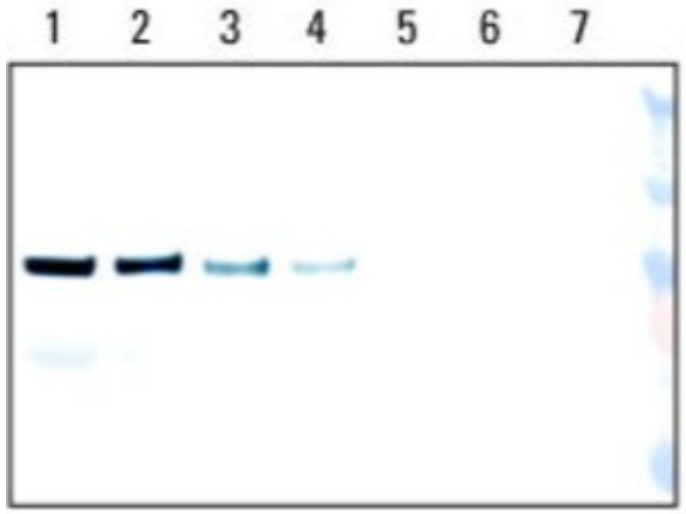


# Colometric Imaging




3,3',5,5'- Tetramethylbenzidine (TMB)



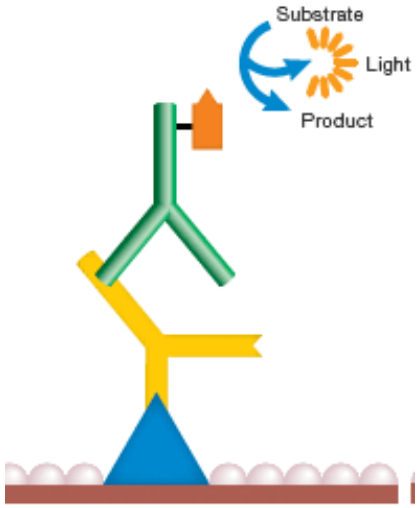

Advantages:

- Cheap, quick and easy to use
- No specialist equipment required

Disadvantages:

- Low sensitivity
- Requires high expression
- Cannot multiplex

# Chemiluminescence-Based Imaging




*Pierce Substrates*

- ***ECL System***
- Supersignal ***West Pico***
- Supersignal ***West Dura*** (Extended duration)
- Supersignal ***West Femto***

*Increasing Sensitivity*

Advantages:

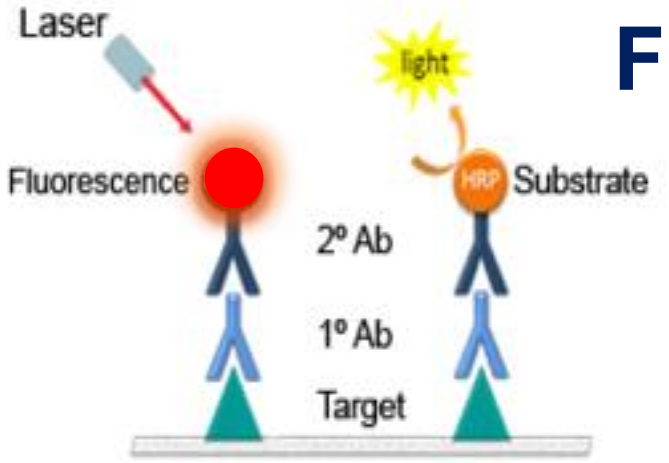
- High sensitivity (and many substrate options to adjust)
- Routinely used
- Widely accepted

or

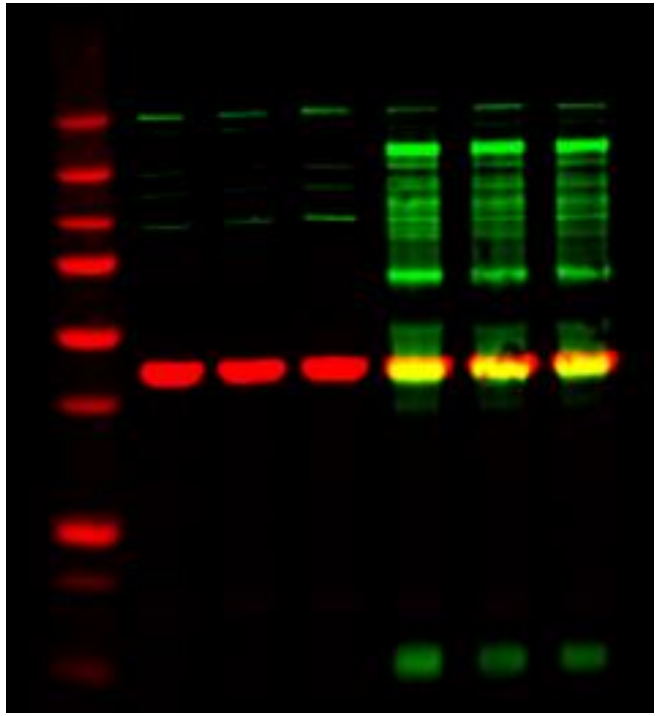
Disadvantages:

- Not fully linear, signal saturates
- Signal not infinitely stable
- Cannot multiplex

