Western Blotting & ELISA

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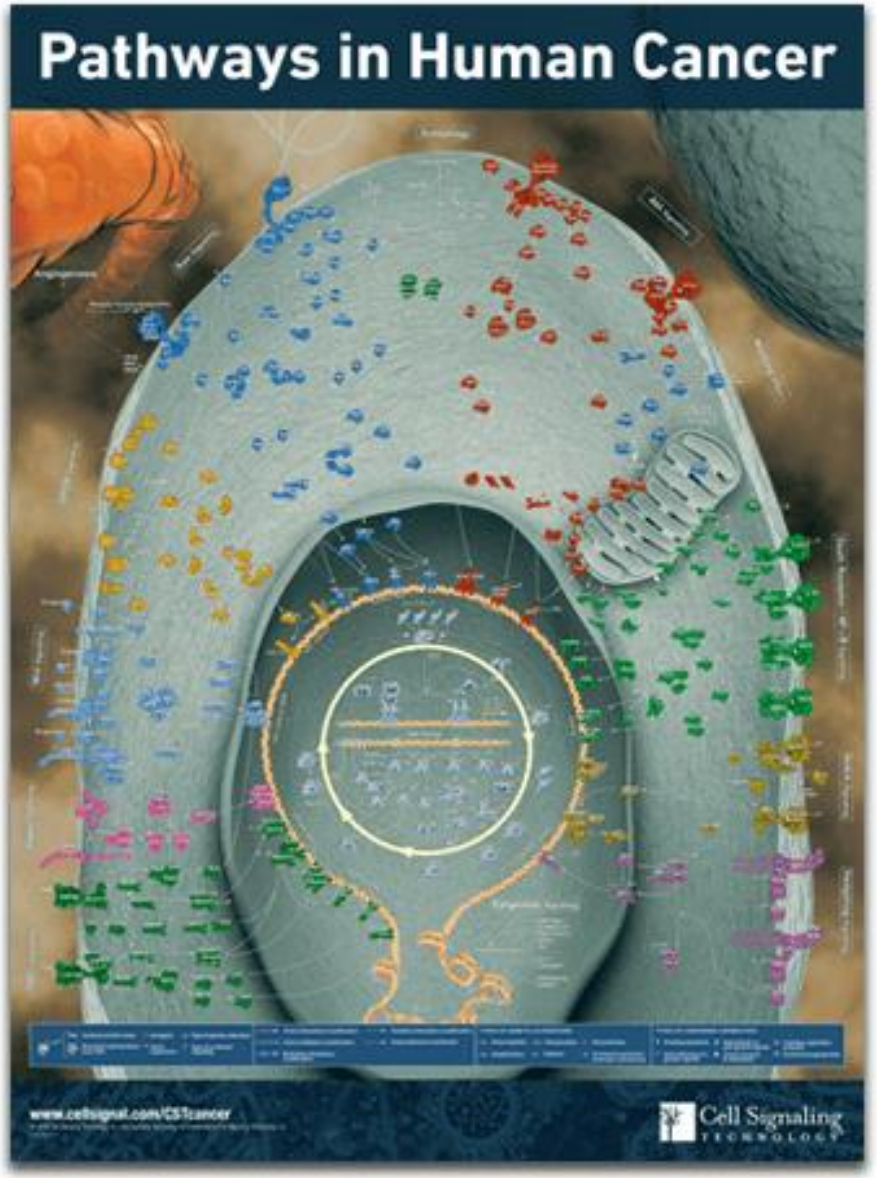
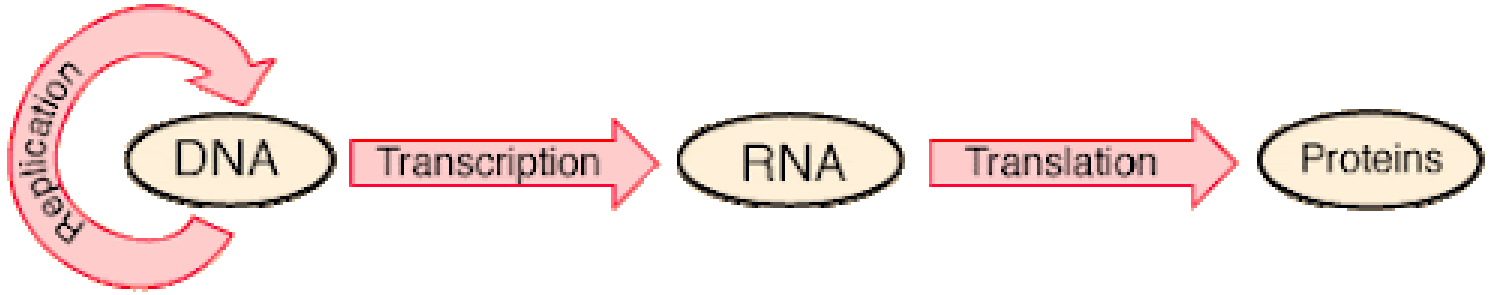
Why Detect and Quantify Proteins?

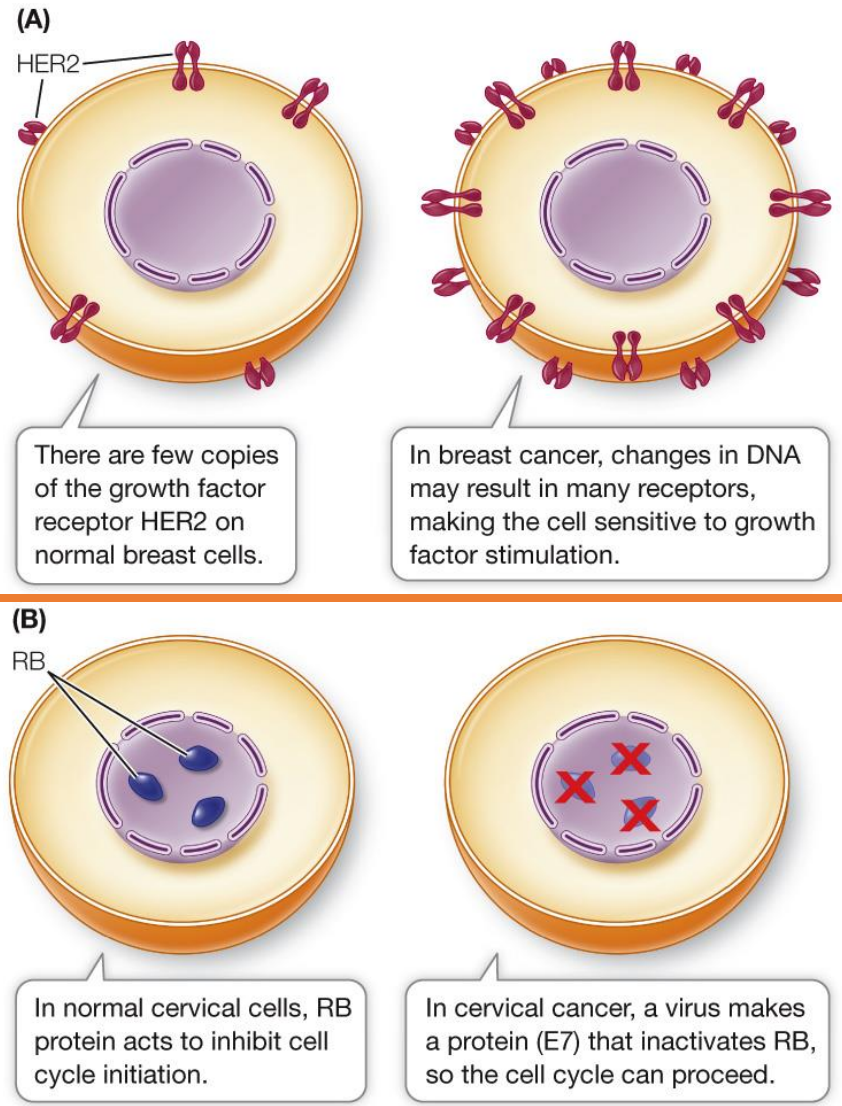




Central dogma of molecular biology

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?



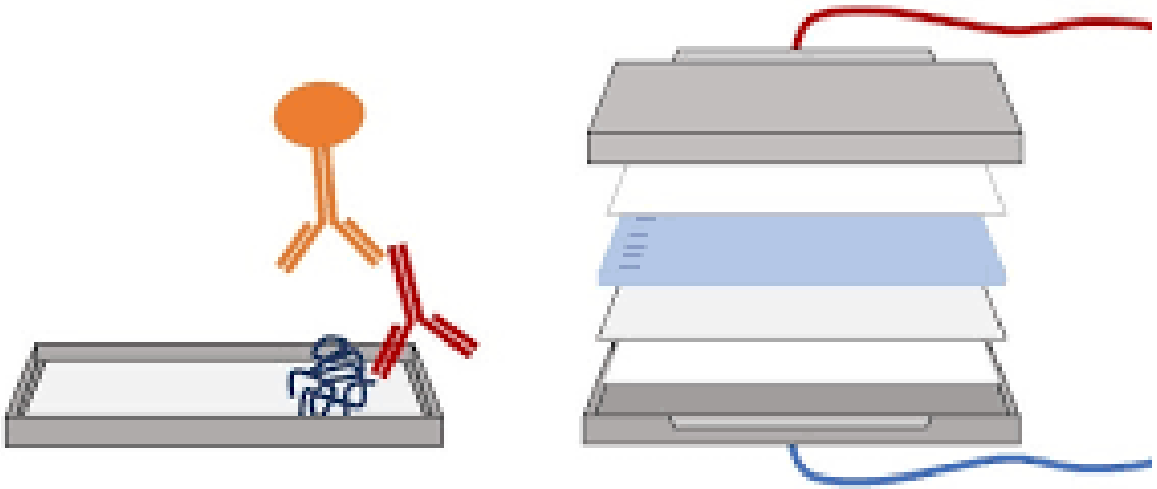
LIFE: THE SCIENCE OF BIOLOGY 11e, Figure 11.23

Oncogenic / Tumour Promoting
Increased in cancer vs. normal

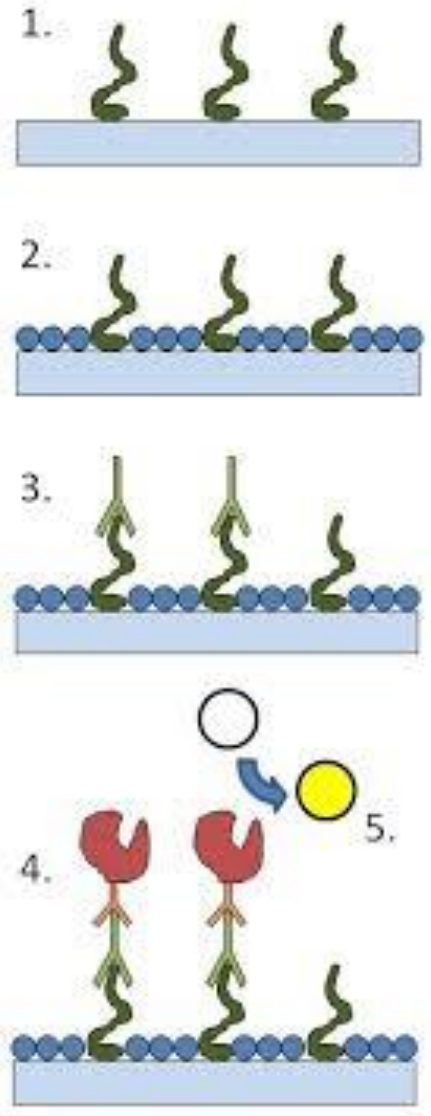
Tumour Suppressive
Decreased in cancer vs. normal

