UNIVERSITY OF CAMBRIDGE

Biochemistry of protein interactions: pull-downs, IPs and ELISA

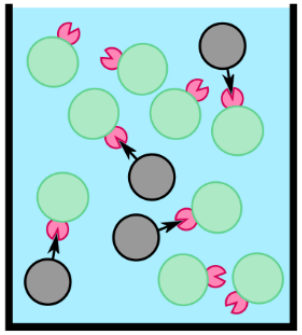
Day 6: Monday 27th March

This talk

- Identifying and characterising protein interactions
  - Pull-downs
  - Co-immunoprecipitation
  - Western blots
  - ELISA

Pull-down experiments

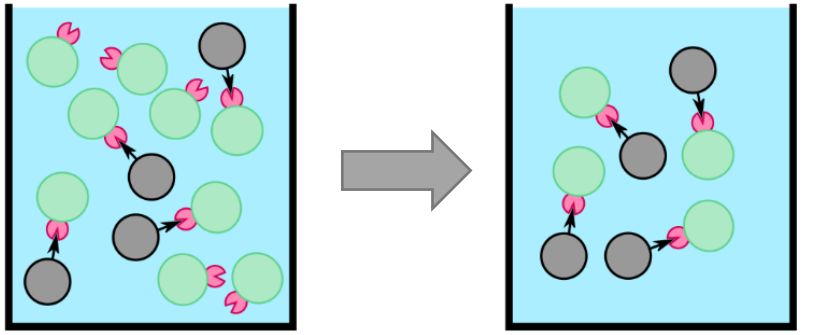
- Immobilise ***bait*** protein onto a resin
  - Pull-downs use purified bait protein



1. Load affinity resin with **bait**

Pull-down experiments

- Immobilise ***bait*** protein onto a resin
  - Pull-downs use purified bait protein
- Wash away unbound bait

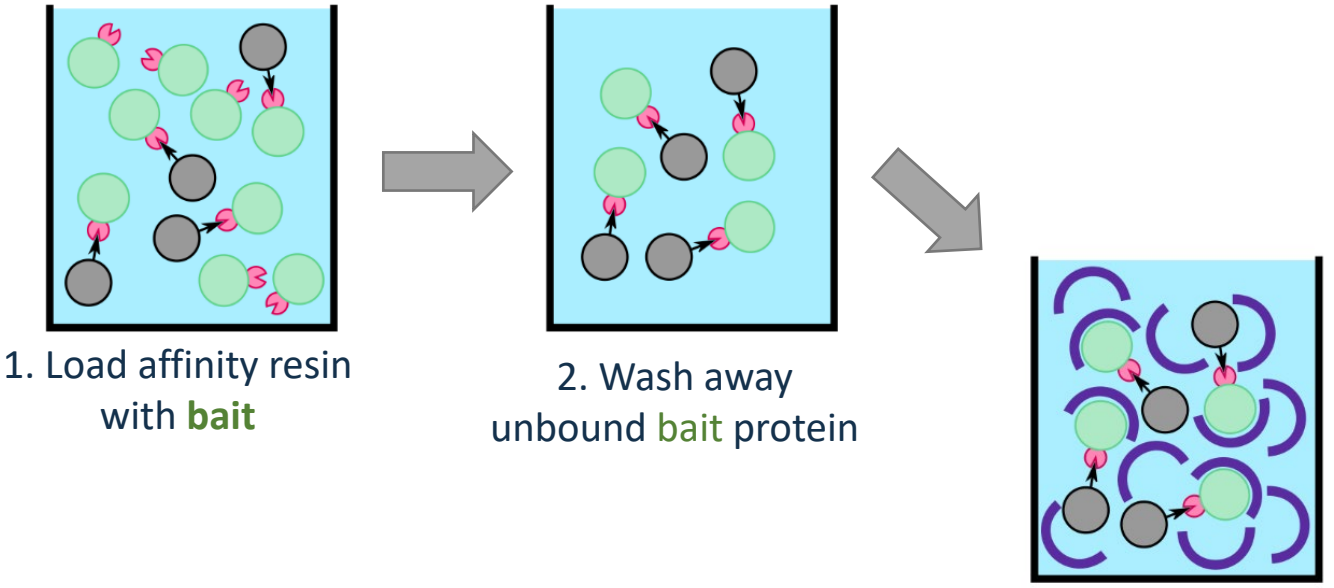


1. Load affinity resin with **bait**

2. Wash away unbound bait protein

Pull-down experiments

- Immobilise ***bait*** protein onto a resin
  - Pull-downs use purified bait protein
- Wash away unbound bait
- Incubate the bait with a ***prey*** protein
  - Can be purified or as part of a complex mixture