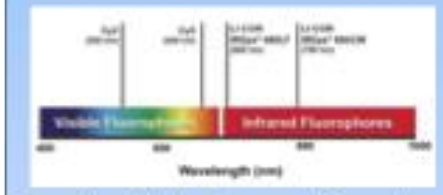
# Fluorescence-Based Imaging



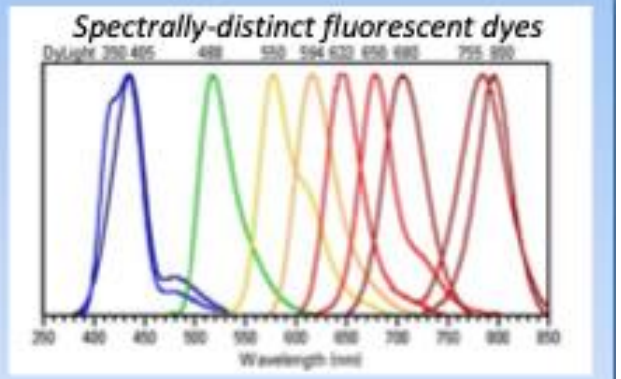




## Spectrally distinct fluorescent dyes for WB

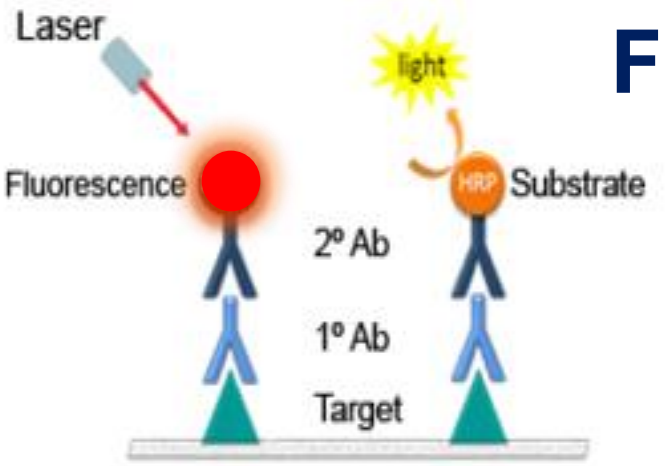


*Span the visible to near infrared range*



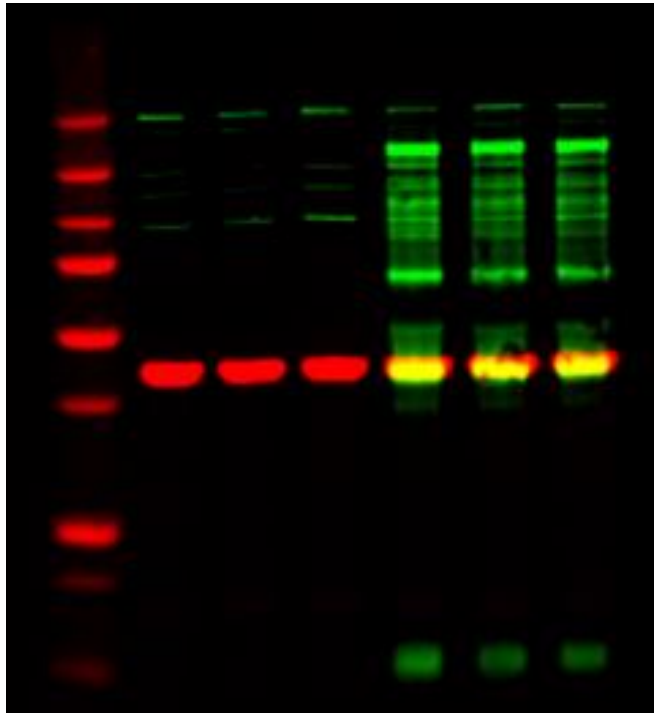

- Li-Cor Infra-red dyes
- DyLight dyes
- AlexaFluors
- ECL Plex antibodies (Cy3/Cy5)
- Qdots

# Fluorescence-Based Imaging




Advantages:

- Wide linear dynamic range, good for quantification
- The most sensitive method
- Signal infinitely stable (though light sensitive)
- Multiplexing capabilities



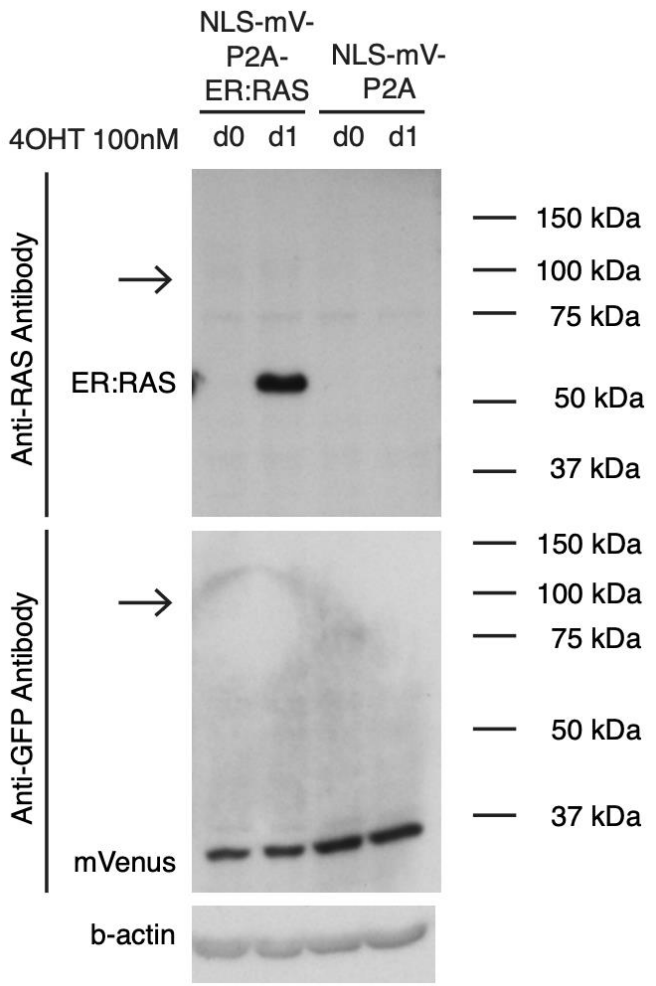
Disadvantages:

- Requires the most specialized equipment and reagents

# Step 7: Quantification & Analysis

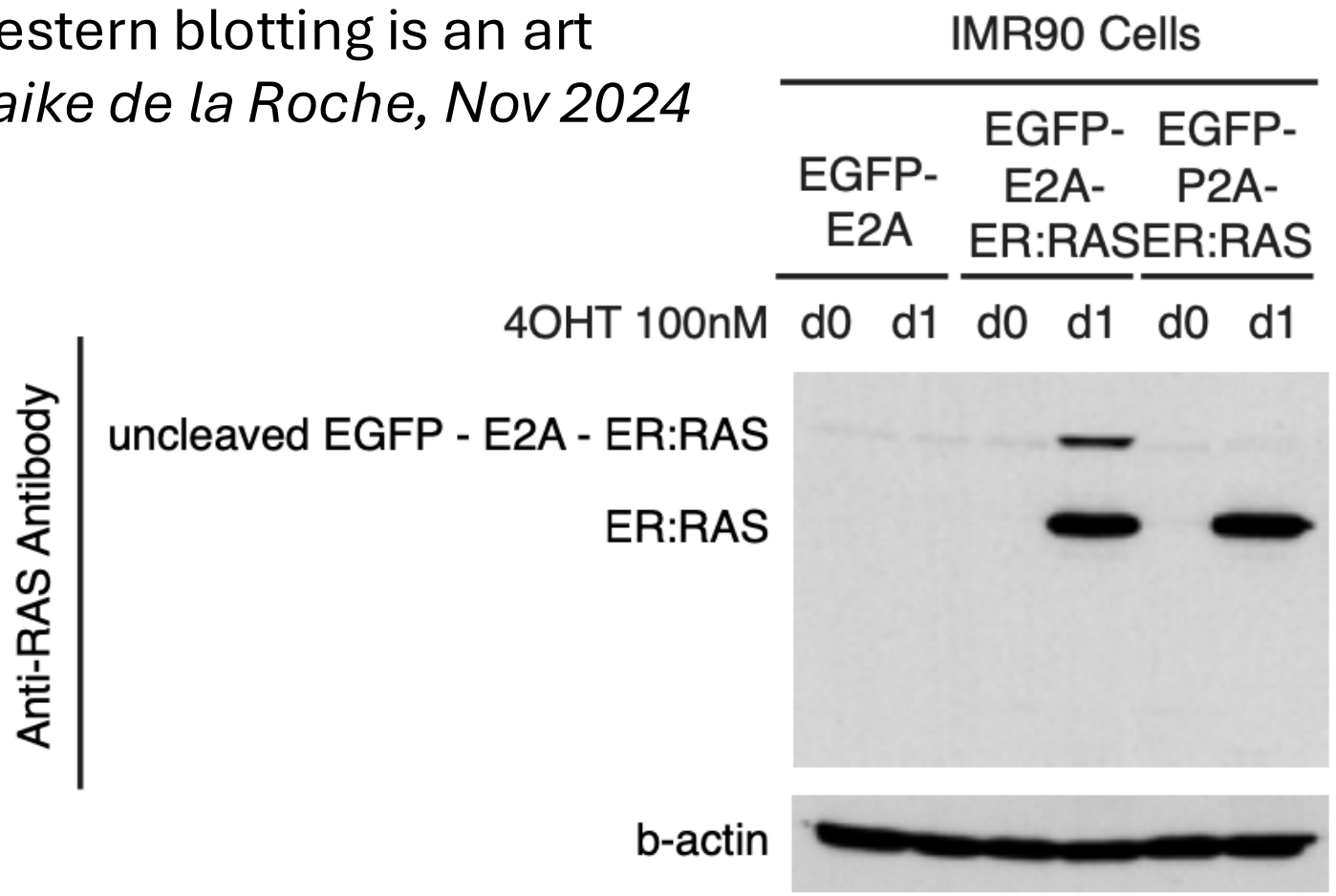
# Quantifying Western Blots

Western blotting is inherently a qualitative technique!