Methods of Protein Detection

Western Blotting

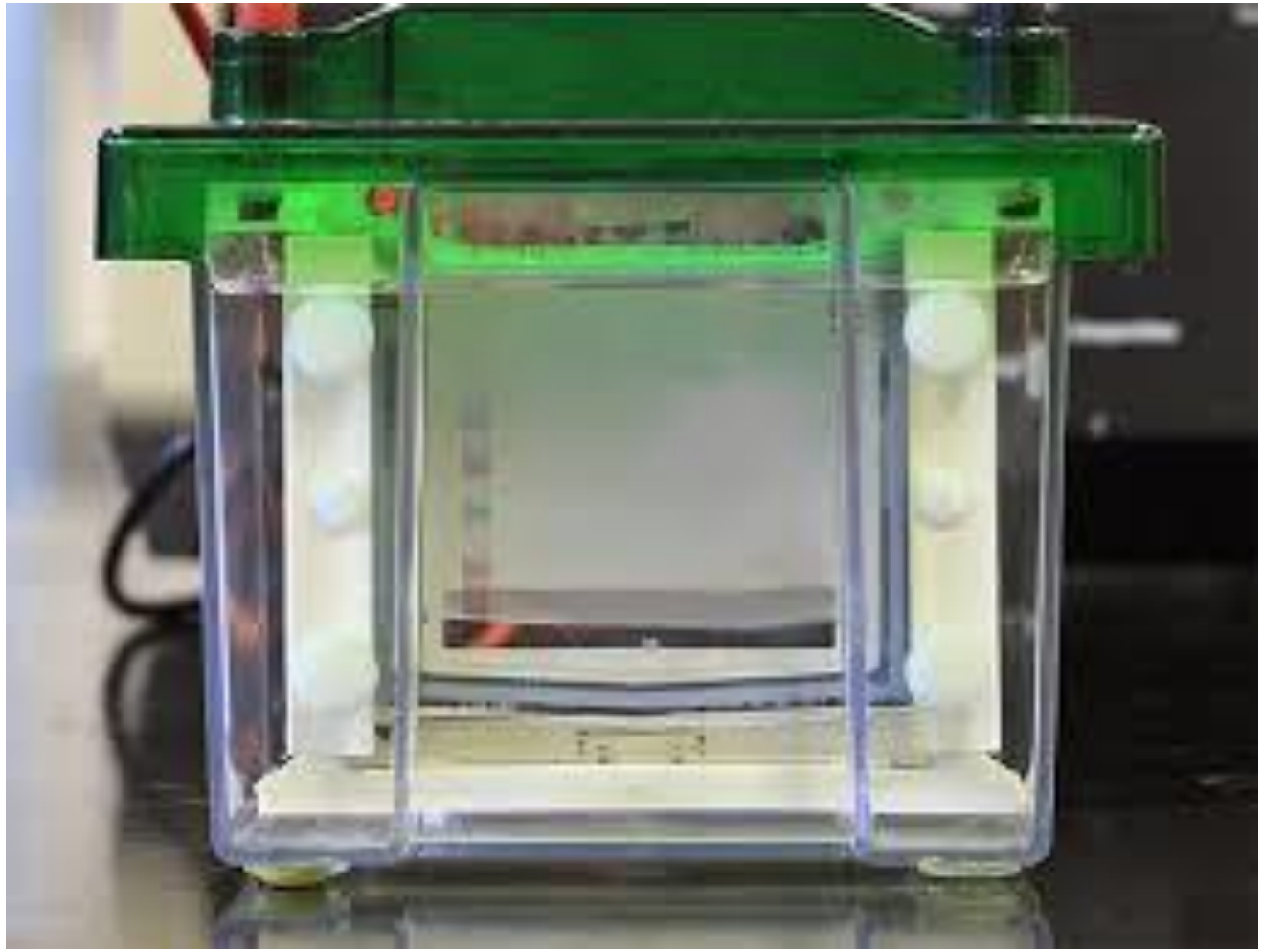


ELISA



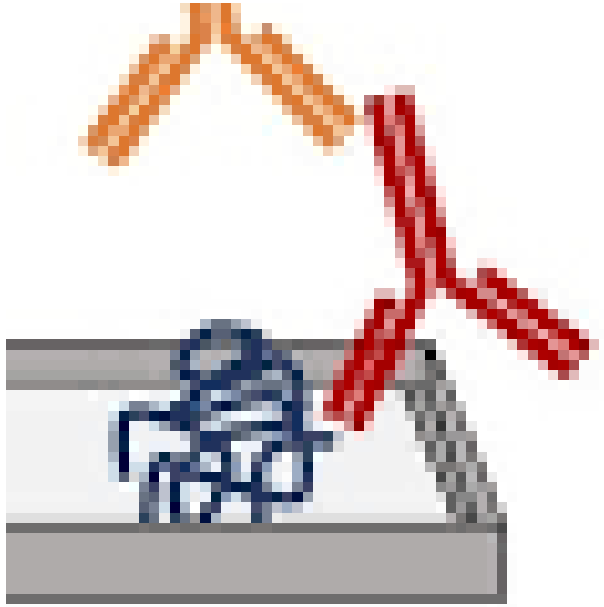
Western Blotting: The Theory

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

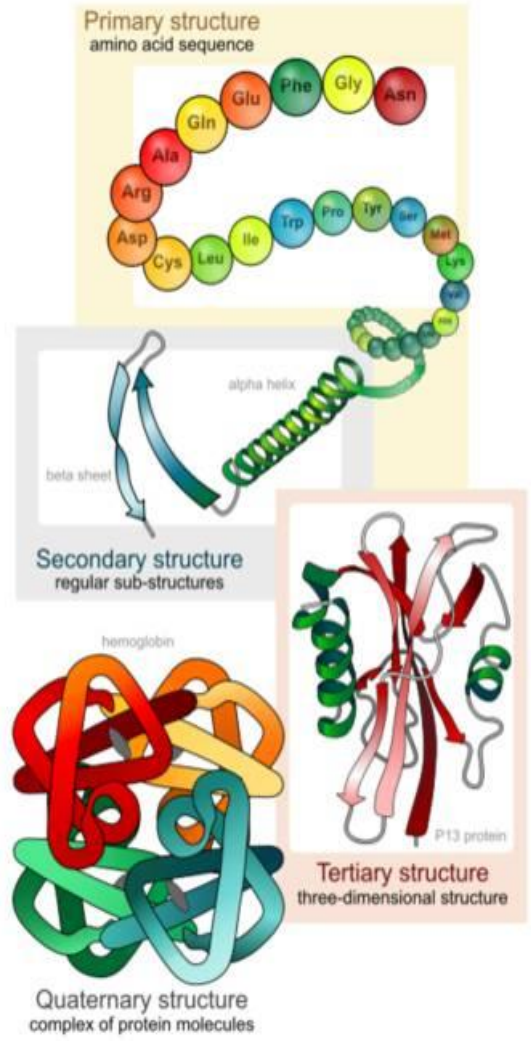


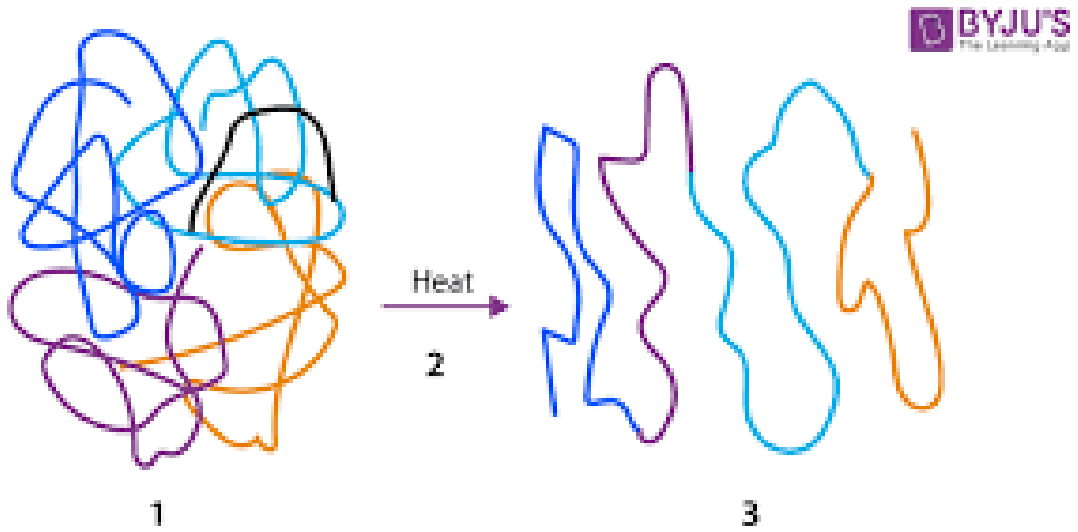
Factors usually affecting electrophoretic mobility:

- Size
- Charge



Western Blotting: The Theory



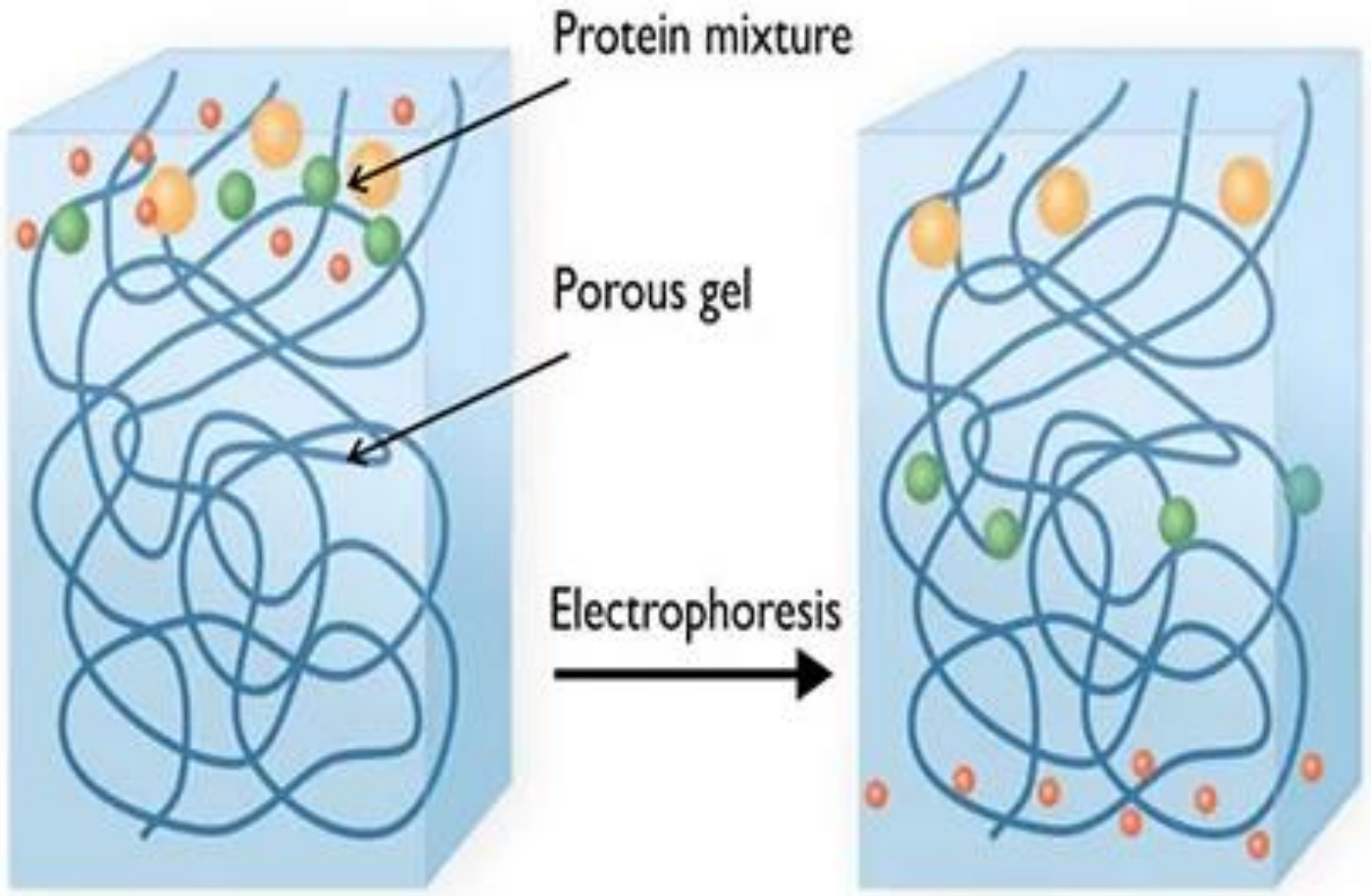


1. Anionic detergent, commonly SDS
    - Disrupts protein structure into a linear polypeptide
    - Imparts uniform negative charge
2. Reducing agent e.g. DTT, β-mercaptoethanol
    - Disrupt disulphide bonds

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

Western Blotting: The Theory

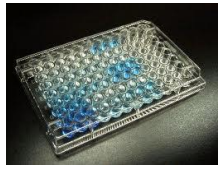
Polyacrylamide Gels



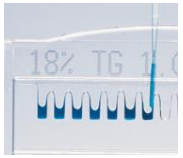
Running a Western Blot

Sample preparation

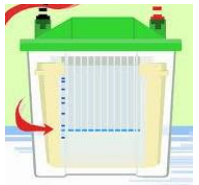
Protein quantification



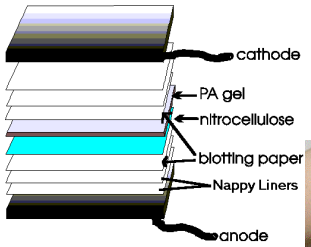
Load a gel



Run the gel



Transfer to a membrane blot



Block membrane

Immunodetection and washes

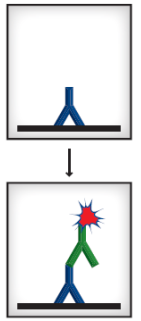
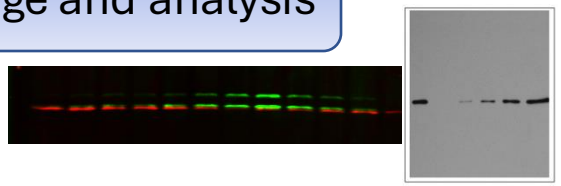
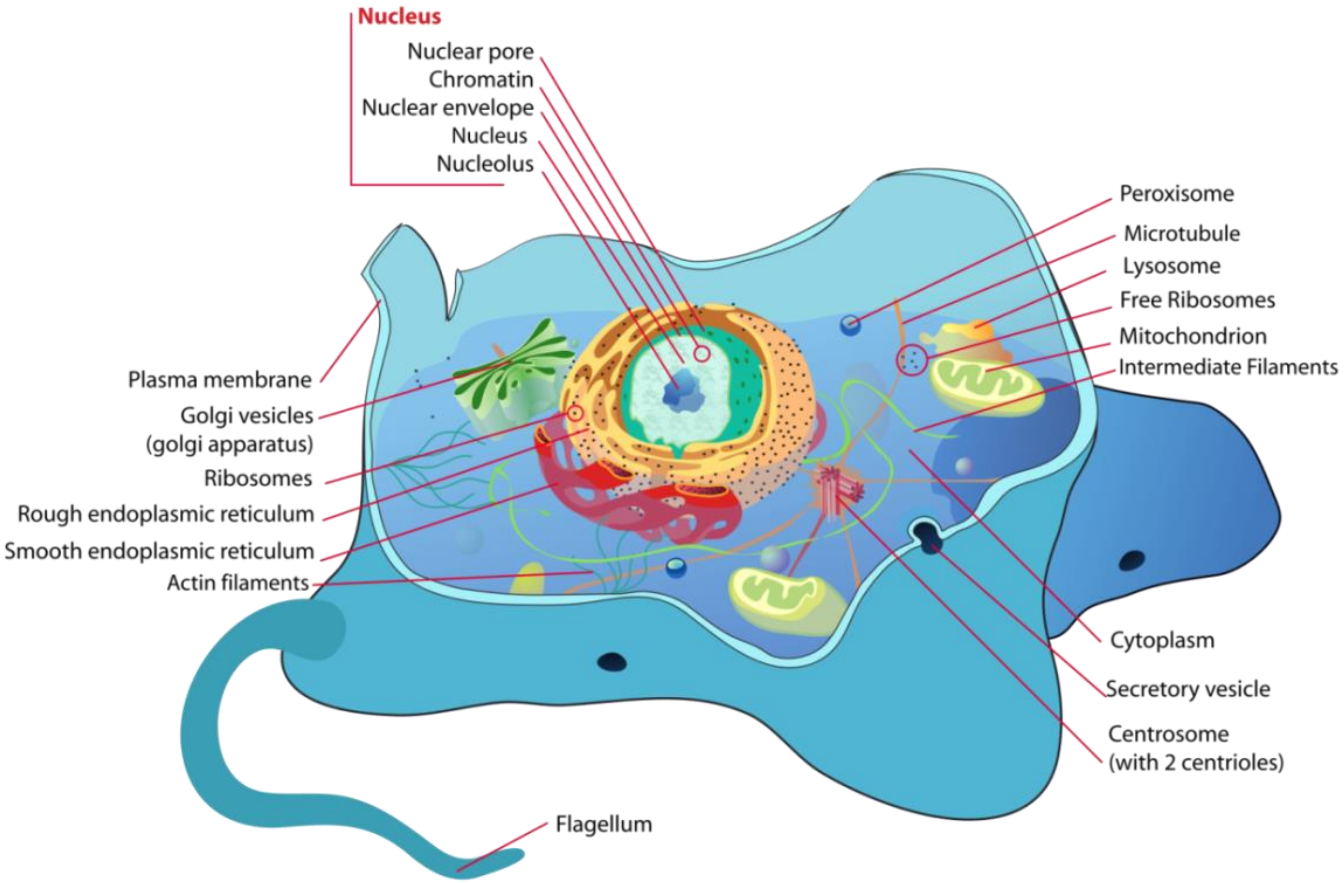