1. Load affinity resin with **bait**

2. Wash away unbound bait protein

3. Add **prey** protein

Pull-down experiments

- Immobilise ***bait*** protein onto a resin
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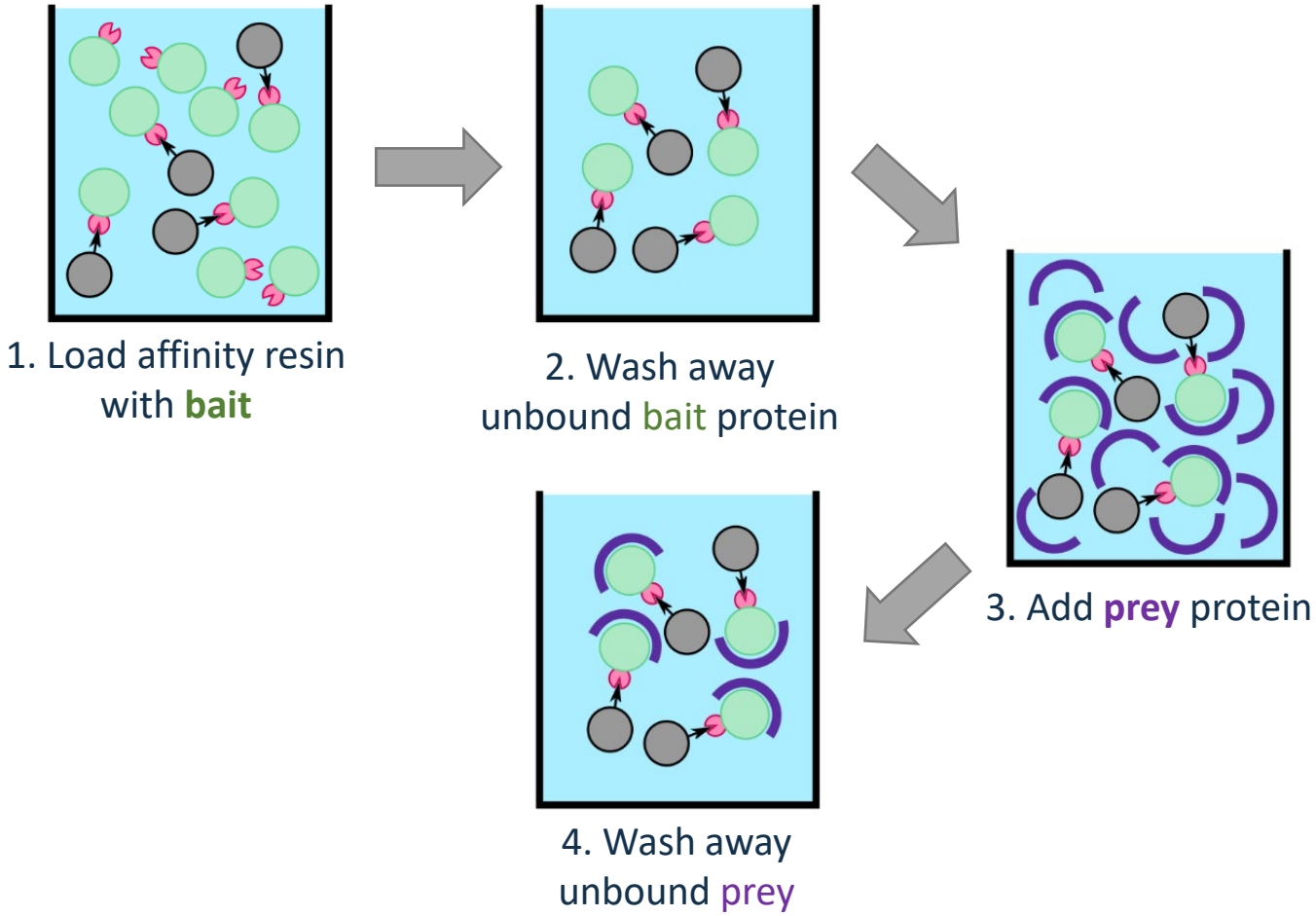
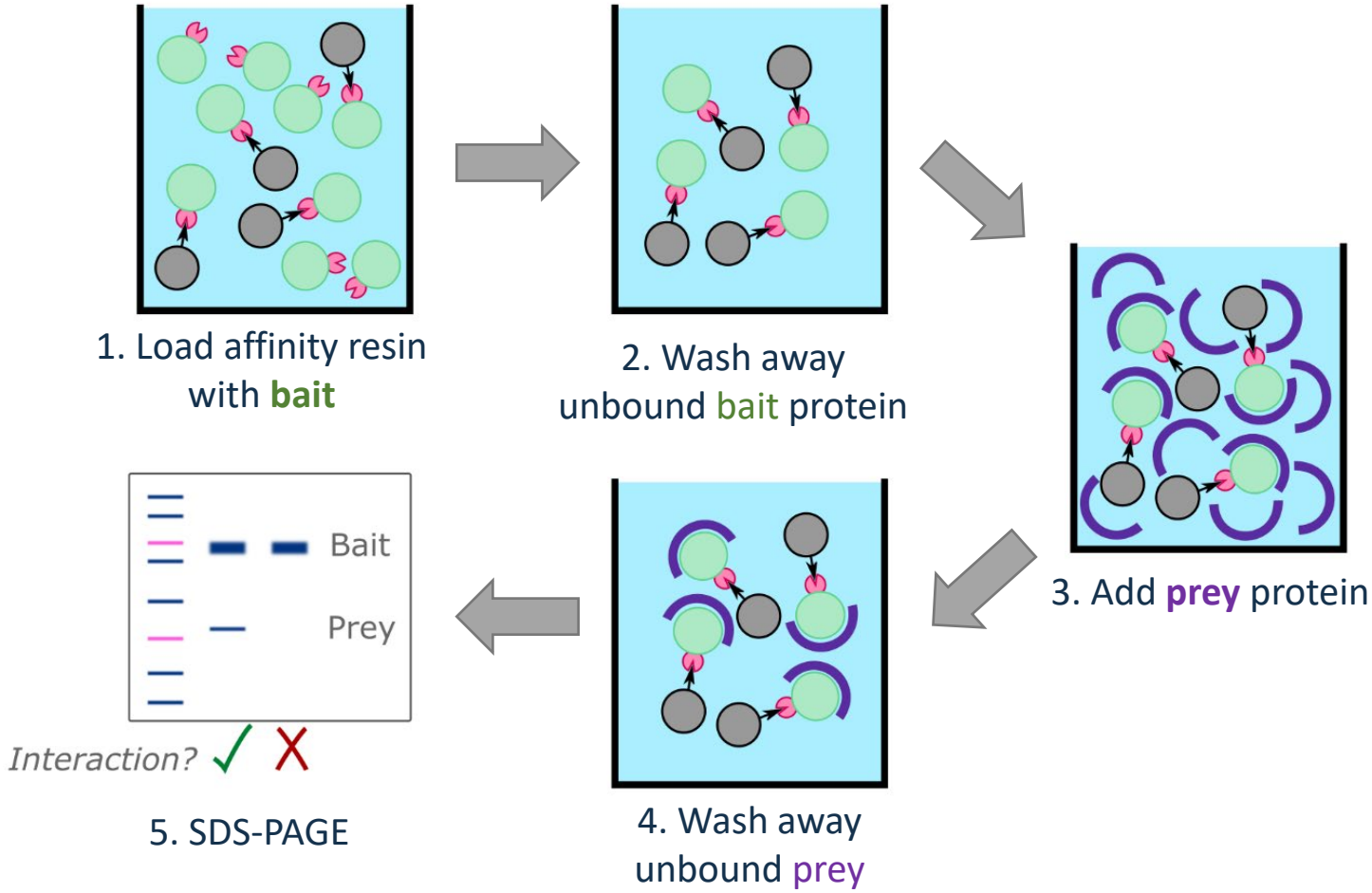
- Wash away unbound bait
- Incubate the bait with a ***prey*** protein
  - Can be purified or as part of a complex mixture
- Wash away unbound prey proteins