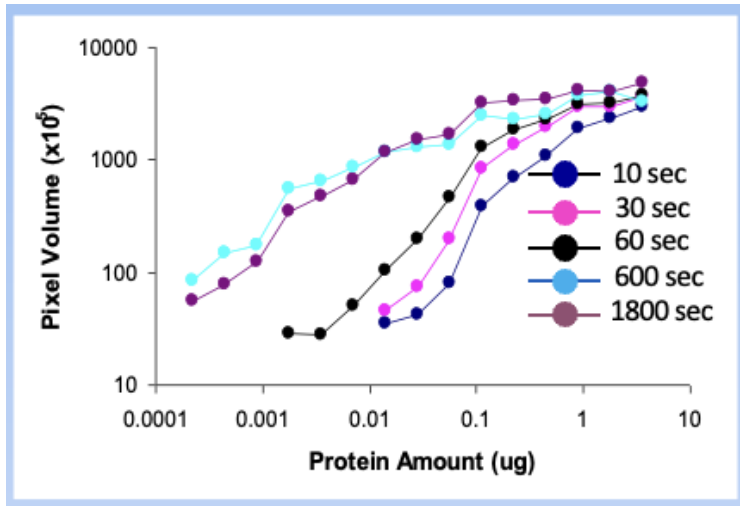

Western blotting is an art

*Maike de la Roche, Nov 2024*

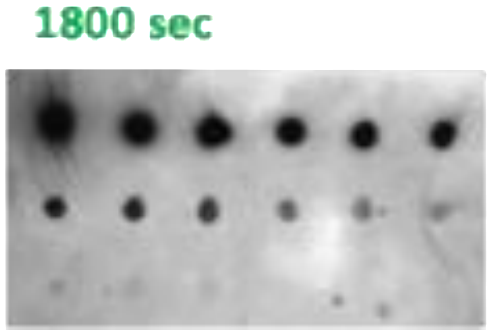


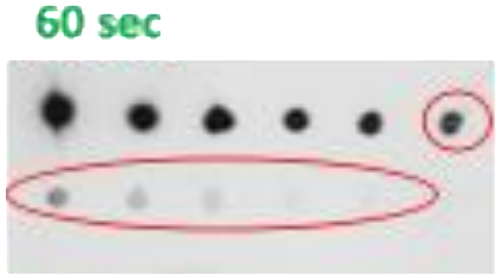
# The Importance of Linearity

## Chemiluminescence (with film)

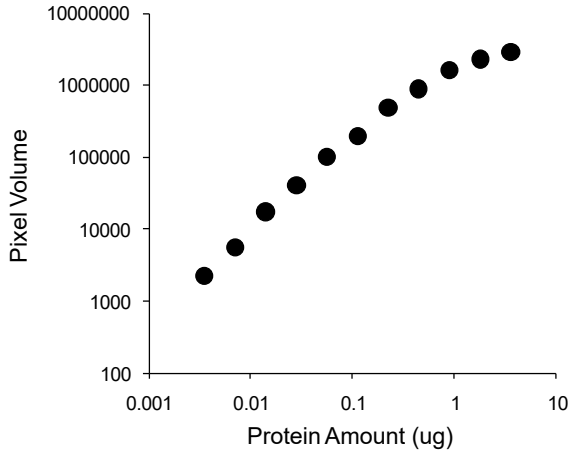


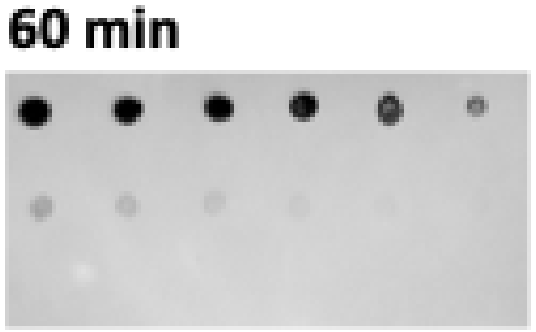




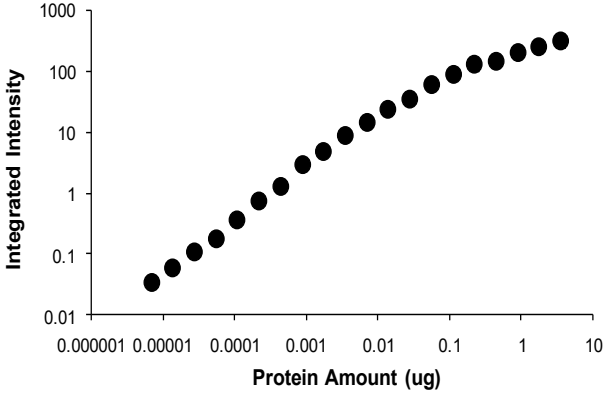



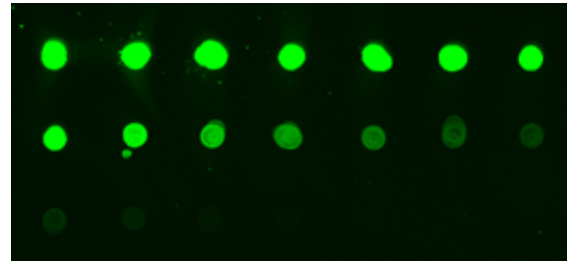

## ECL (digital camera)



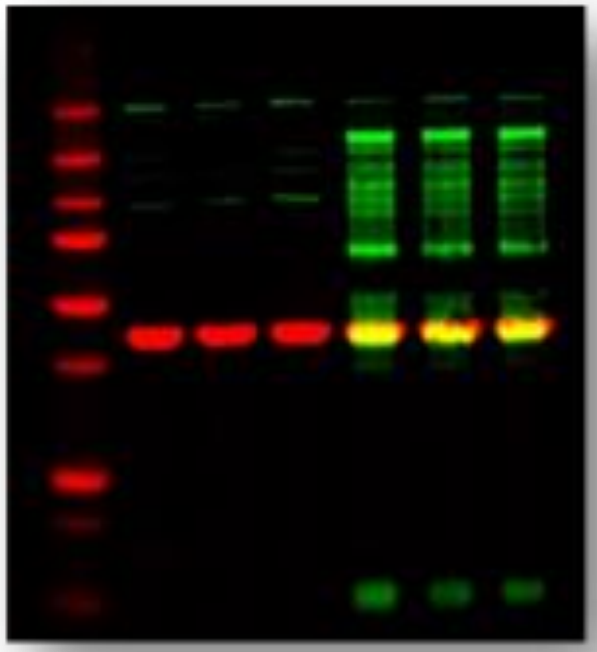