Image and analysis

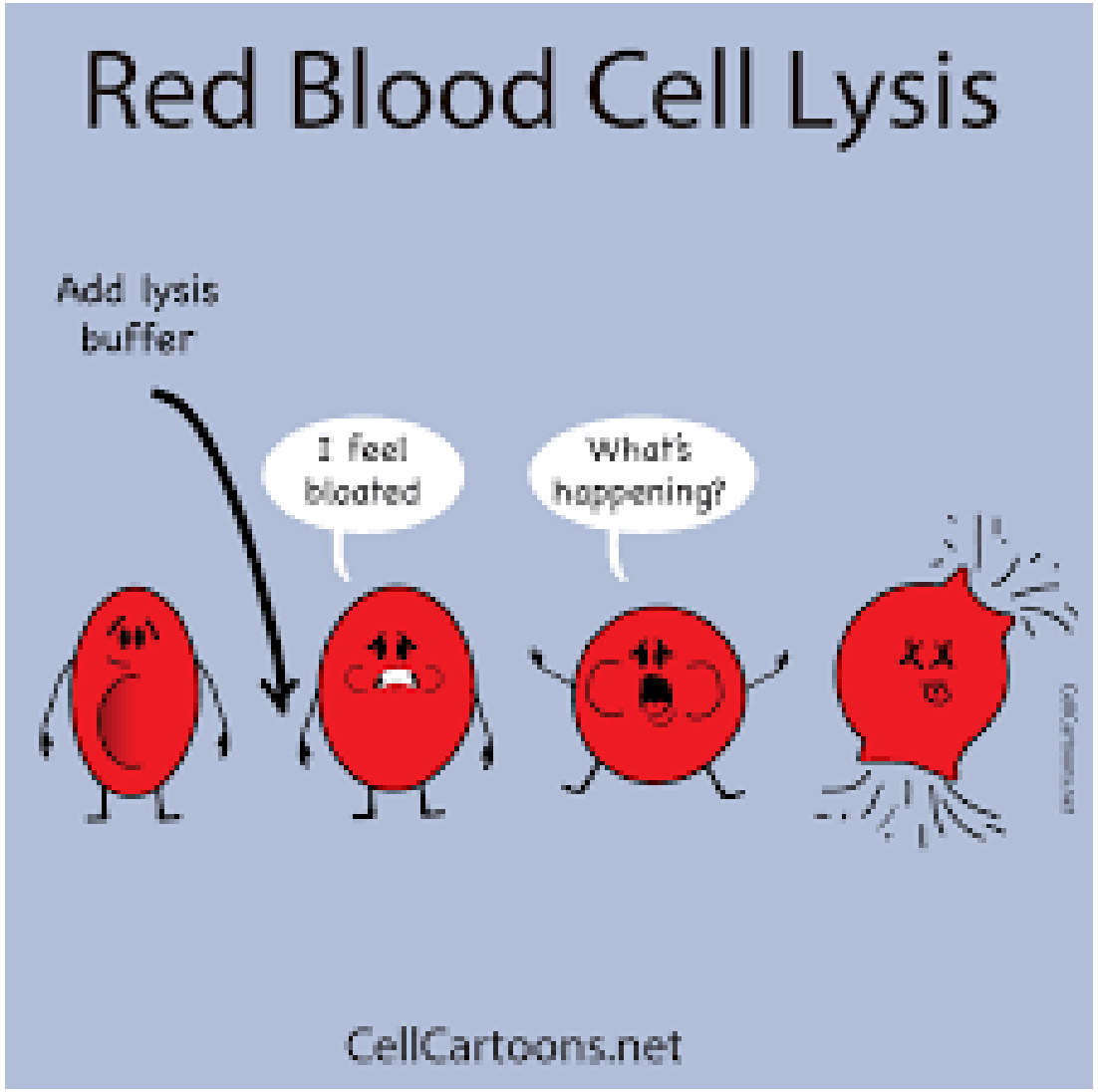


Properties of a Protein-of-Interest



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

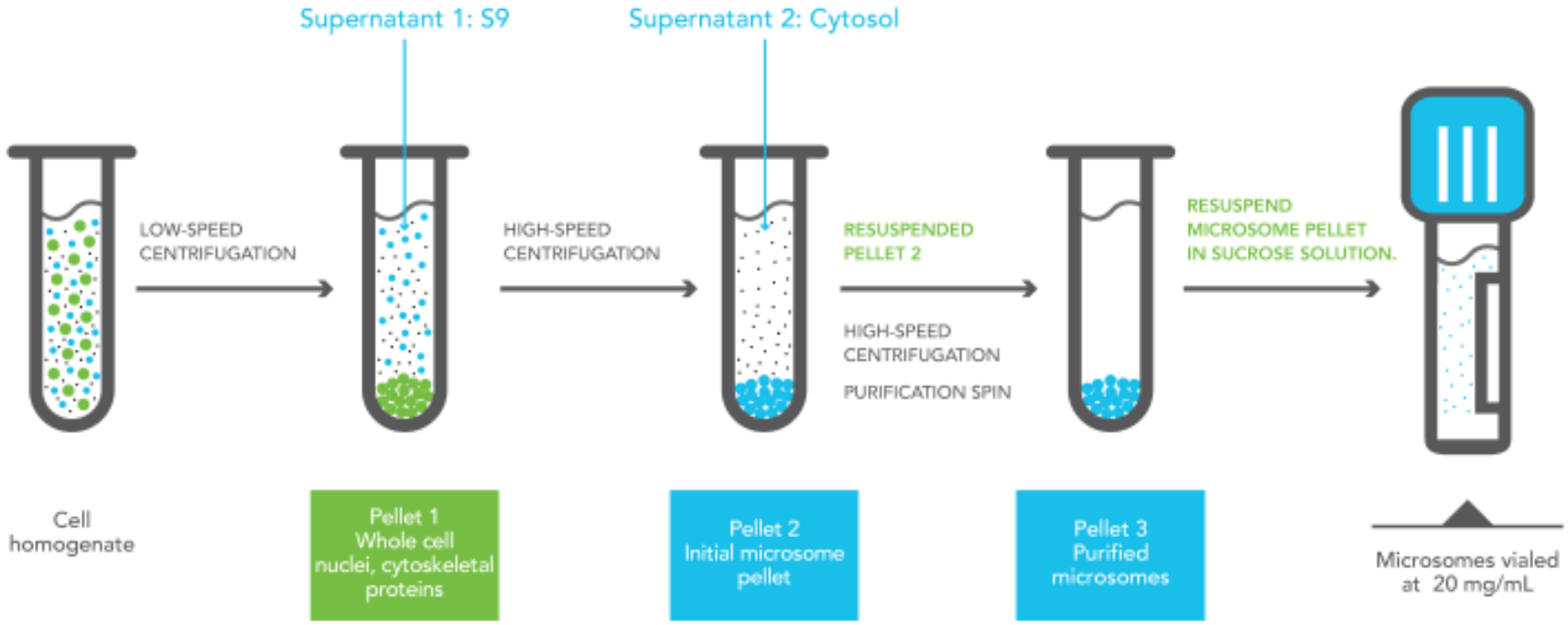
Step 1: Cell Lysis



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

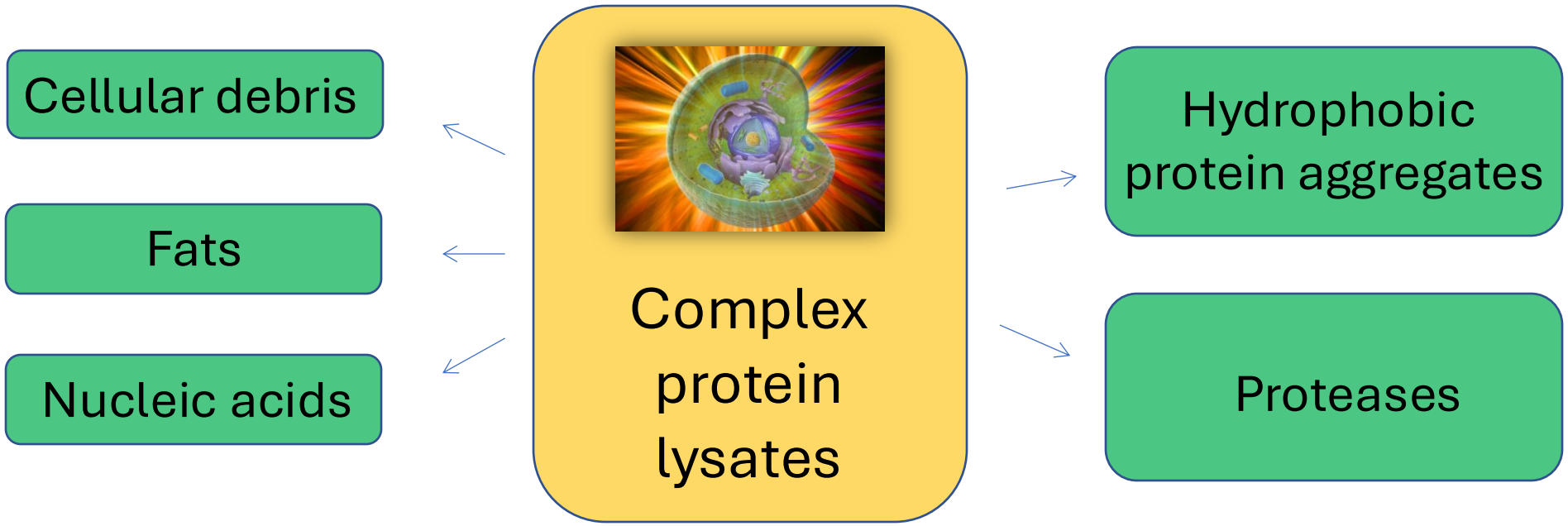
Cell Lysis: Things to Think About

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

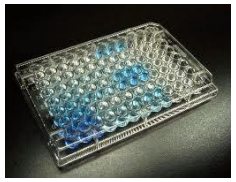
Sonicate sample on ice (3x10s)

Agitate at 4°C for 30 min

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

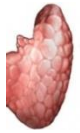
Carefully aspirate supernatant into a pre-cooled clean tube (discard any pellet)

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)

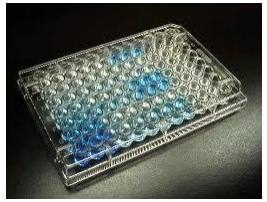
Homogenise (e.g. using Precellys)

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)

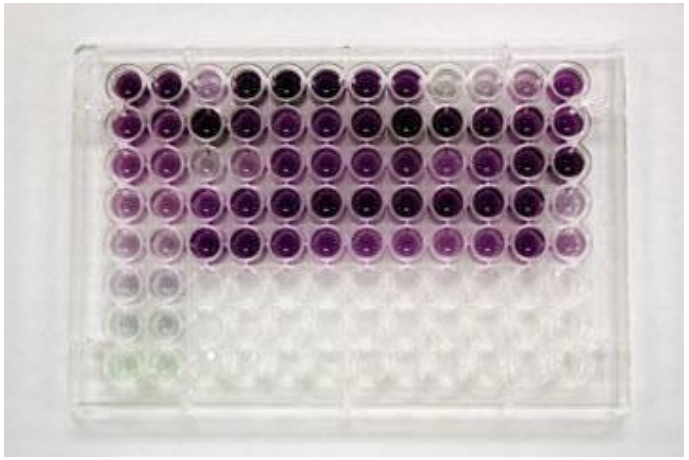


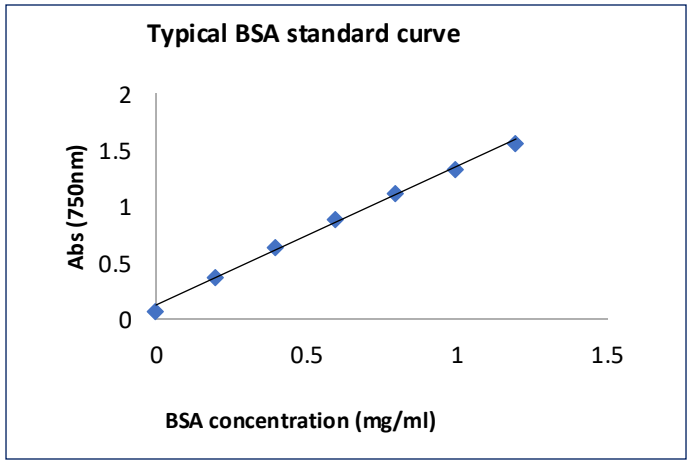
Keep aliquot for protein assay (~150mg wet brain tissue = ~15 mg protein)