## Fluorescence (digital scanner)



# Normalisation and Loading Controls



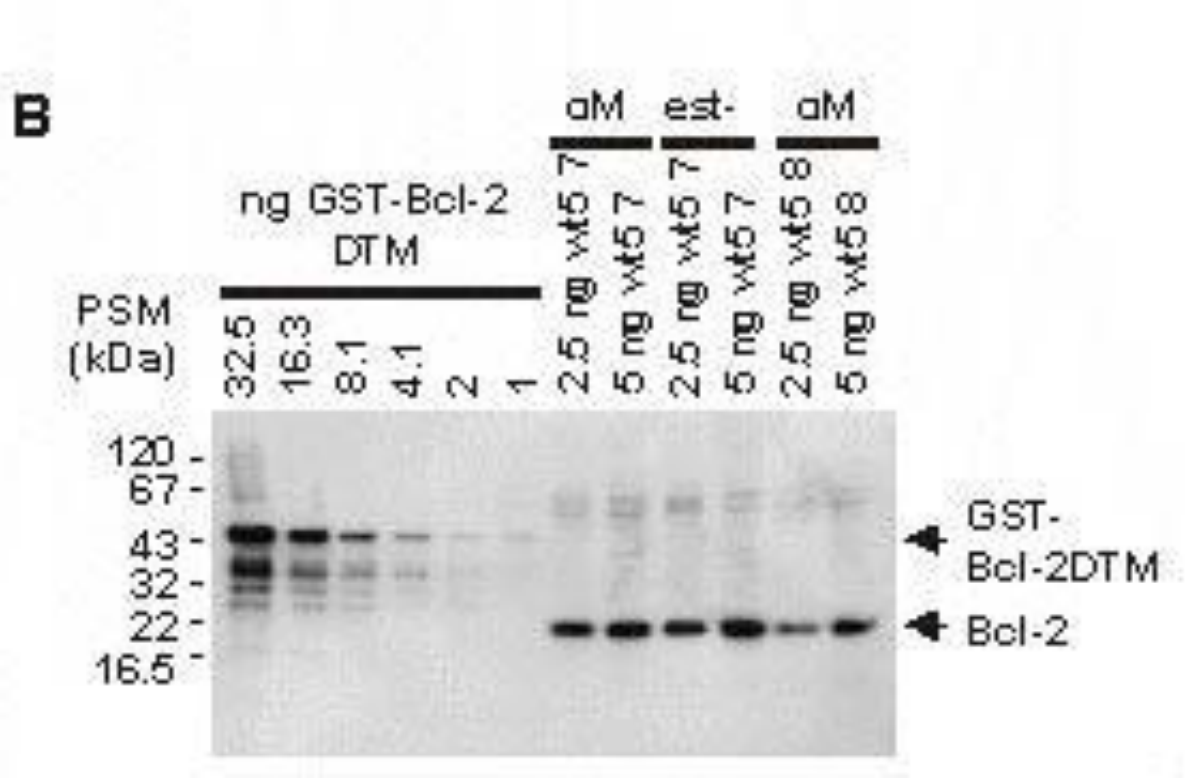
*Housekeeping protein (Red)*

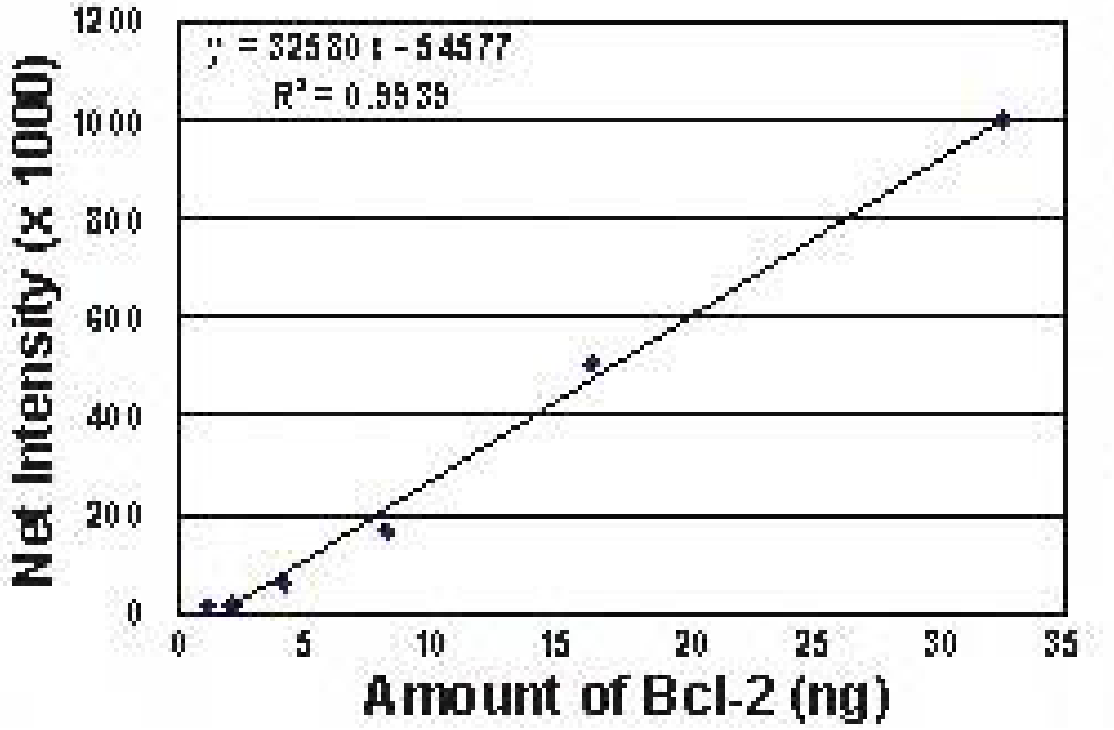
Ensuring that quantification is not due to:

1. Lane differences e.g. if signal is better in one lane
2. Loading differences

$$\text{Sample Signal} = \frac{\text{Signal from Protein-of-Interest}}{\text{Signal from Housekeeping / Total Protein}}$$

# Absolute Quantification



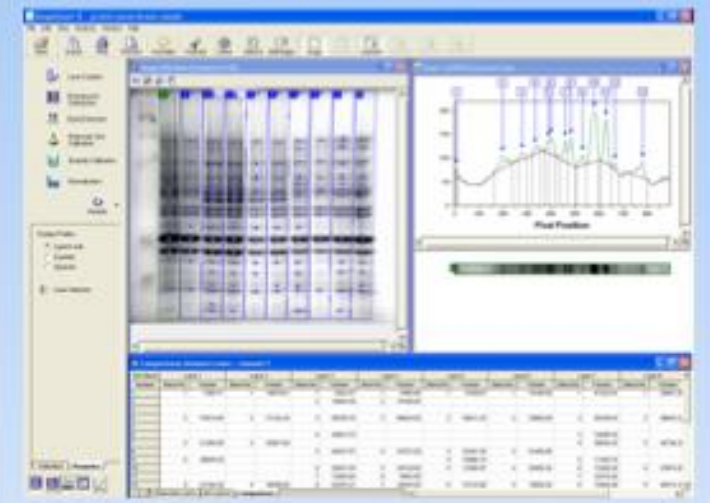




Requires purified protein of your target protein, and a standard curve on the same blot

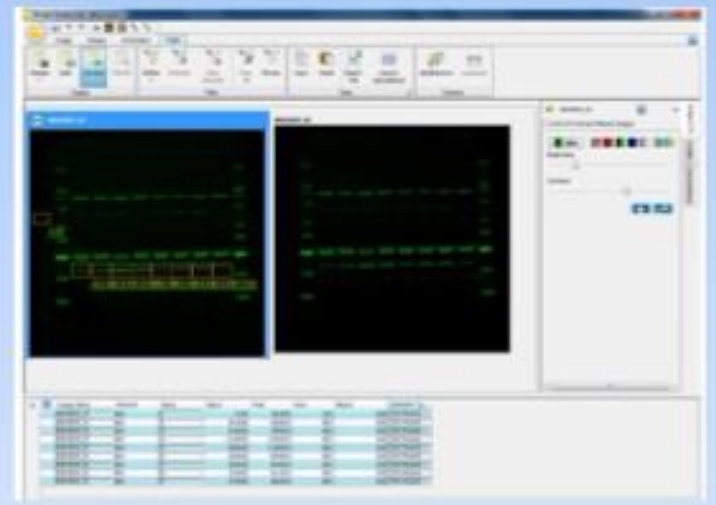
# Analysis Software: Options

**Licenced**

***ImageQuant***



***ImageStudio***



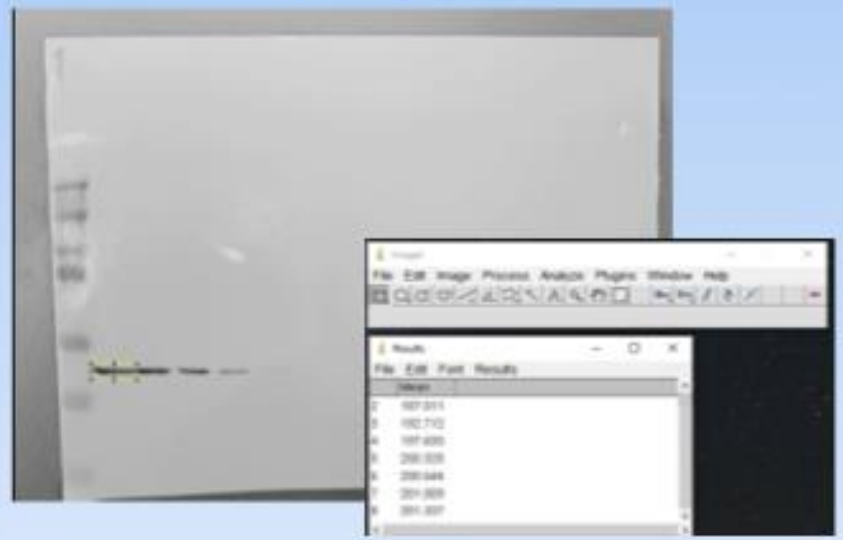
Require 16 bit .tif files

Generated from digital camera or scanner

Wizard-based software

**Free!**


***Image J***



Very basic software

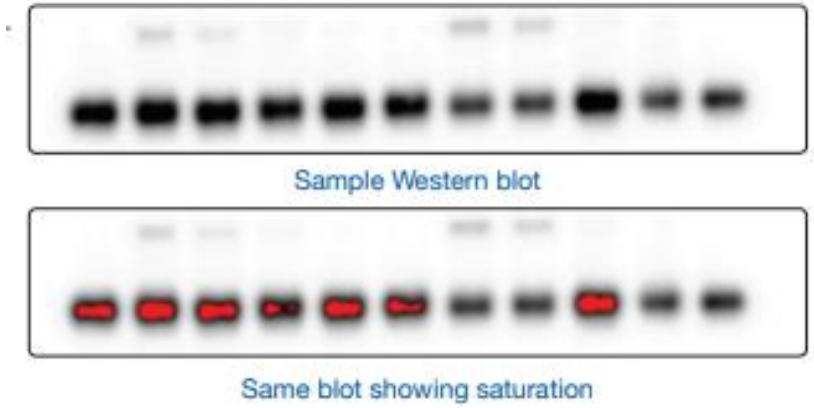
Manual analysis

# Analysis: Pitfalls

Band boundaries

- Can be tricky – especially if bands are close together
- Depending on downstream analysis factors e.g. different sizes of quantified area may affect

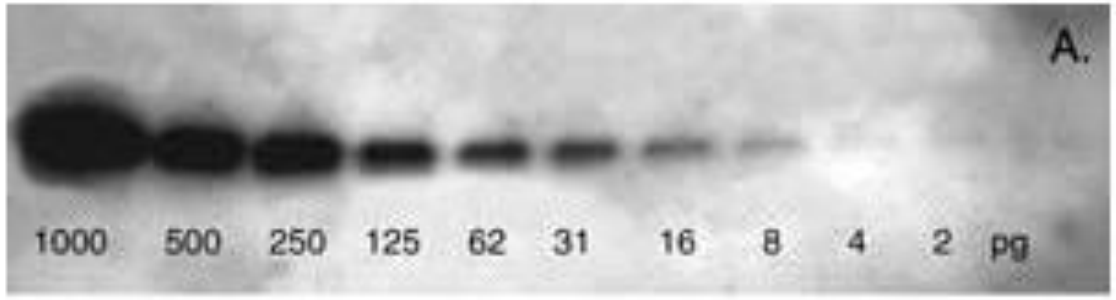
Saturation – remember linearity is key!



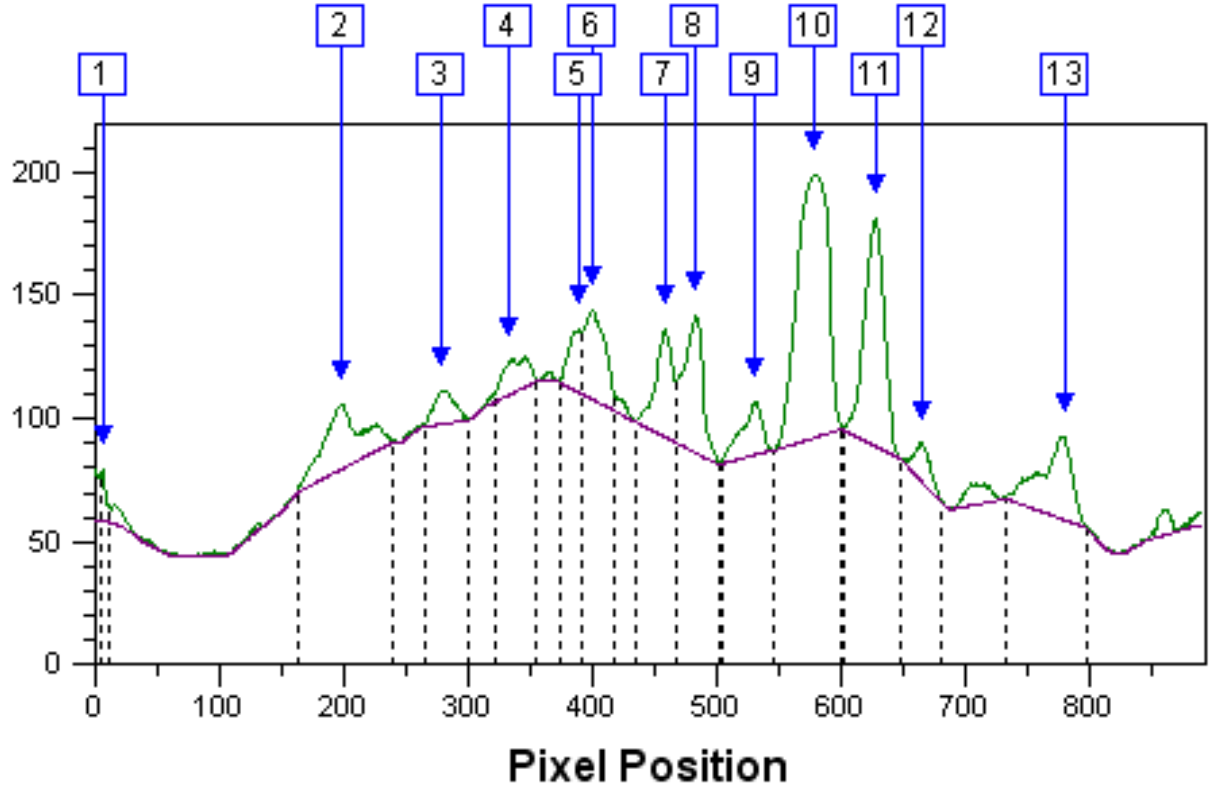

- NOT POSSIBLE to quantify saturated samples
- Some analysis software integrate saturation detection
- Others don’t (need to use image analysis software to check this beforehand)

# Analysis: Pitfalls

Background subtraction




- Varies from lane to lane
- “Rolling background” recommended



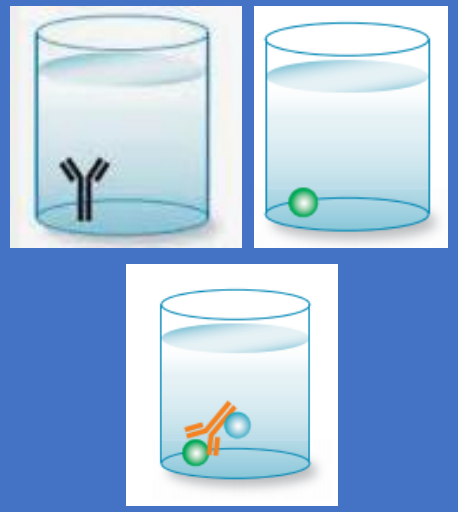

Replicates, replicates, replicates!!