Step 2: Protein Quantification

Methods for Protein Quantification

1. Colorimetric Assays

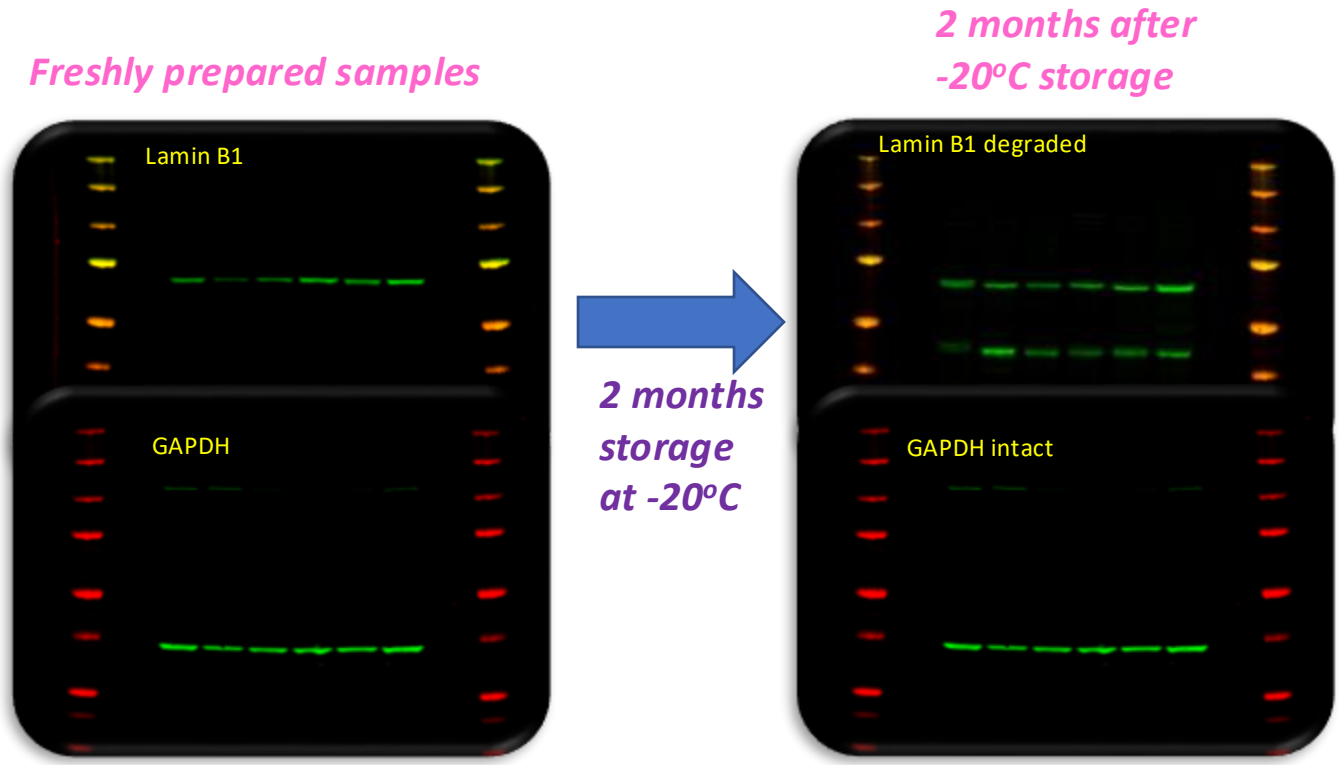




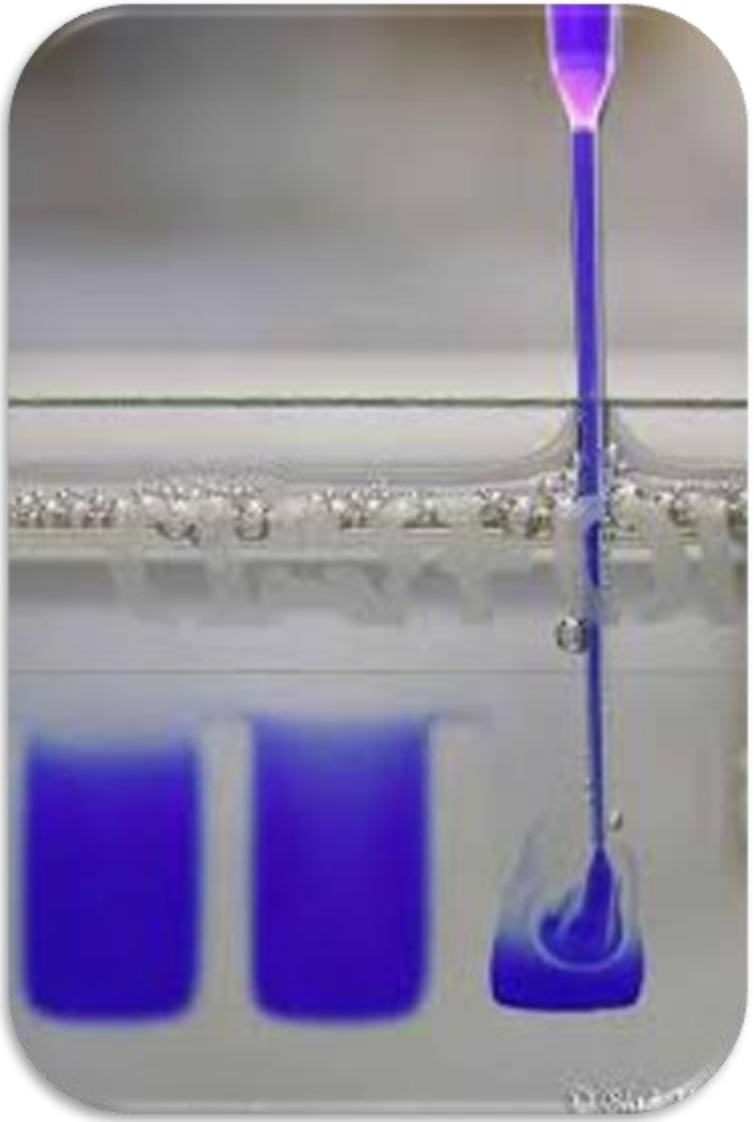
2. Fluorescent Qubit

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

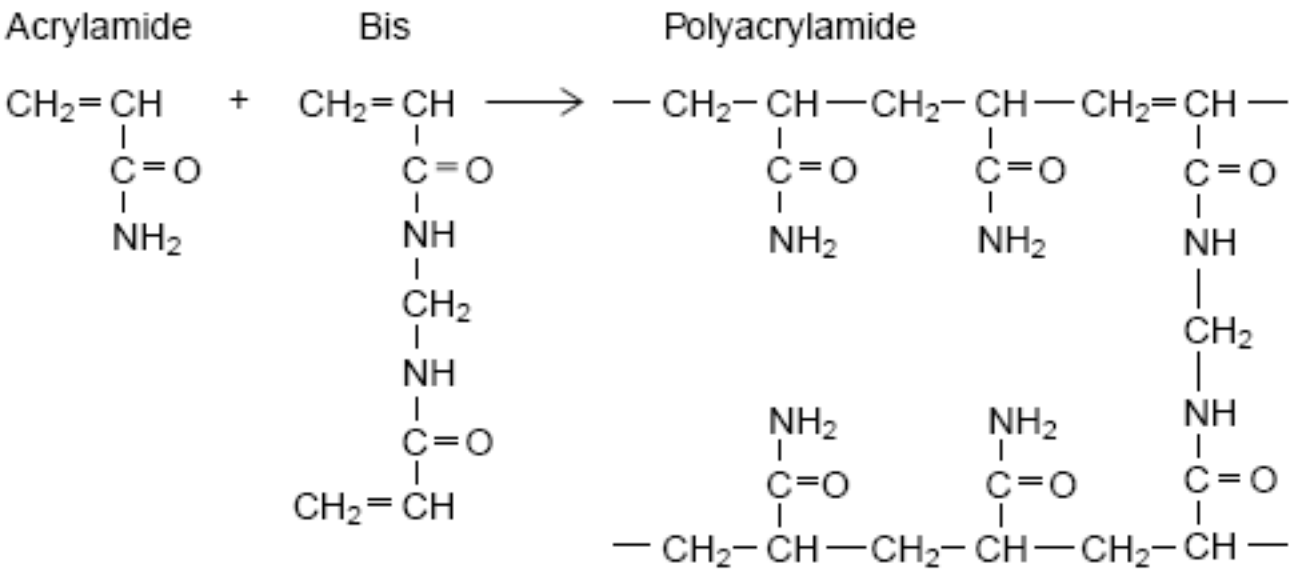
Preparing Samples for Loading

2. Heating

3. Vortex

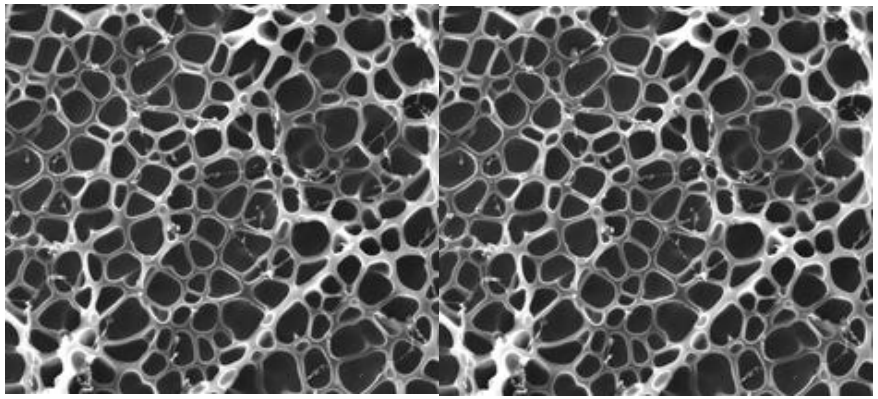
Preparing Polyacrylamide Gel

Cross-linker



+ Ammonium Persulphate (APS, initiator)

+ TEMED (catalyst)



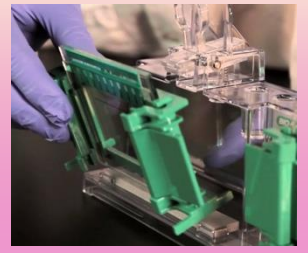
Pore size determined by

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



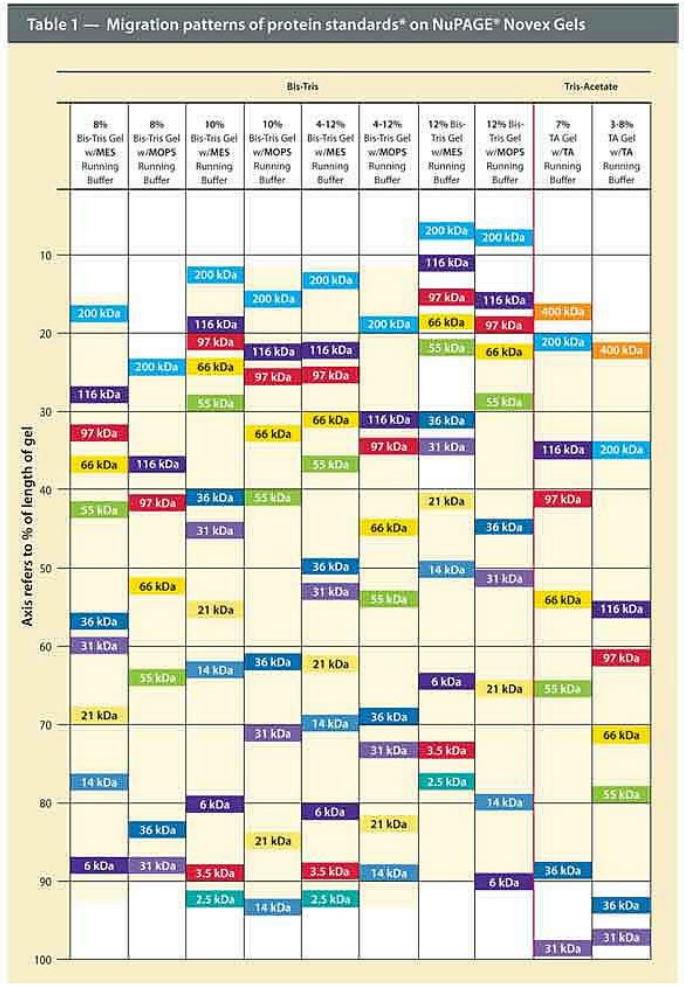
Manually poured

Pre-cast

Sizes (mini, midi, large-format)

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

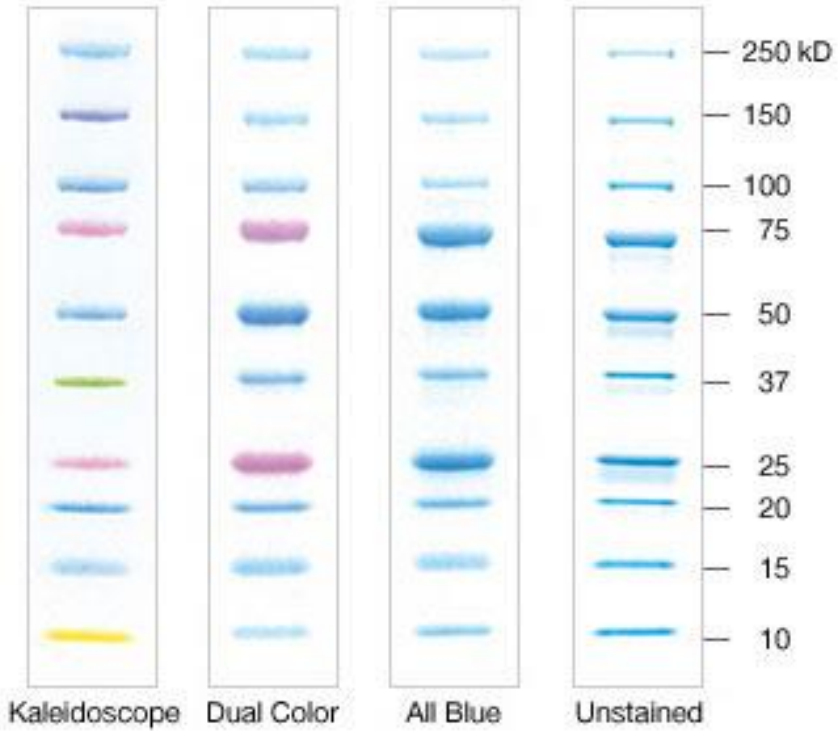
Typical migration patterns



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

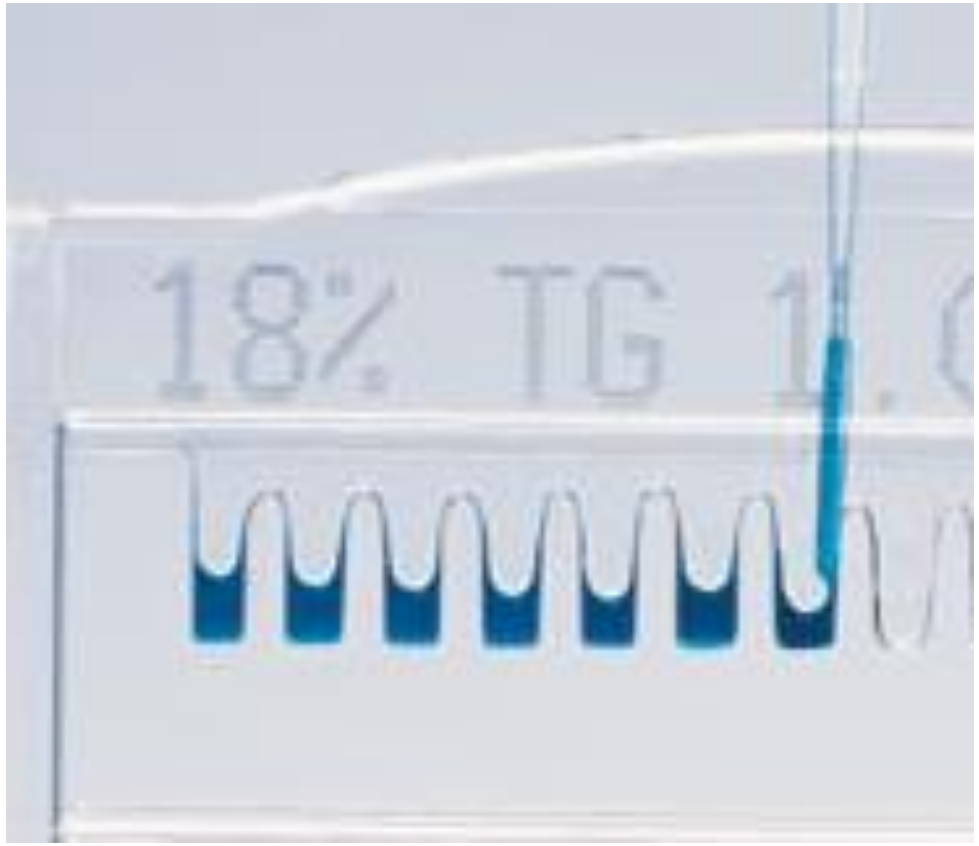


2. Loading controls

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

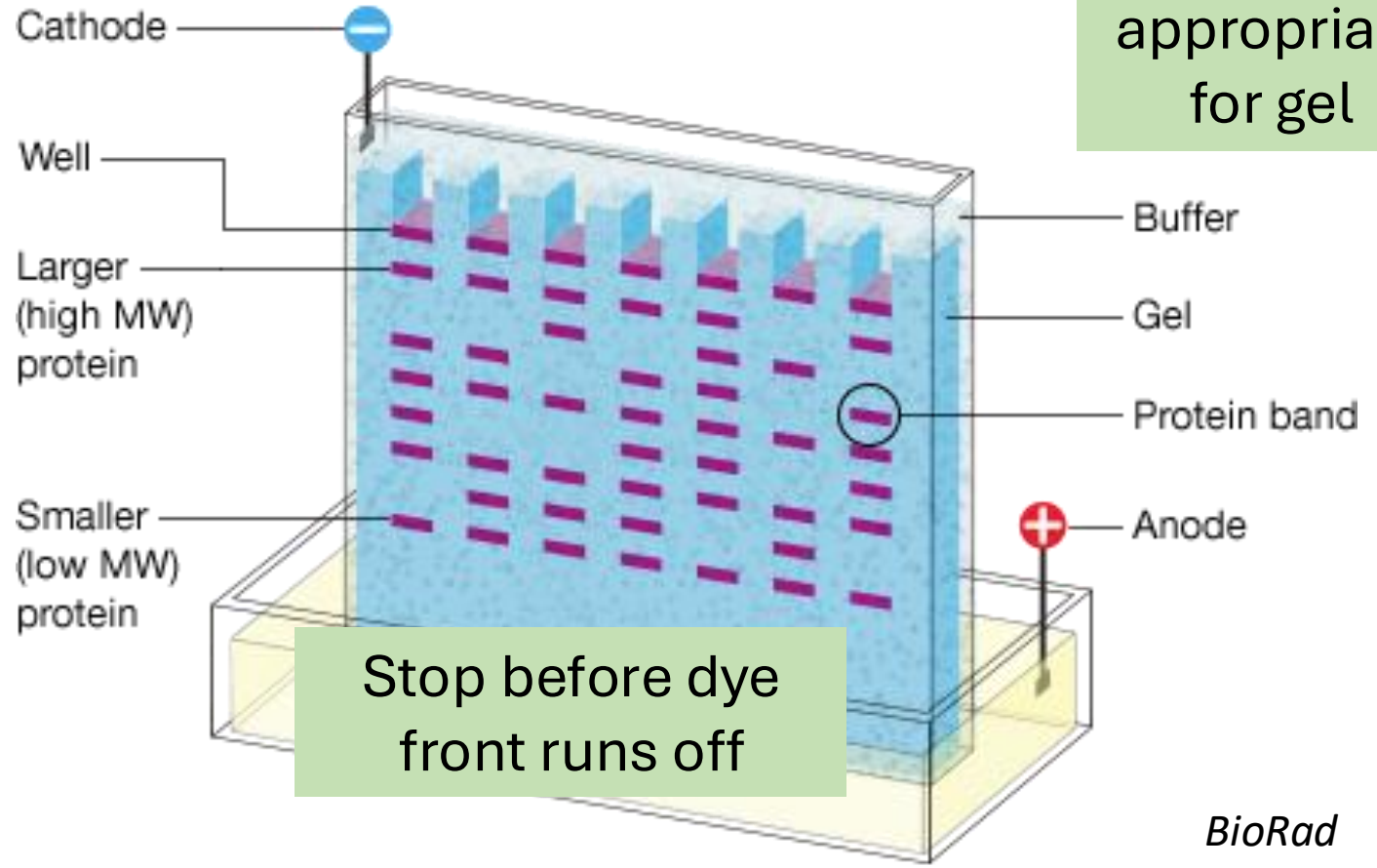
Well Types	Maximum Load Volume
1 well 1.0 mm	700 µL
2D well 1.0 mm 1.5 mm	400 µL 600 µL
IPG well 1.0 mm	7 cm IPG Strip
9 well 1.0 mm	28 µL
10 well 1.0 mm 1.5 mm	25 µL 37 µL
12 well 1.0 mm	20 µL
15 well 1.0 mm 1.5 mm	15 µL 25 µL
17 well 1.0 mm	15 µL



Running the Gel

Appropriate voltage (generally voltage limit)