# ELISA: The Theory

# Key Steps

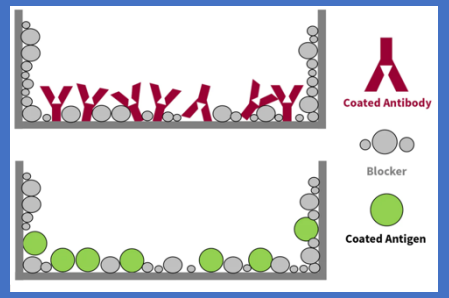
## Capture

.... of analyte and antibodies



## Blocking

.... to prevent non-specific binding of your analyte or antibody




## Washing

.... to remove unbound materials from the wells between steps

## Detection and quantification

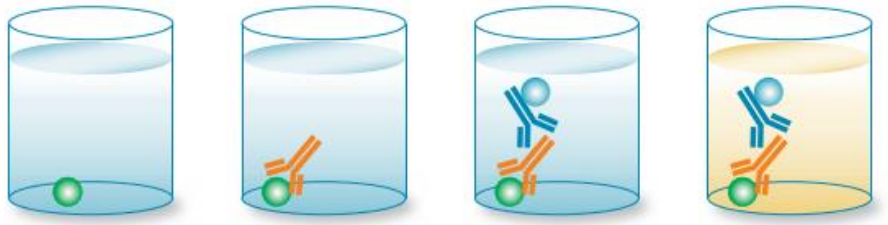
.... To measure your protein signal

# Different Types

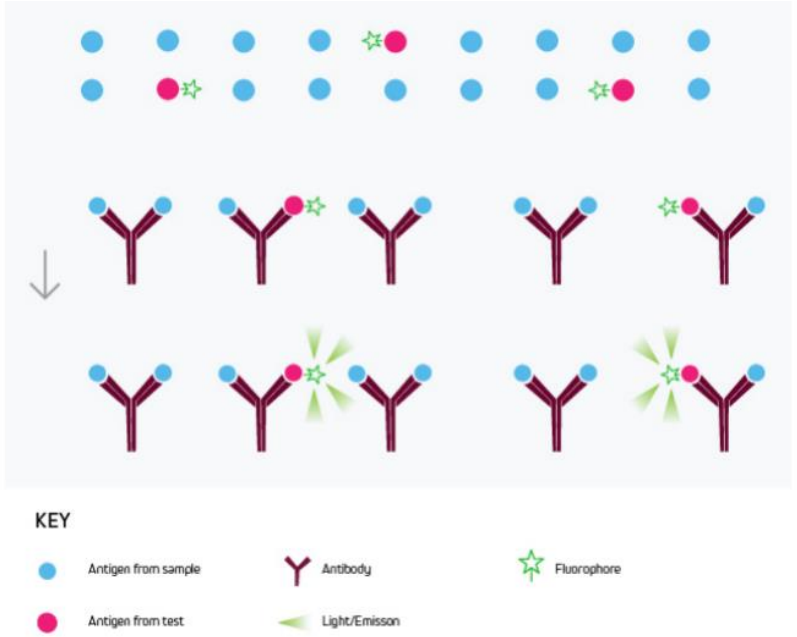
## Direct ELISA

## Sandwich ELISA

## Indirect ELISA



## Competitive ELISA



# Capture

Capture onto high-binding microtiter plates

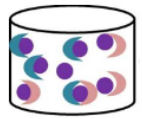
| Detection method | Typical microtitre plate used |
|---|---|
| Colorimetric | Clear |
| Chemiluminescence | Black or white |
| Fluorescence | Black plates |

Typical coating conditions:

- 50-100µl per well
- Antigen / Ab conc 1-10 µg/ml
- Incubation overnight at 4°C or 1-3 hrs at 37°C
- Typical coating buffer = bicarbonate buffer (pH9.6) or PBS

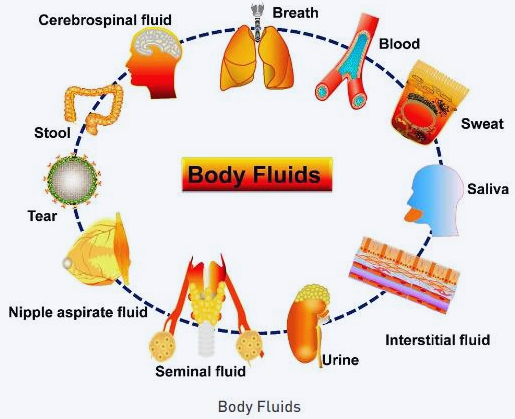
# Sample Preparation – Not Just Lysates!

Consider sample matrix and purify if needed (e.g. lipids / carbohydrates can confound analysis)




Consider biosafety level of samples


Food stuffs (e.g. milk)




Body Fluids

**Samples analysed by ELISA vary extensively……**

Tissue homogenates

Cell lysates

Cell culture supernatants

Environmental material

Avoid free-thaw cycles

Aliquot samples

Store samples at -80°C

- **Sample preparation will vary depending on material**
- **Handle carefully to maintain sample integrity**

# Experimental Controls

- Wavelength correction: corrects for changes in background correction
- Non-specific binding control: wells containing no analyte. Subtract form all data points (blanking)
- Secondary / detection antibody controls: Evaluates secondary binding the absence of primary / capture antibodies
- Total activity controls: substrate and enzyme incubated in absence of everything else to ensure no non-specific signal

# Comparison: ELISA and Western Blots

| | ELISA | Western Blot |
|---|---|---|
| Format / capture | Microtitre plate | SDS-PAGE and membrane binding |
| Readout | Single number | Image with molecular weight bands |
| Quantitation | Absolute quantitation (using standard curve) | Semi-quantitative |
| Optimisation required | Extensive if from scratch, less if commercial | Some usually required |
| Sample throughput | High (typically 96-well) | Low to medium (although 'In-Cell Westerns' are higher throughput) |
| Versatility | Excellent | Less versatile |
| Detection | Colorimetric, chemiluminescence and fluorescence | |
| Ease of technique | Quick and easy once set up | Some skill required |
| Time taken to complete | Typically a few hours | Longer: typically 2 days |
| Specificity | Generally less specific | Generally more specific (can see non-specific binding) |
| Sensitivity | Extremely sensitive (amplification methods): Low fmole levels | Generally less sensitive |

# Key take-home message.....

Western blotting and ELISA and are complimentary methods and both have their place in research!

# Thank You!!!


CANCER RESEARCH UK

UNIVERSITY OF CAMBRIDGE

UNIVERSITY OF CAMBRIDGE

CANCER RESEARCH UK | CAMBRIDGE CENTRE

# Immunohistochemistry (IHC)

Gideon Nsubuga

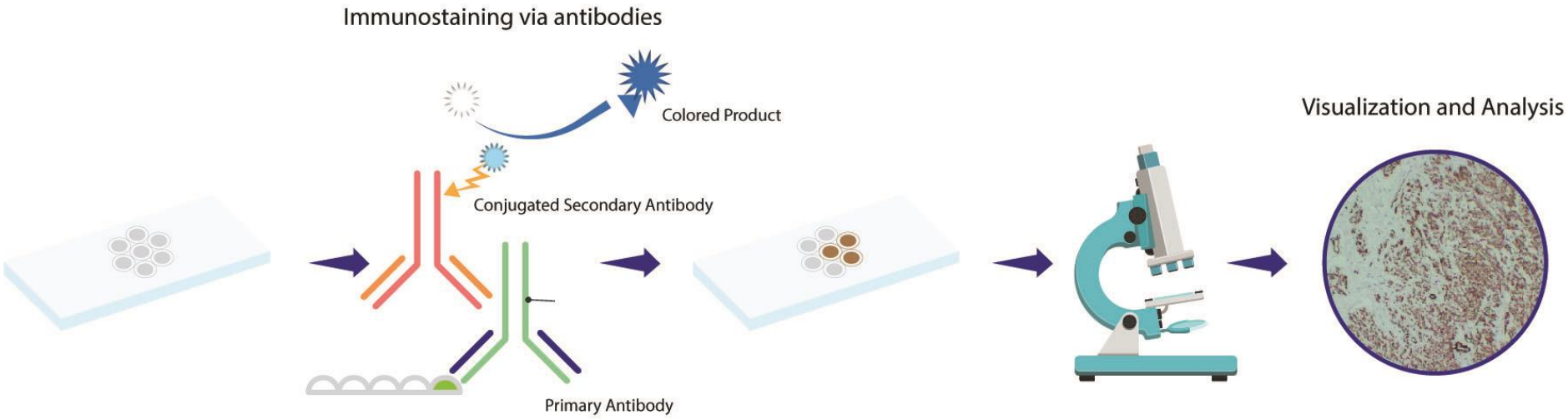
Cancer Biology Summer School

Uganda Cancer Institute/Makerere/CRUK Cambridge Institute

01 October 2025

# What is IHC?

- IHC is a widely used laboratory technique that combines histological, immunological, and biochemical methods
- Visualization of the presence and localisation of specific proteins (antigens) in tissue sections
- Antigen–antibody interactions

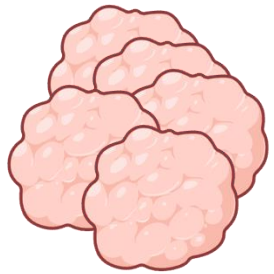


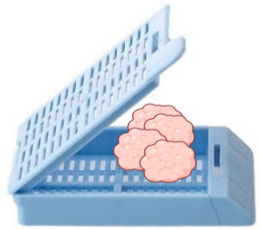
# Applications of IHC

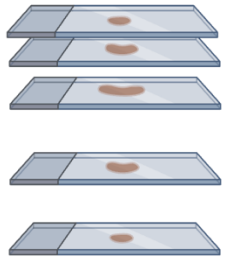
1. Clinical diagnostics
    - Cancer diagnosis and classification
    - Prognostic and predictive markers
2. Research applications
    - Protein localisation
    - Disease mechanism studies
3. Drug development
    - Biomarker validation
    - Pharmacodynamics
    - Toxicology

# Sample preparation: Pre-IHC Steps

Fixation → Dehydration → Embedding → Sectioning





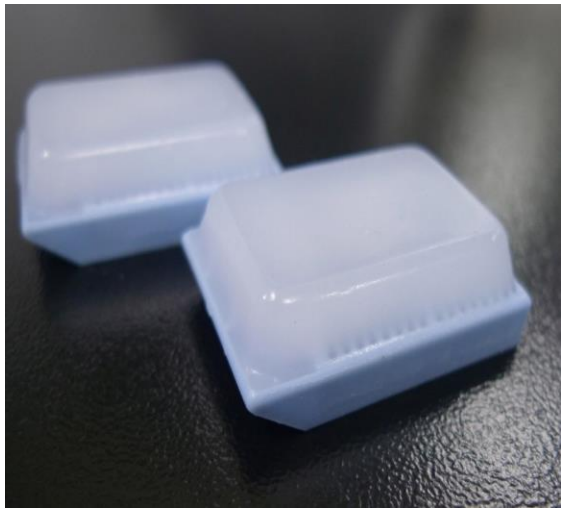



# Fixation

- Purpose: Preserve structure & prevent degradation
- Common fixative: Formaldehyde/paraformaldehyde
- Actions:
  - Crosslinks proteins → stabilises tissue architecture
  - Prevents autolysis & bacterial growth
  - Preserves epitopes (though may mask them)
- Caveat: Over-fixation = epitope masking

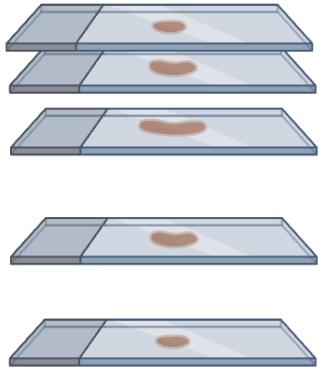
# Dehydration & Embedding

- **Dehydration**
  - Removes water with graded ethanol (70–100%)
  - Xylene (or substitute) replaces ethanol
- **Embedding**
  - Paraffin infiltration → solid block
  - Provides rigidity for sectioning



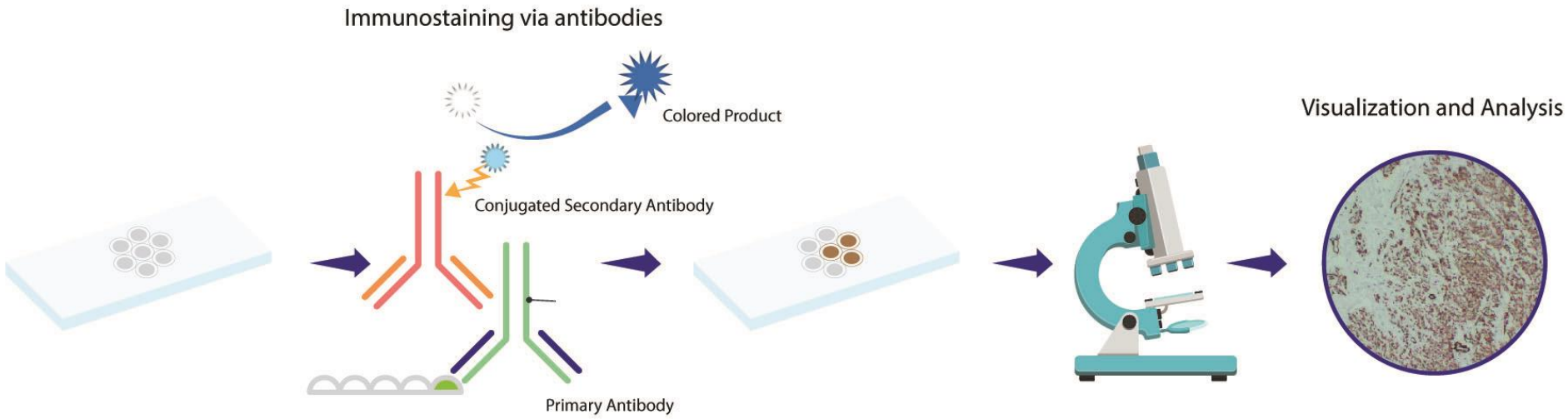
# Sectioning

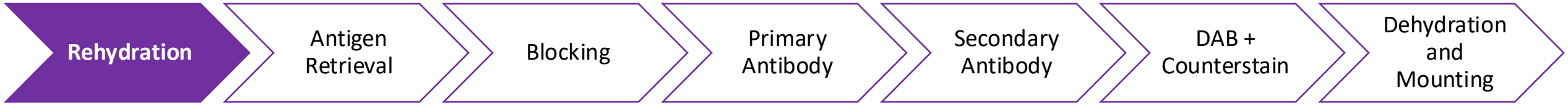
- Performed with a microtome (3–5 µm slices)
- Sections mounted on slides
- Importance: Thin, uniform slices → antibody penetration + clear imaging



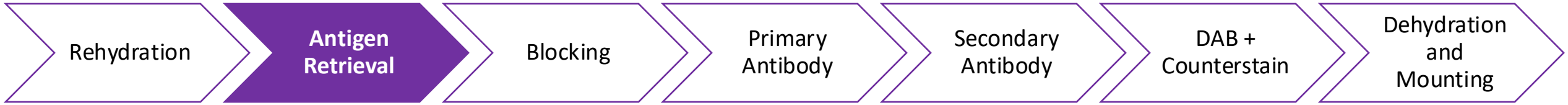
# IHC Process

Rehydration → Antigen Retrieval → Blocking → Primary Antibody → Secondary Antibody → DAB + Counterstain → Dehydration and Mounting






- **Deparaffinisation**: Xylene removes paraffin
- **Rehydration**: Graded alcohols (100% → 70%) replace xylene with water
- Final step: Slides in distilled water/PBS
- Outcome: Tissue ready for aqueous IHC staining