Buffer appropriate for gel



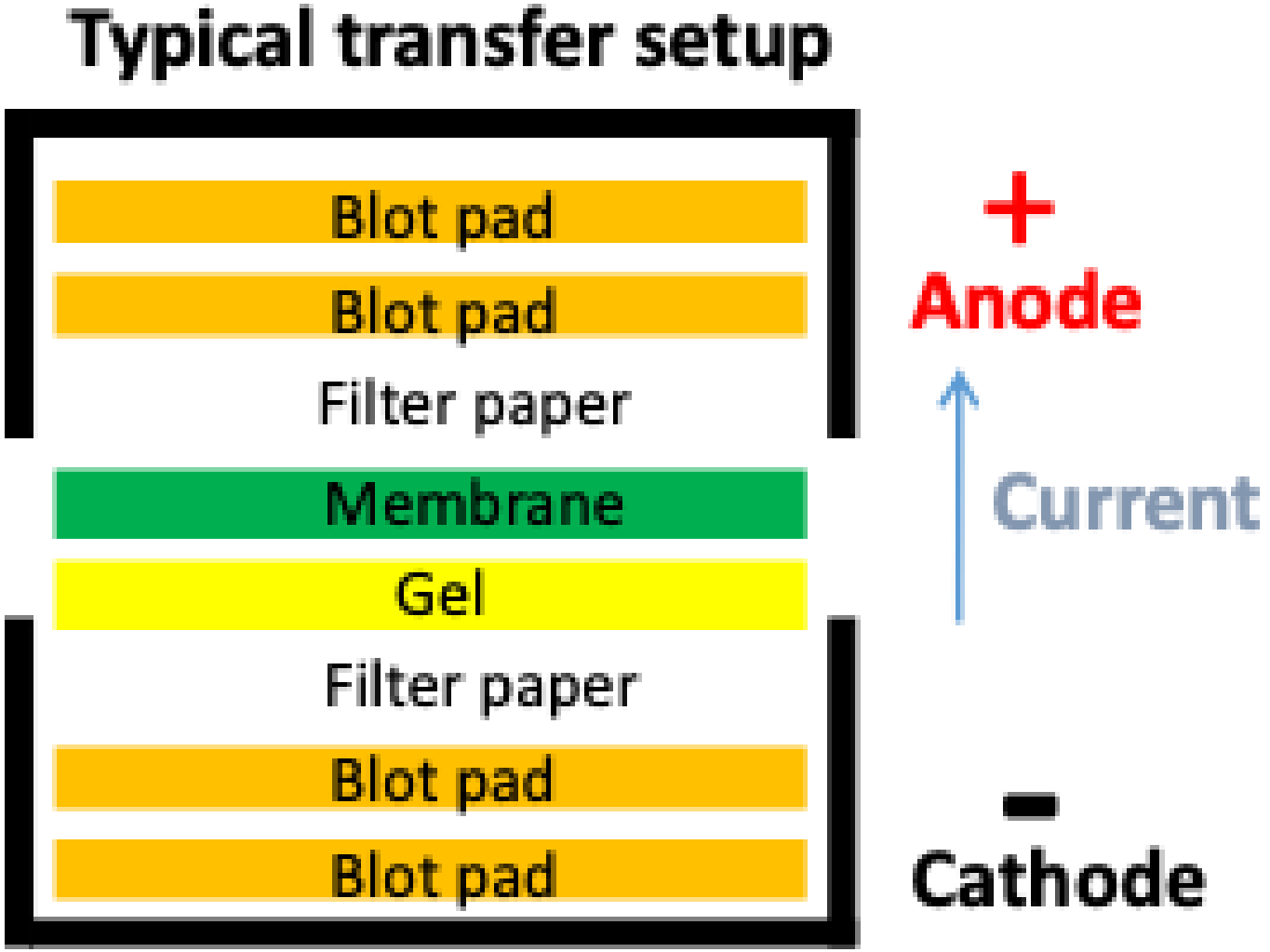
Stop before dye front runs off

BioRad

Transfer immediately to avoid diffusion!

Step 4: Transferring

Setting up the ”Transfer Sandwich”

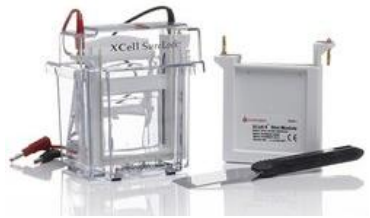


Remember membranes bind proteins!

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

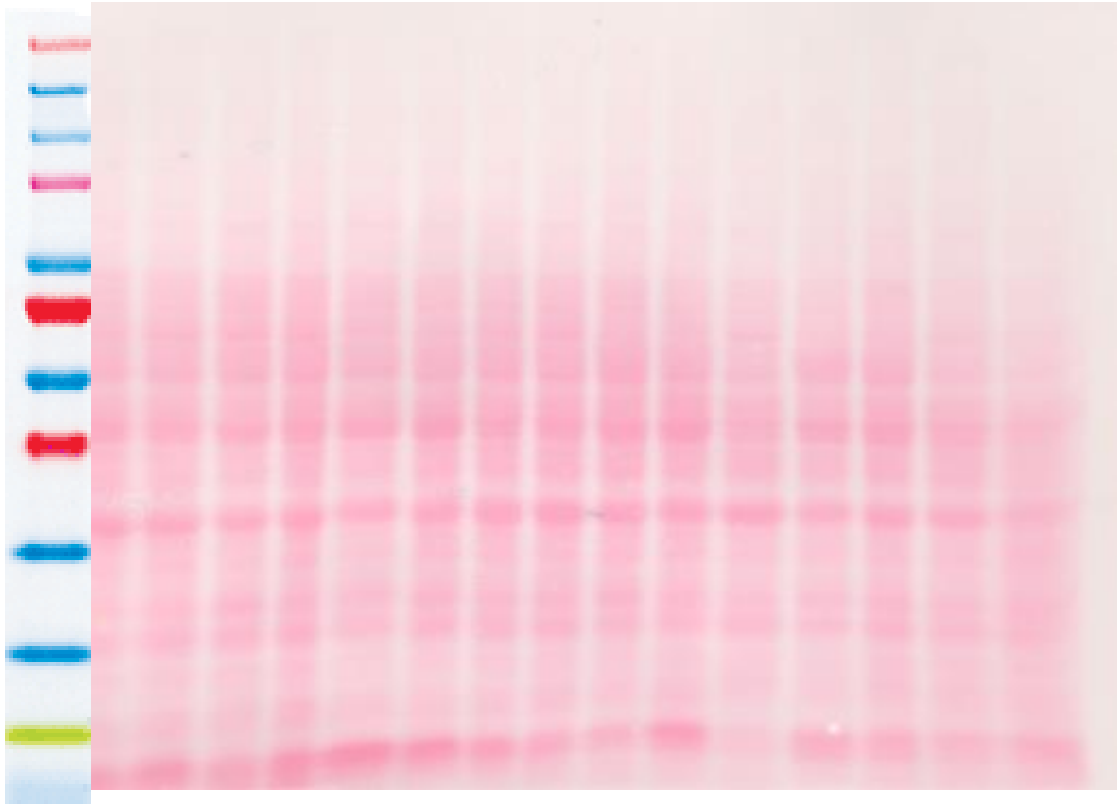
- Faster transfer (~15-60mins) but not as efficient and cannot transfer large proteins (>150kDa).
- Low buffering capacity means its no good for prolonged transfers.
- Prone to current leakage.

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.

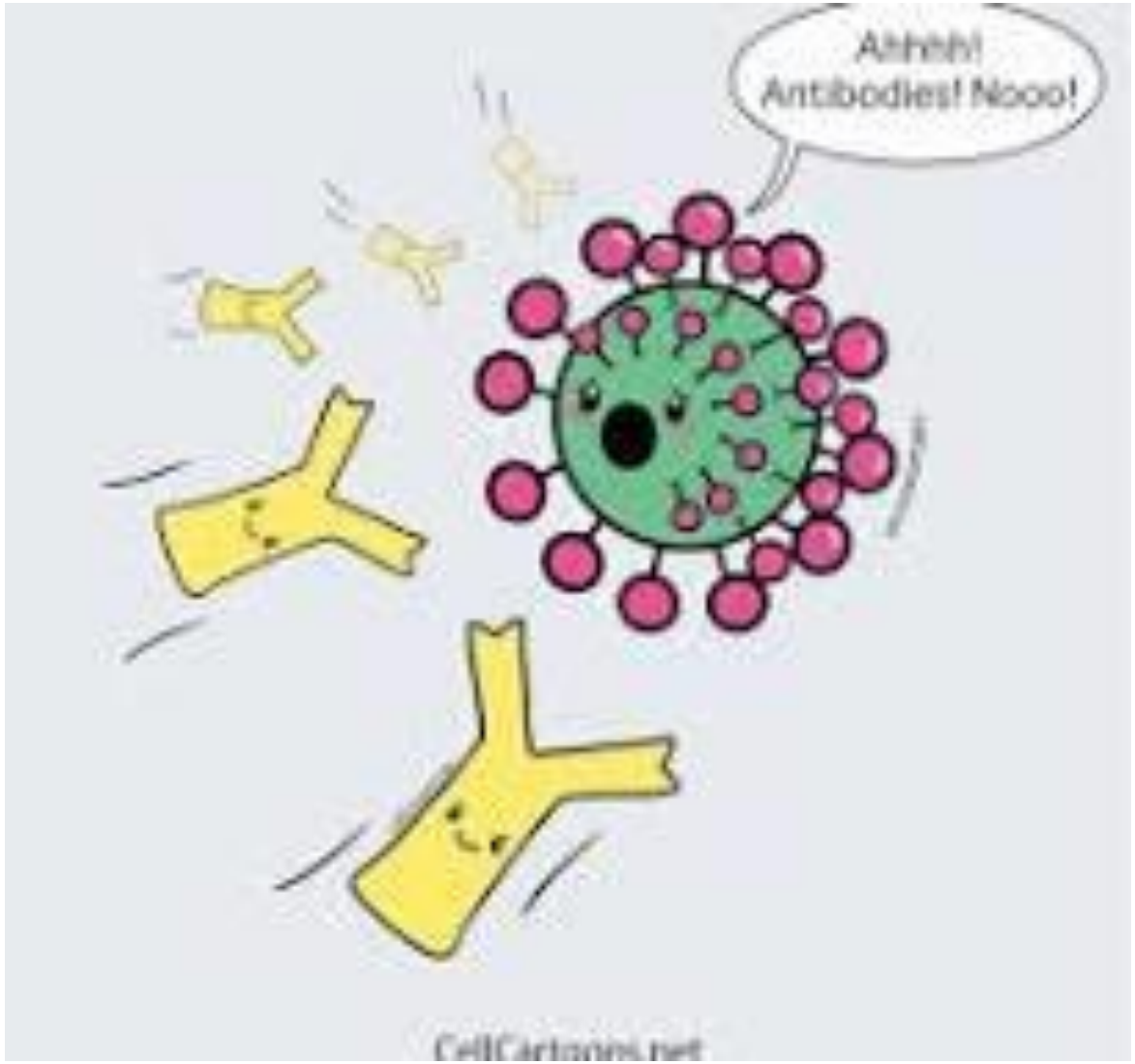
Staining Proteins on Membrane

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



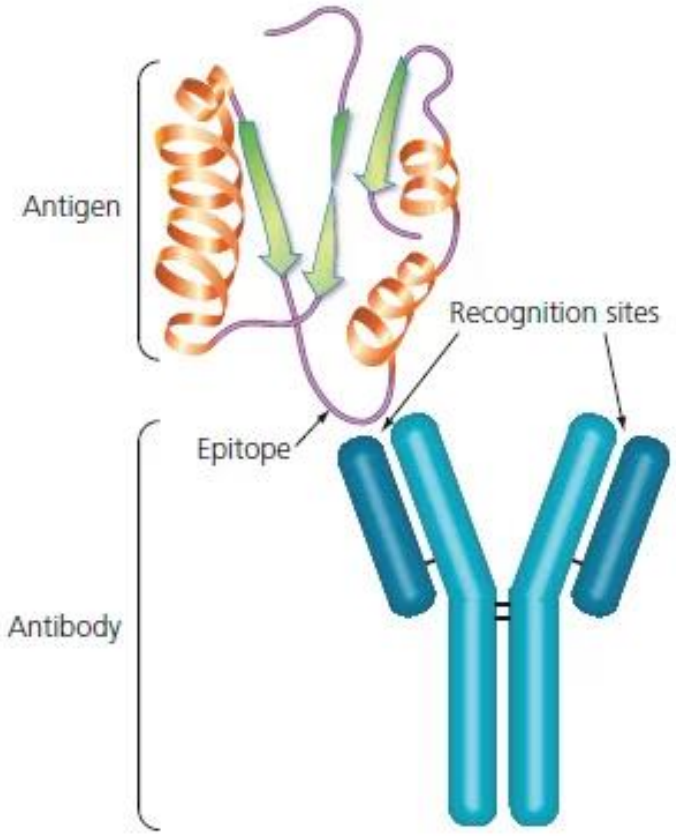
Blocking the Membrane

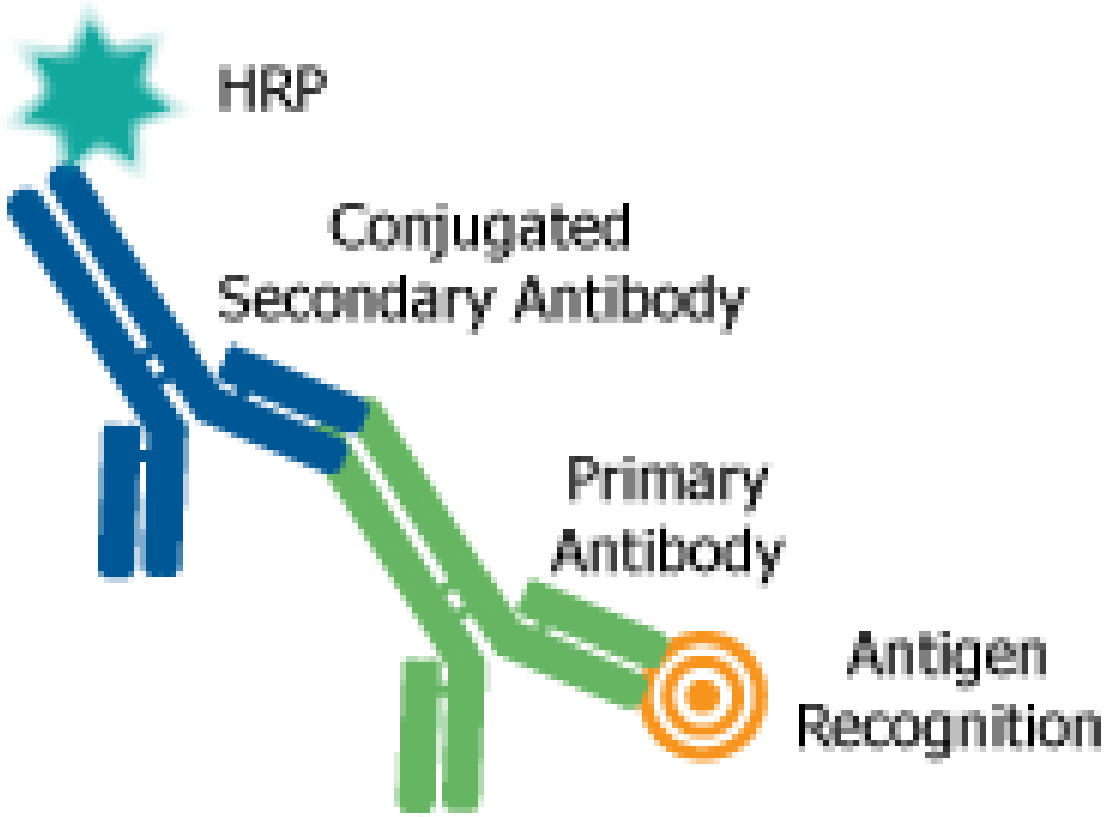
Typical block buffers:

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

Made up in the same buffer as antibody

Probing with Antibody



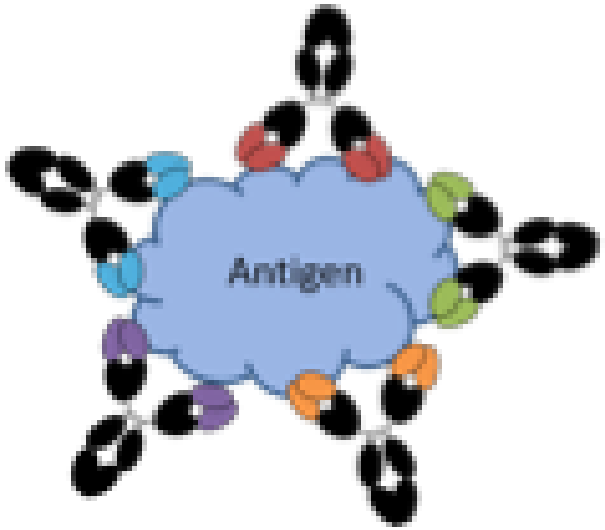


Typically use a primary-secondary combination:

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

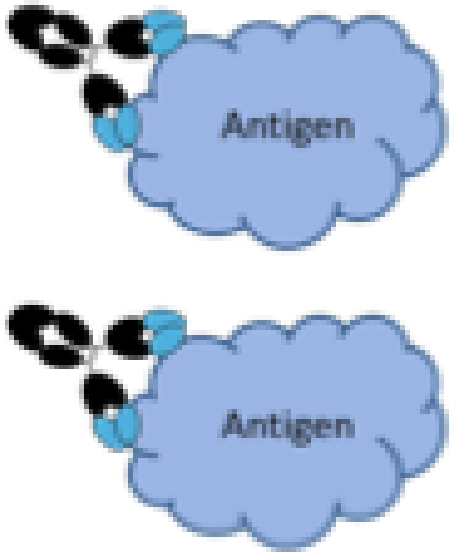
Antibodies: Polyclonal vs. Monoclonal

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

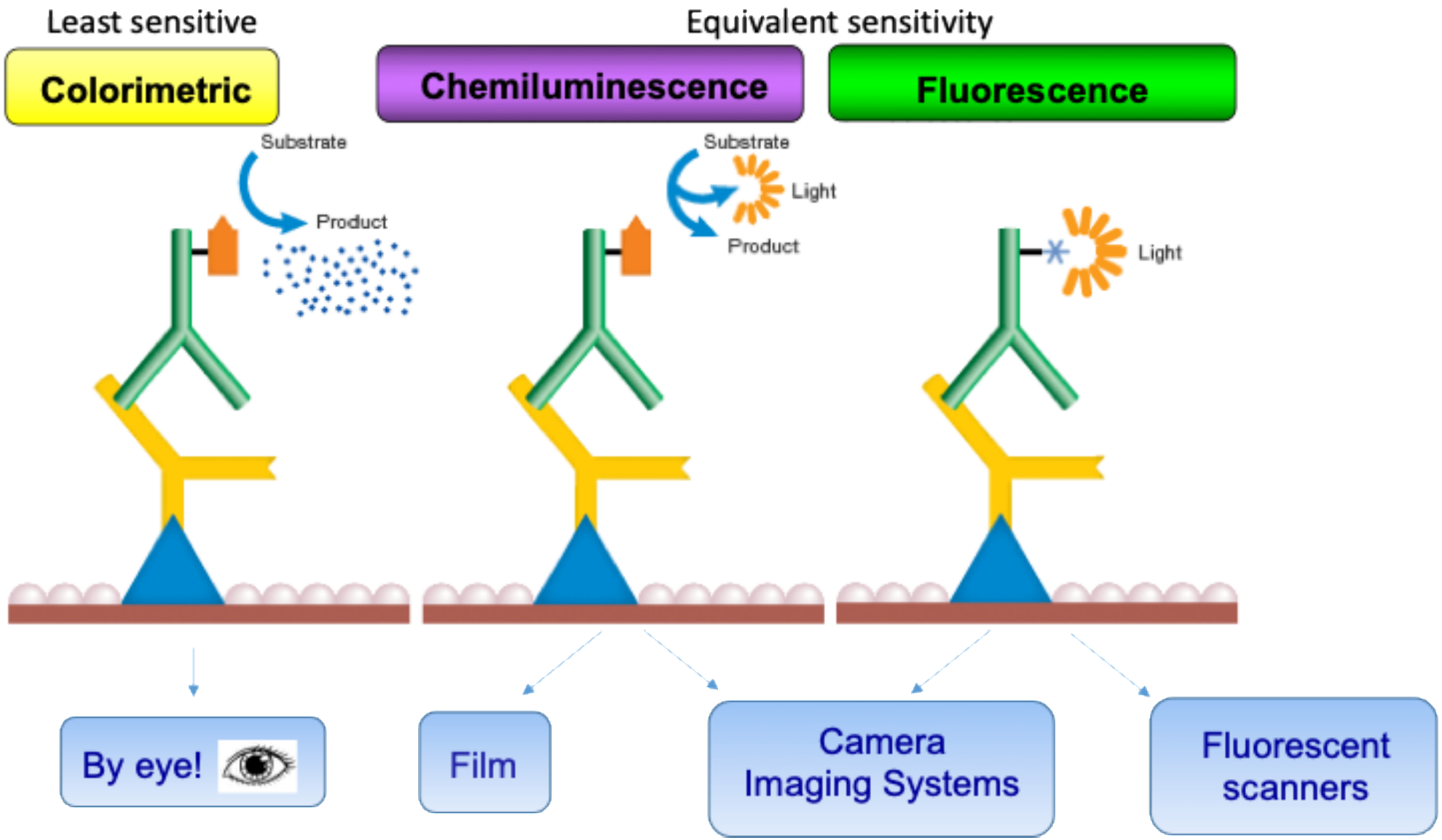
Concentration of primary to use

- 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

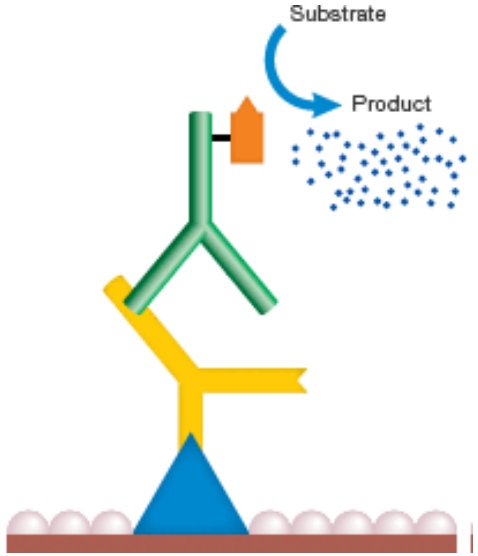
For multiplexing, use primary antibodies derived from different species

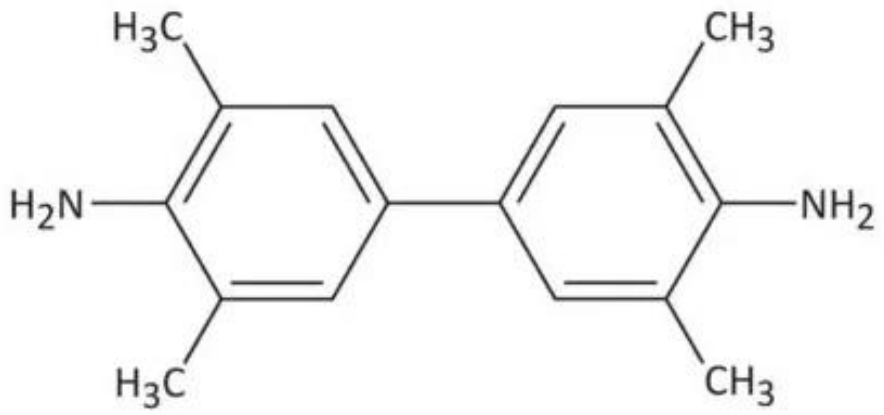
Step 6: Imaging

Options for Imaging

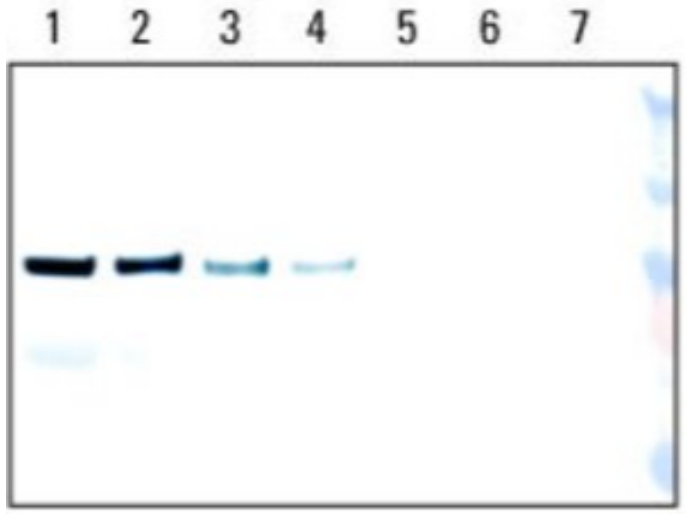


Colometric Imaging





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



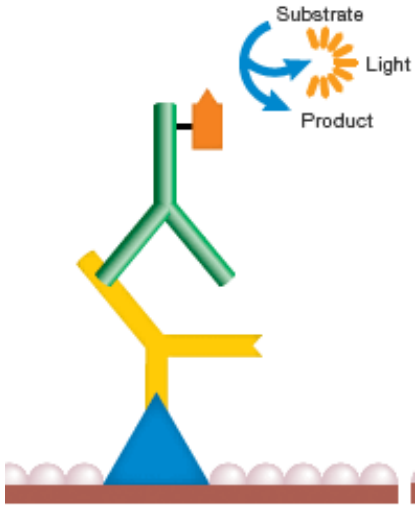
Advantages:

- 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:

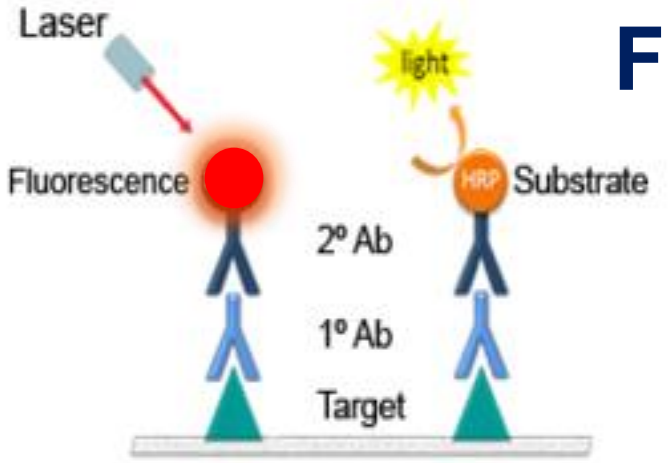
- High sensitivity (and many substrate options to adjust)
- Routinely used
- Widely accepted

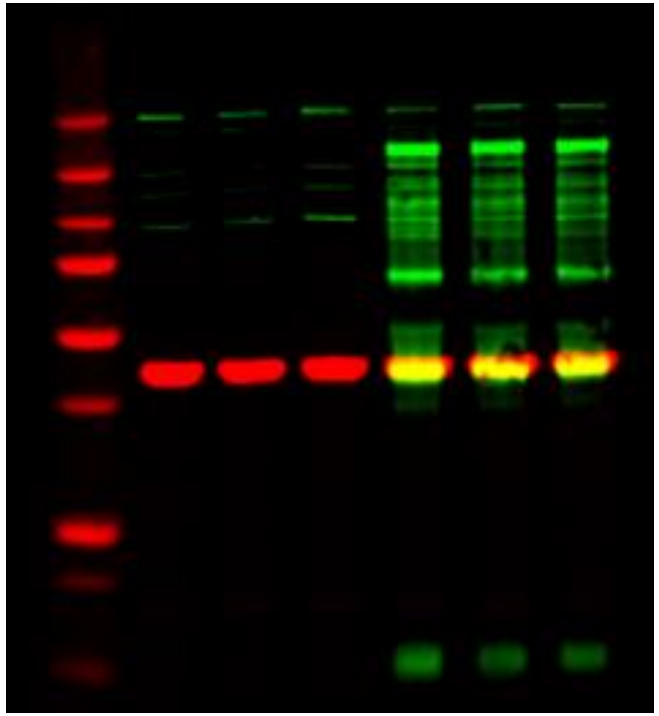
or

Disadvantages:

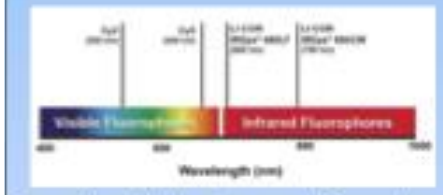
- Not fully linear, signal saturates
- Signal not infinitely stable
- Cannot multiplex

Fluorescence-Based Imaging

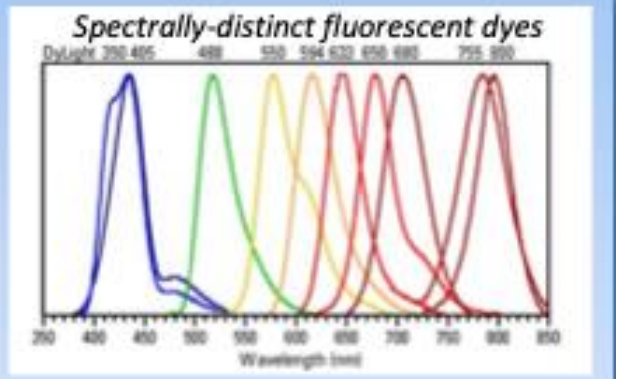




Spectrally distinct fluorescent dyes for WB

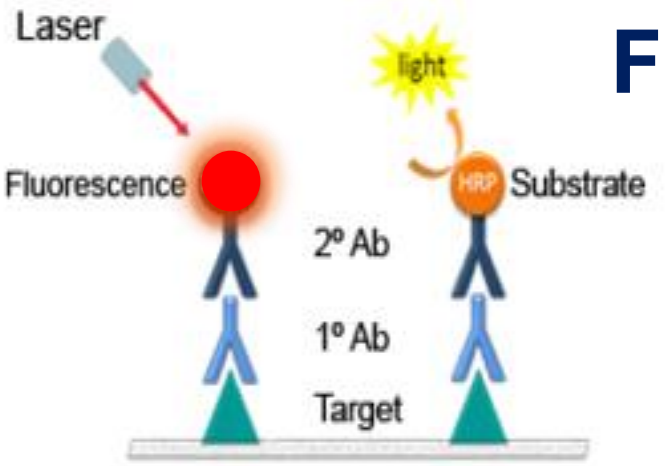


Span the visible to near infrared range



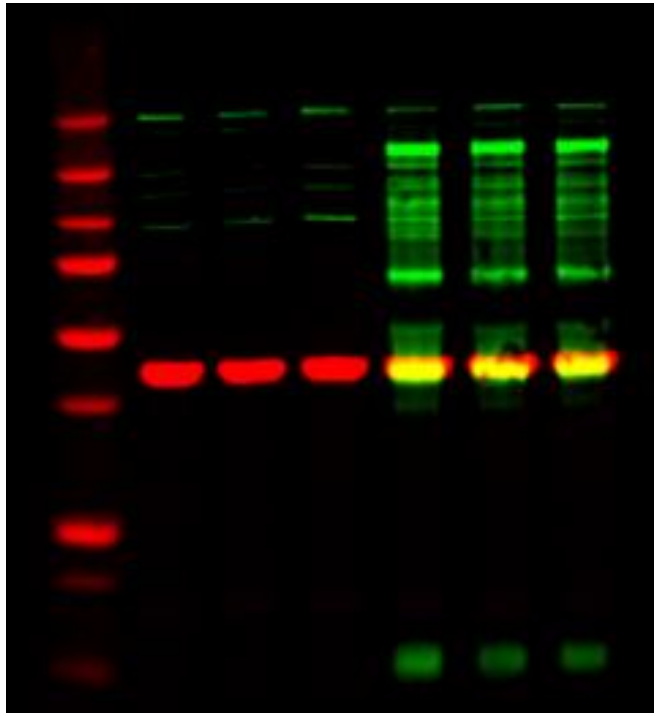
- Li-Cor Infra-red dyes
- DyLight dyes
- AlexaFluors
- ECL Plex antibodies (Cy3/Cy5)
- Qdots

Fluorescence-Based Imaging



Advantages:

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



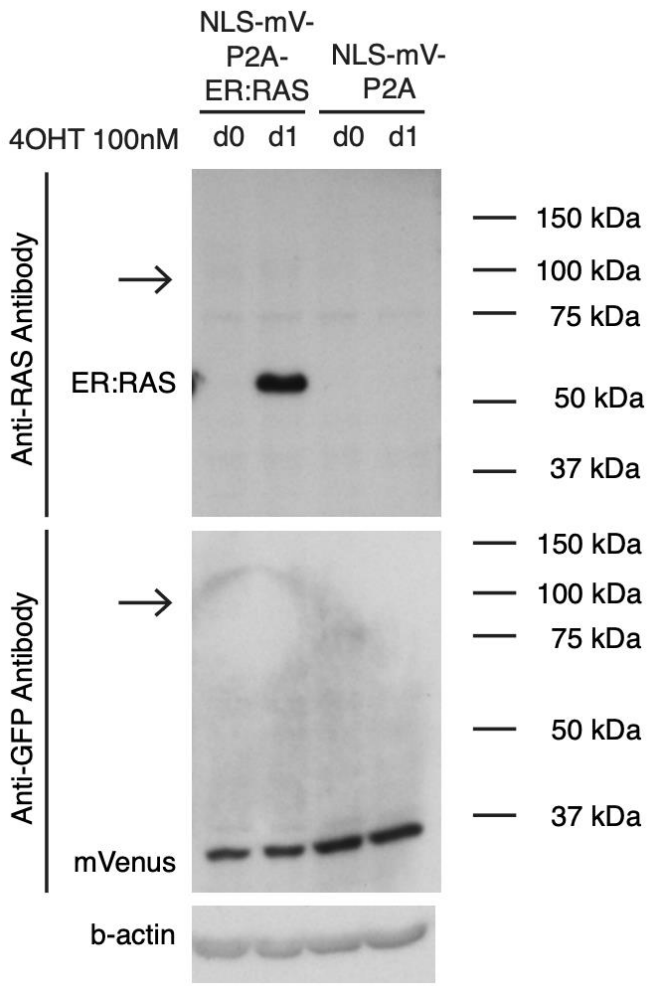
Disadvantages:

- Requires the most specialized equipment and reagents

Step 7: Quantification & Analysis

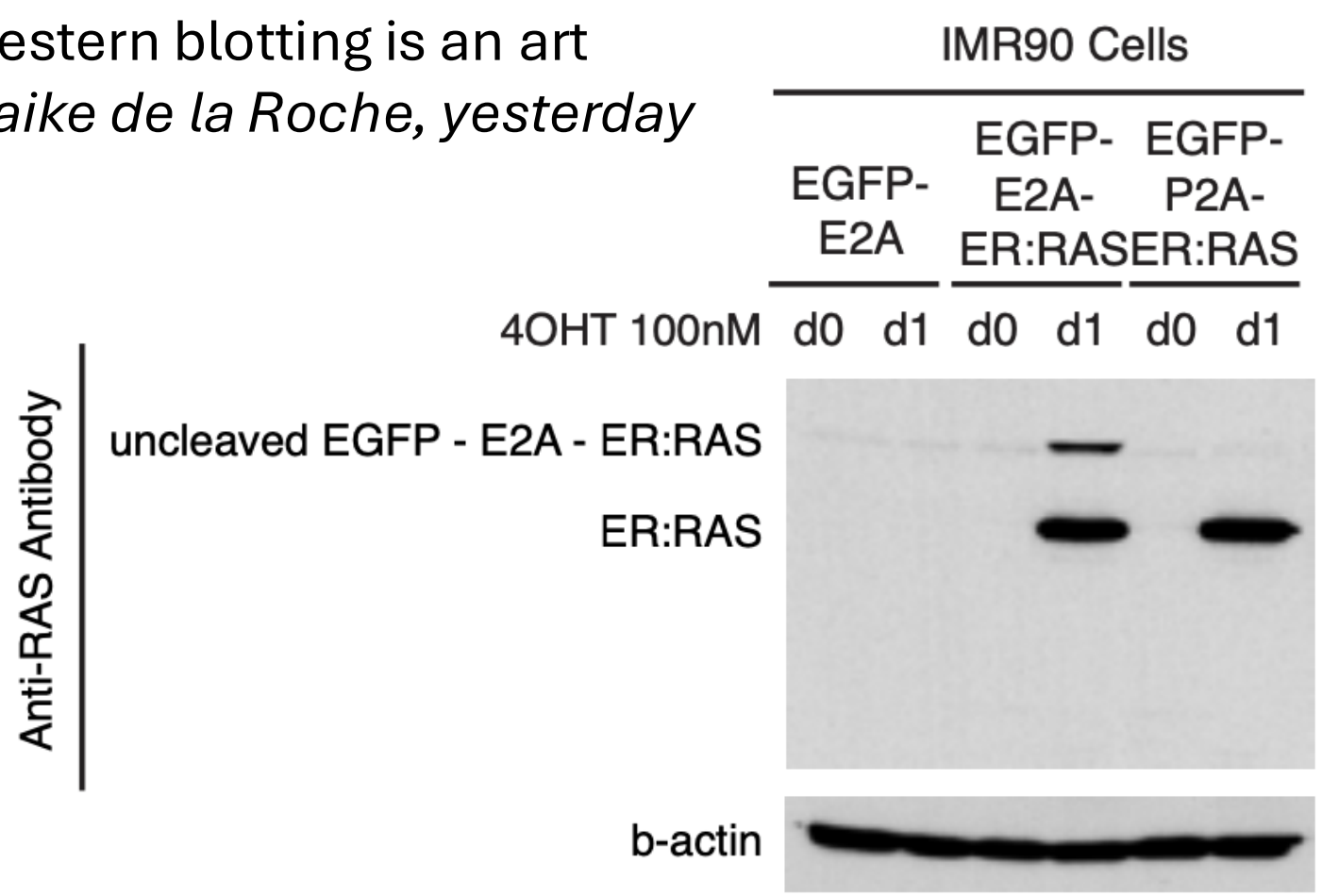
Quantifying Western Blots

Western blotting is inherently a qualitative technique!



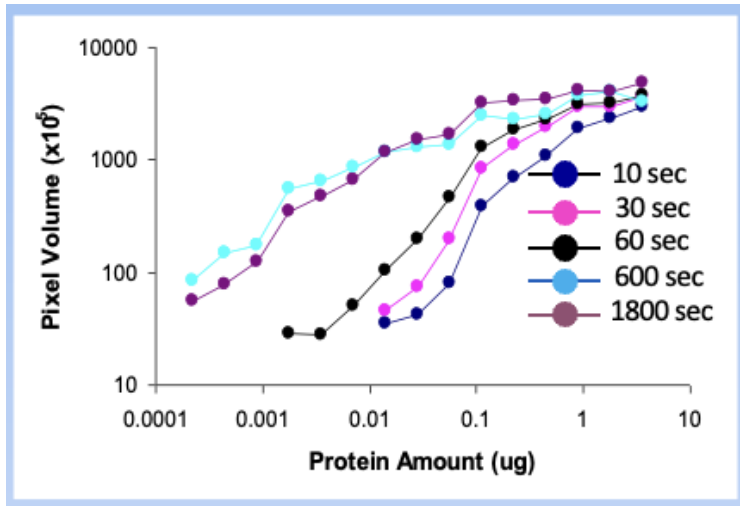
Western blotting is an art

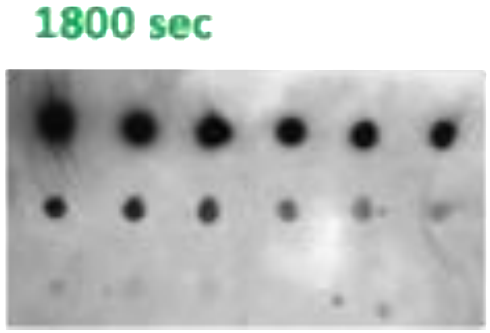
Maike de la Roche, yesterday

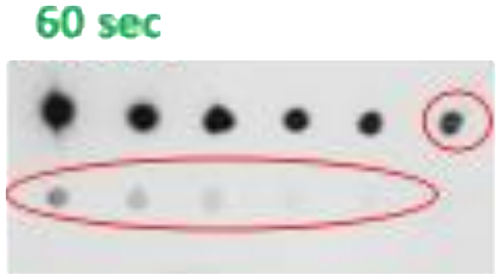


The Importance of Linearity

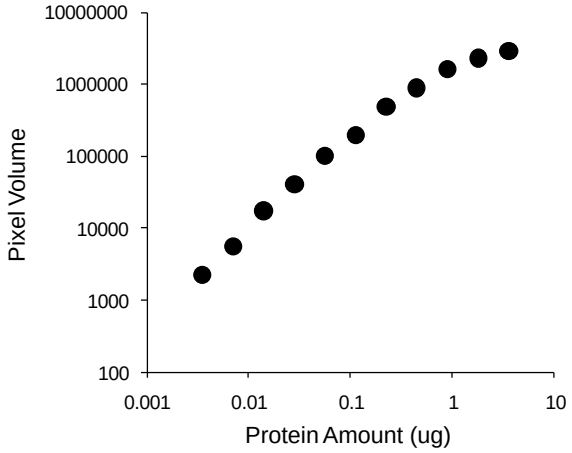
Chemiluminescence (with film)

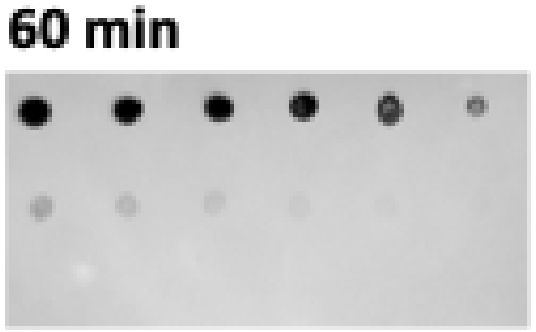




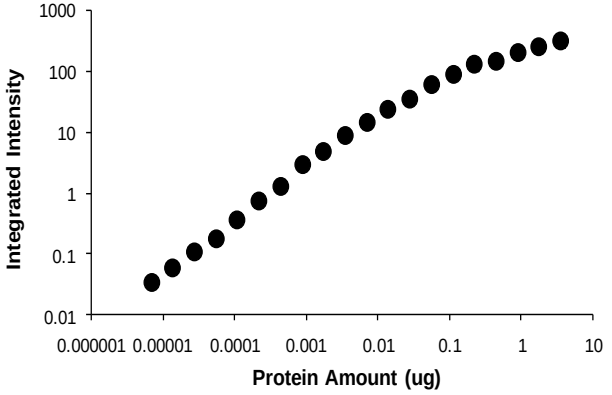


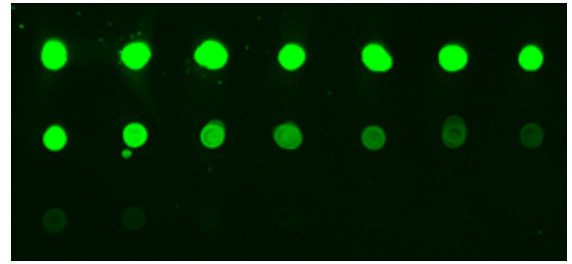
ECL (digital camera)



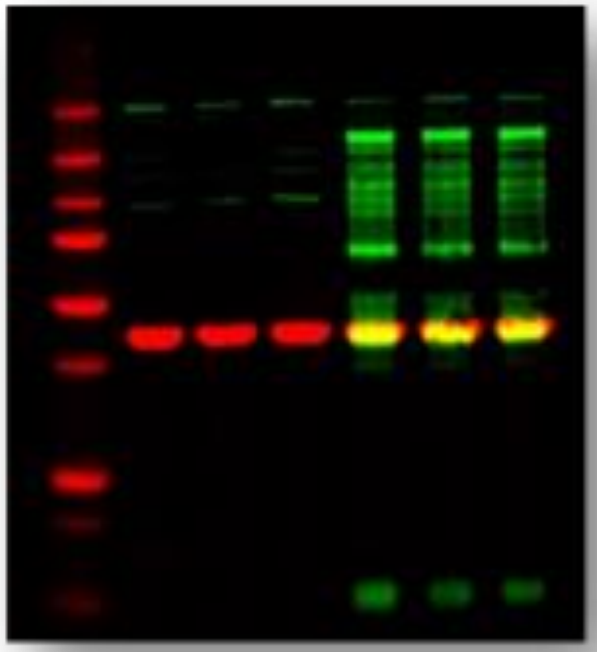


Fluorescence (digital scanner)





Normalisation and Loading Controls



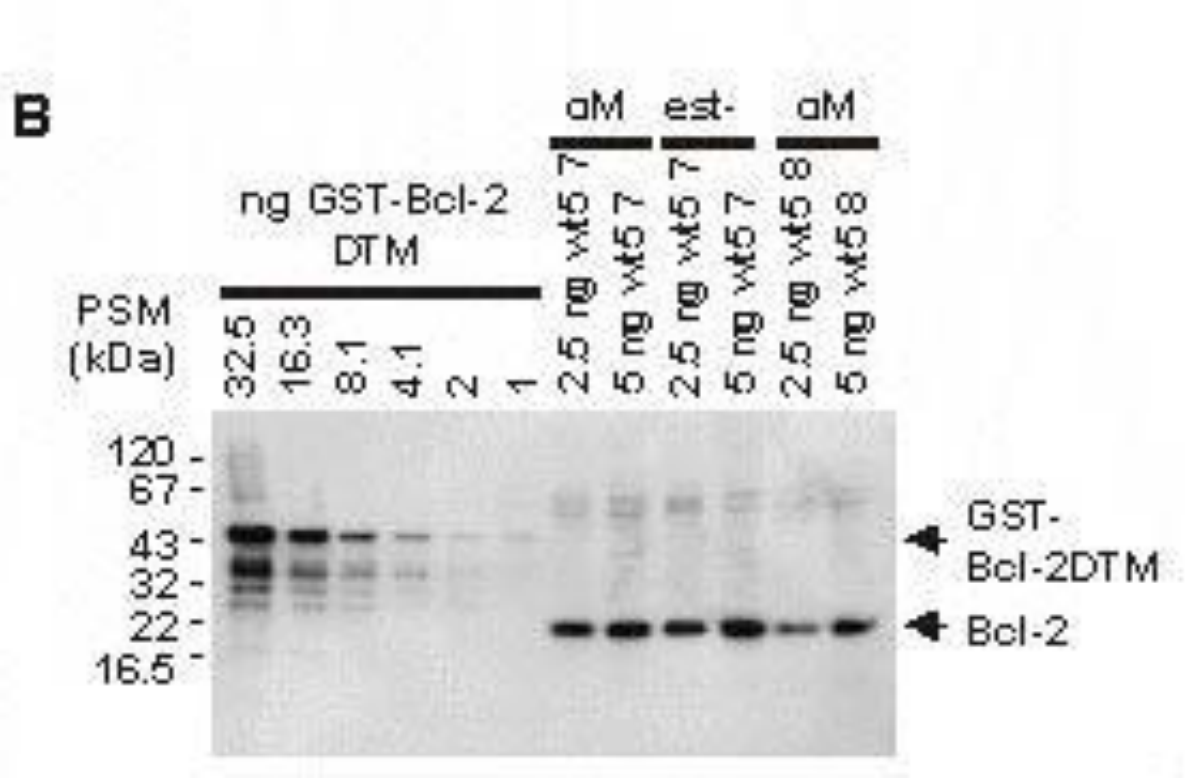
Housekeeping protein (Red)

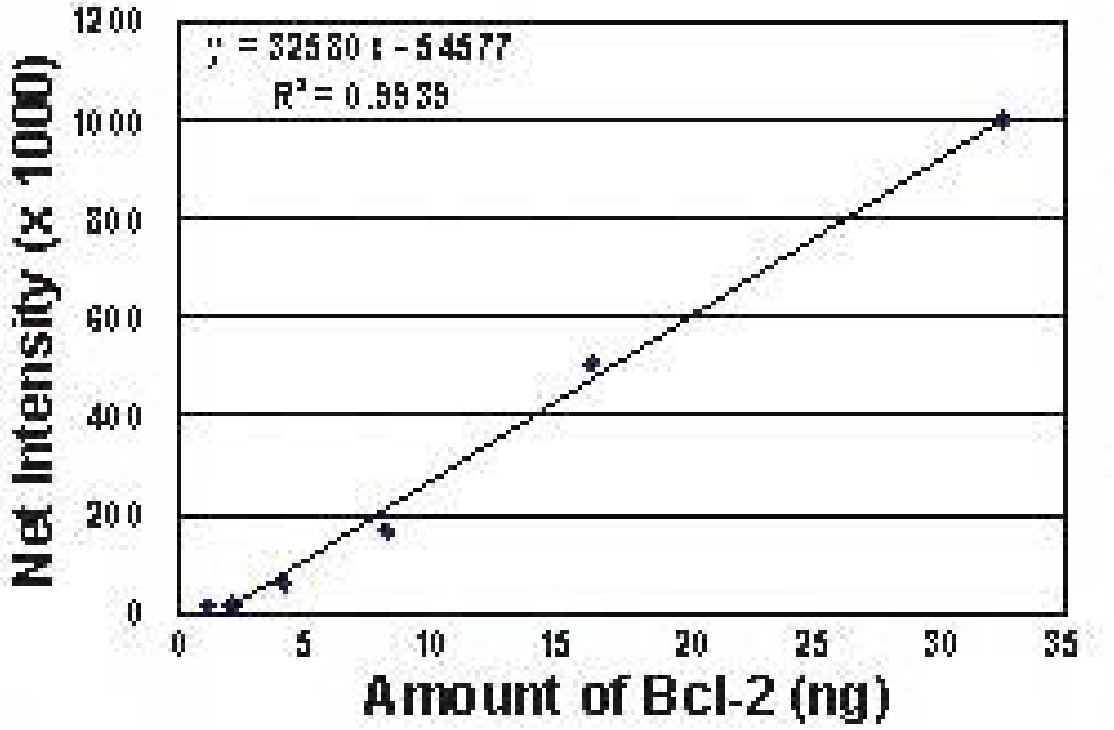
Ensuring that quantification is not due to:

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



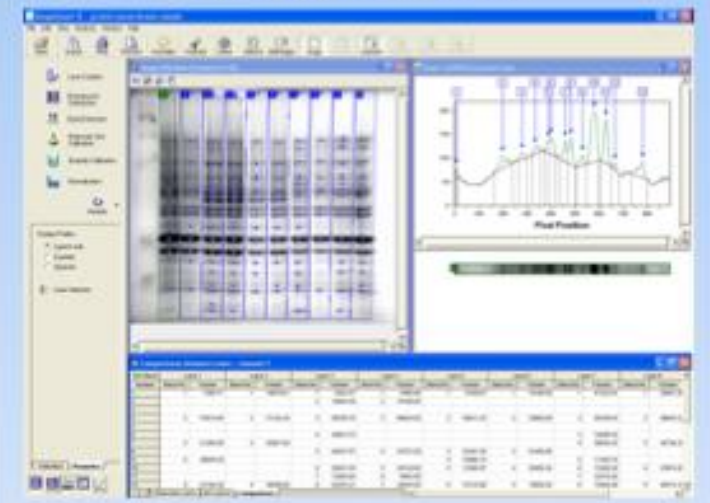


Requires purified protein of your target protein, and a standard curve on the same blot

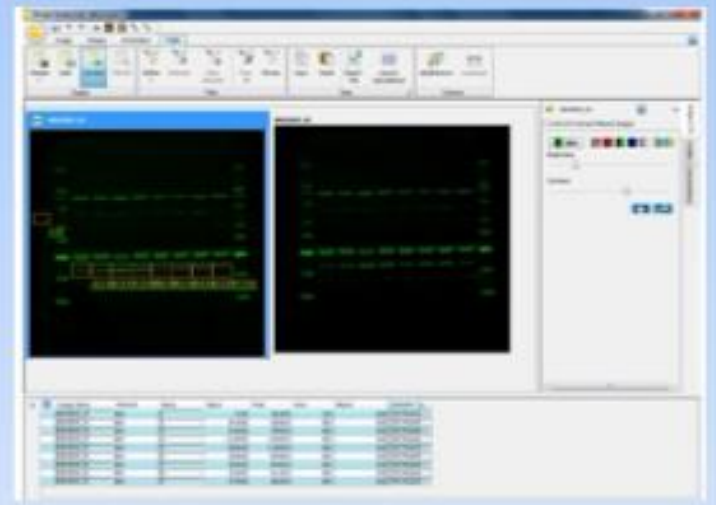
Analysis Software: Options

Licenced

ImageQuant



ImageStudio



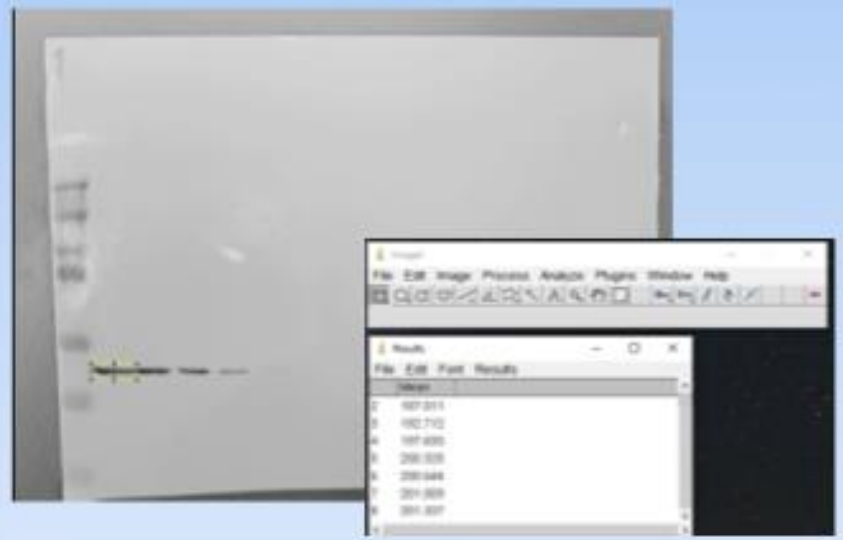
Require 16 bit .tif files

Generated from digital camera or scanner

Wizard-based software

Free!

Image J



Very basic software

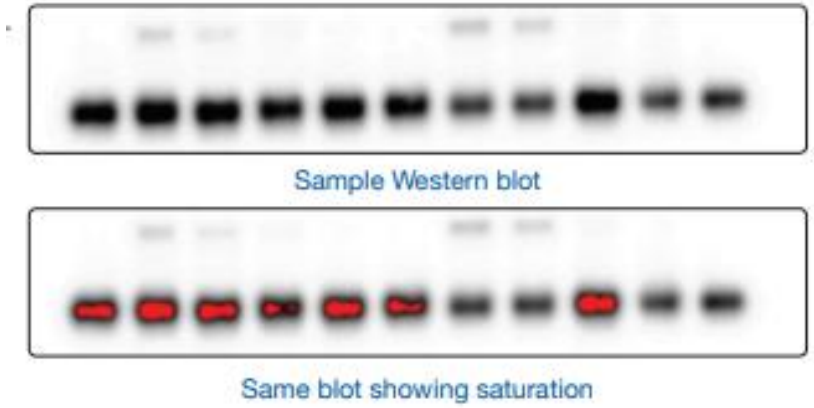
Manual analysis

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

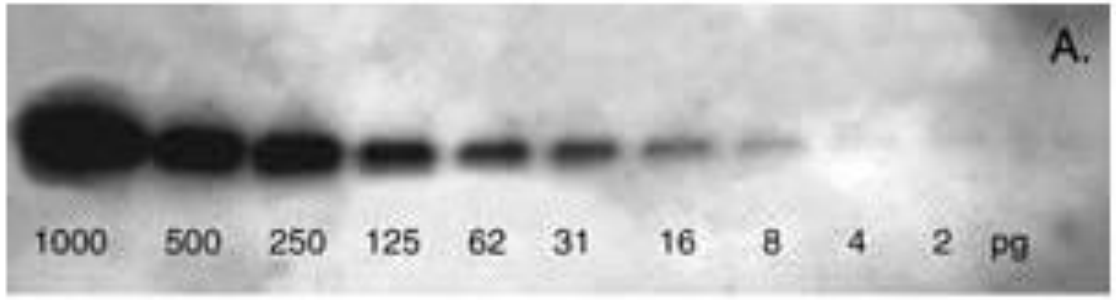
Saturation – remember linearity is key!



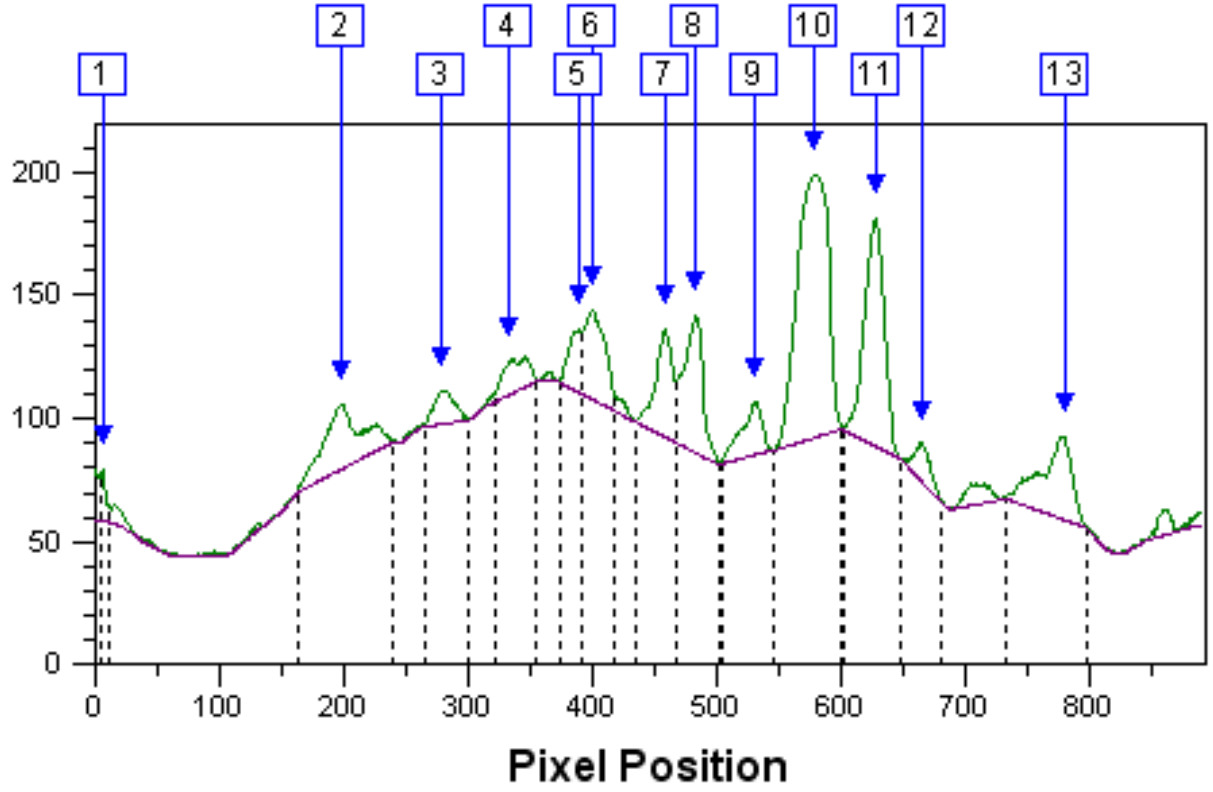
- NOT POSSIBLE to quantify saturated samples
- Some analysis software integrate saturation detection
- Others don’t (need to use image analysis software to check this beforehand)

Analysis: Pitfalls

Background subtraction



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



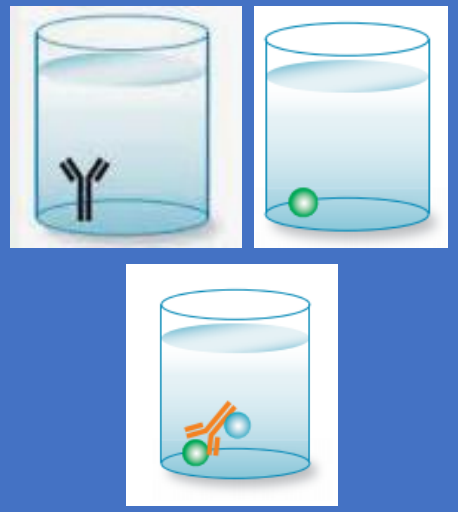
Replicates, replicates, replicates!!

ELISA: The Theory

Key Steps

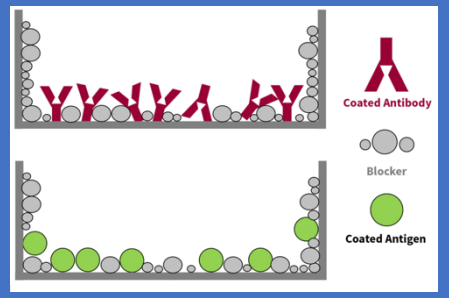
Capture

.... 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

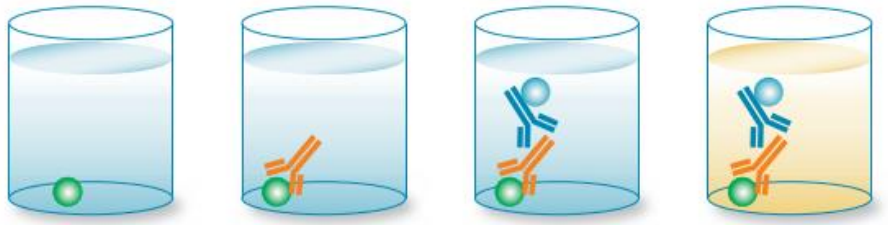
.... To measure your protein signal

Different Types

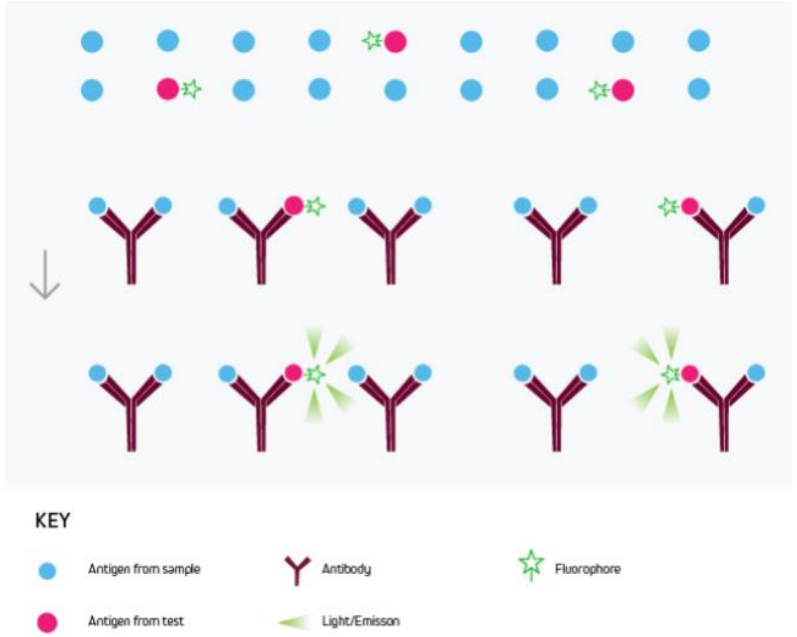
Direct ELISA

Sandwich ELISA

Indirect ELISA



Competitive ELISA



Capture

Capture onto high-binding microtiter plates

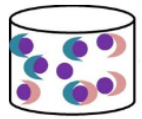
Detection method	Typical microtitre plate used
Colorimetric	Clear
Chemiluminescence	Black or white
Fluorescence	Black plates

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

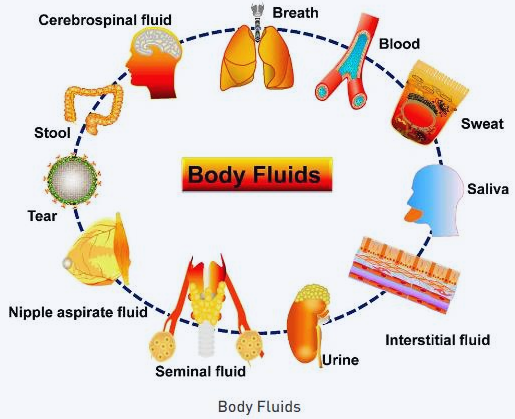
Sample Preparation – Not Just Lysates!

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



Consider biosafety level of samples

Food stuffs (e.g. milk)



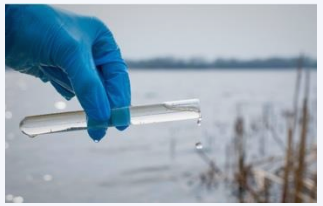
Body Fluids

Tissue homogenates

Samples analysed by ELISA vary extensively……

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