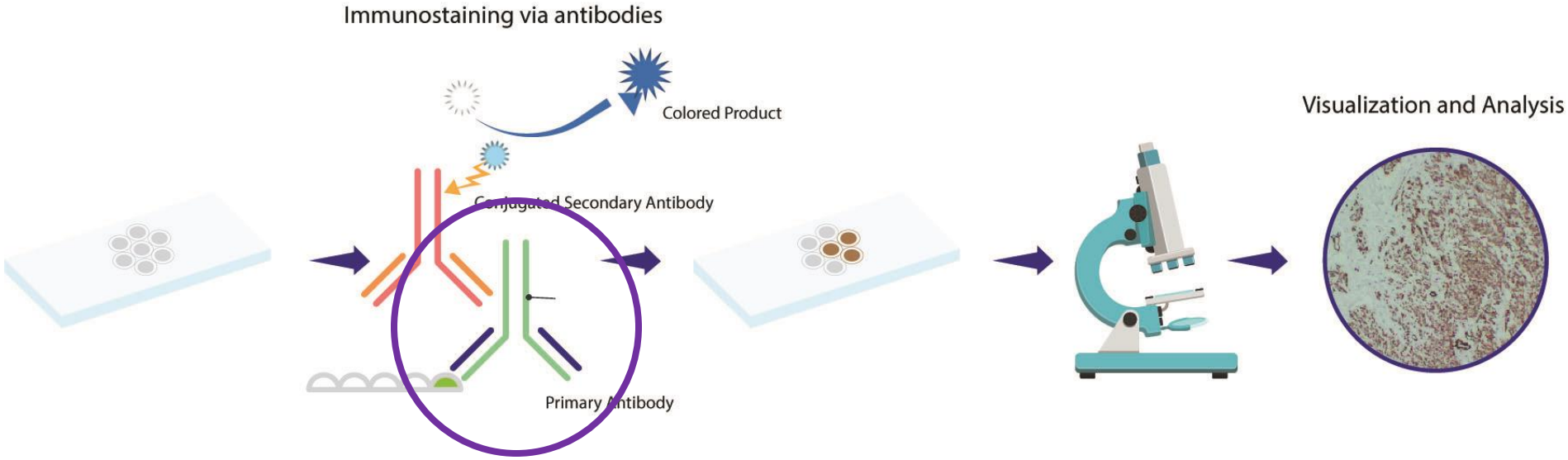

- Fixation masks epitopes → reduces antibody binding
- Antigen retrieval allows to open up the proteins giving the Ab the access to bind
- **Heat-Induced (HIER):** Heat + buffer (e.g. citrate pH 6, EDTA pH 9)
- **Enzymatic (PIER):** Proteolytic digestion (trypsin, proteinase K)
- Restores accessibility of antigens for antibody recognition

Rehydration → Antigen Retrieval → **Blocking** → Primary Antibody → Secondary Antibody → DAB + Counterstain → Dehydration and Mounting

- **Purpose:** Prevent non-specific staining
- **Materials:**
  - Normal serum (from species of secondary antibody)
  - Hydrogen peroxide (0.3–3%) → blocks endogenous peroxidase
- **Outcome:** Reduced background signal

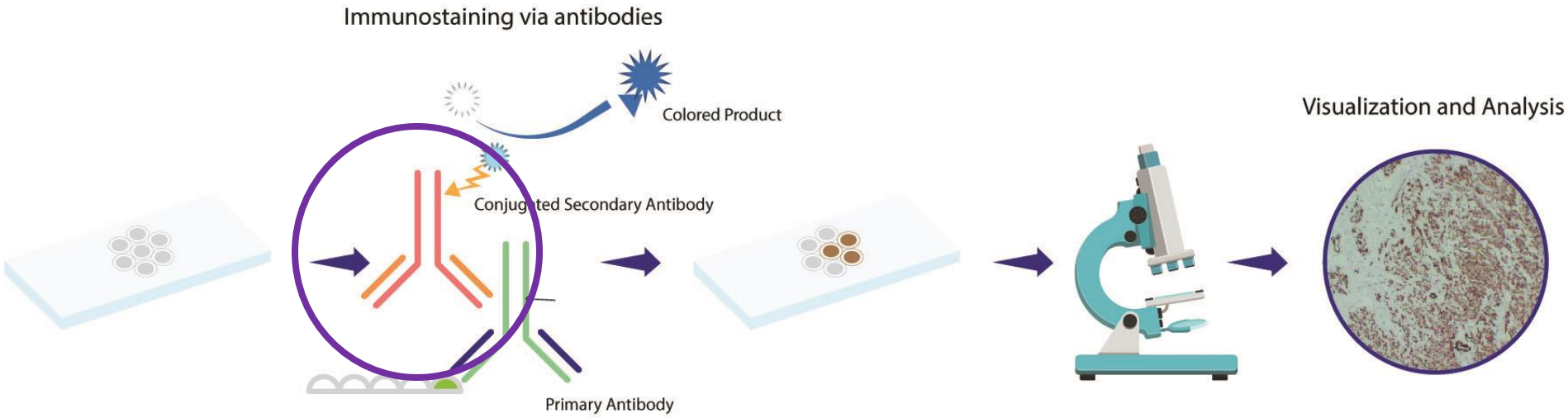
Rehydration | Antigen Retrieval | Blocking | **Primary Antibody** | Secondary Antibody | DAB + Counterstain | Dehydration and Mounting

- **Purpose:** Specific recognition of target antigen
- **Materials:**
  - Primary antibody (host species + validated for IHC)
  - Antibody diluent (PBS/TBS + 1% BSA or serum)
- **Outcome:** Antibody-antigen binding defines specificity



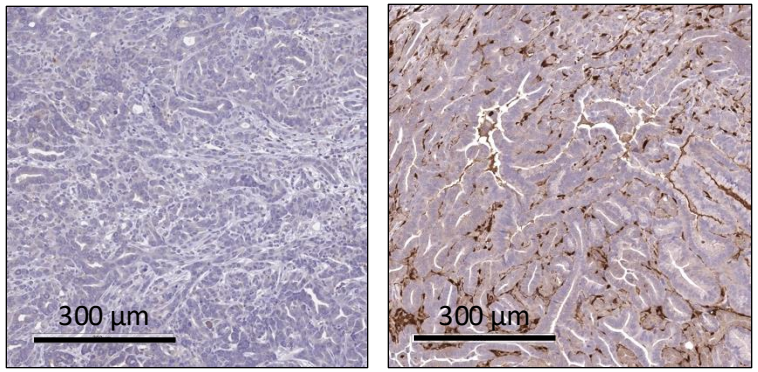
Rehydration | Antigen Retrieval | Blocking | Primary Antibody | **Secondary Antibody** | DAB + Counterstain | Dehydration and Mounting

- **Purpose:** Detection & signal amplification
- **Materials:**
  - Secondary antibody (anti-IgG, species-specific)
  - Conjugates: horseradish peroxidase, alkaline phosphatase, Fluorophores
- **Outcome:** Enables chromogenic or fluorescent detection

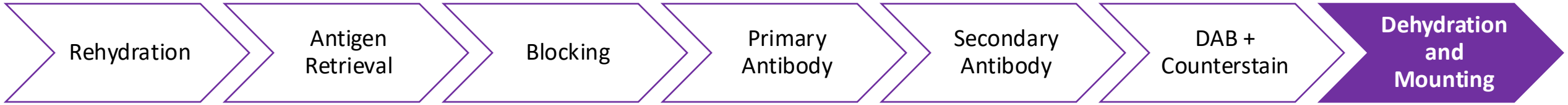


Rehydration → Antigen Retrieval → Blocking → Primary Antibody → Secondary Antibody → **DAB + Counterstain** → Dehydration and Mounting

- **Purpose:** Visualisation of antigen & tissue context
- **Materials:**
  - DAB (3, 3'-diaminobenzidine) substrate kit (HRP + DAB → brown precipitate)
  - Haematoxylin → nuclear counterstain (blue)
- **Outcome:** Brown signal = antigen, blue nuclei = contrast




tdTomato - tdTomato +




- **Purpose:** Preserve stained tissue for microscopy
- **Materials:**
  - Graded alcohol series (70%, 95%, 100%)
  - Xylene (clearing agent)
  - Mounting medium (e.g. DPX, Permount)
  - Coverslips
- **Outcome:** Permanent slide, stable long-term storage

# Thank you