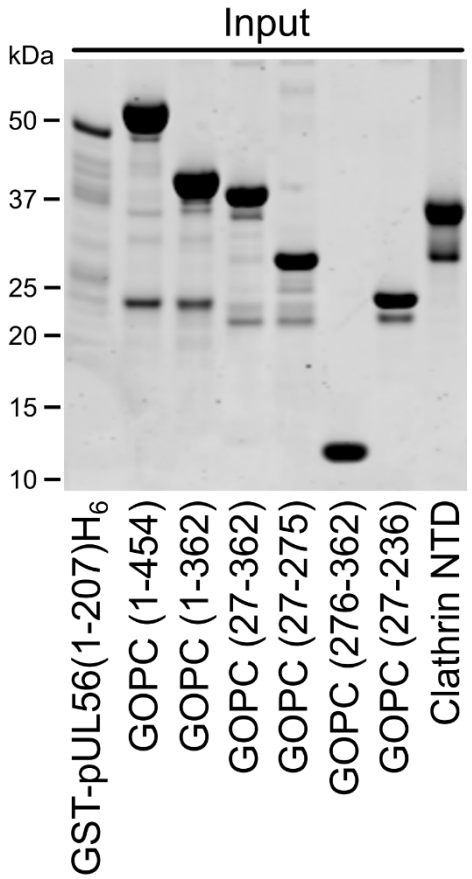


Pull-down experiments

- Immobilise ***bait*** protein onto a resin
  - Pull-downs use purified bait protein
- Wash away unbound bait
- Incubate the bait with a ***prey*** protein
  - Can be purified or as part of a complex mixture
- Wash away unbound prey proteins
- ***Elute*** bait and prey from resin and analyse



Mapping interaction domains by GST pull-down



Adapted from Soh et al. (2020) *Cell Reports* 33, 108235

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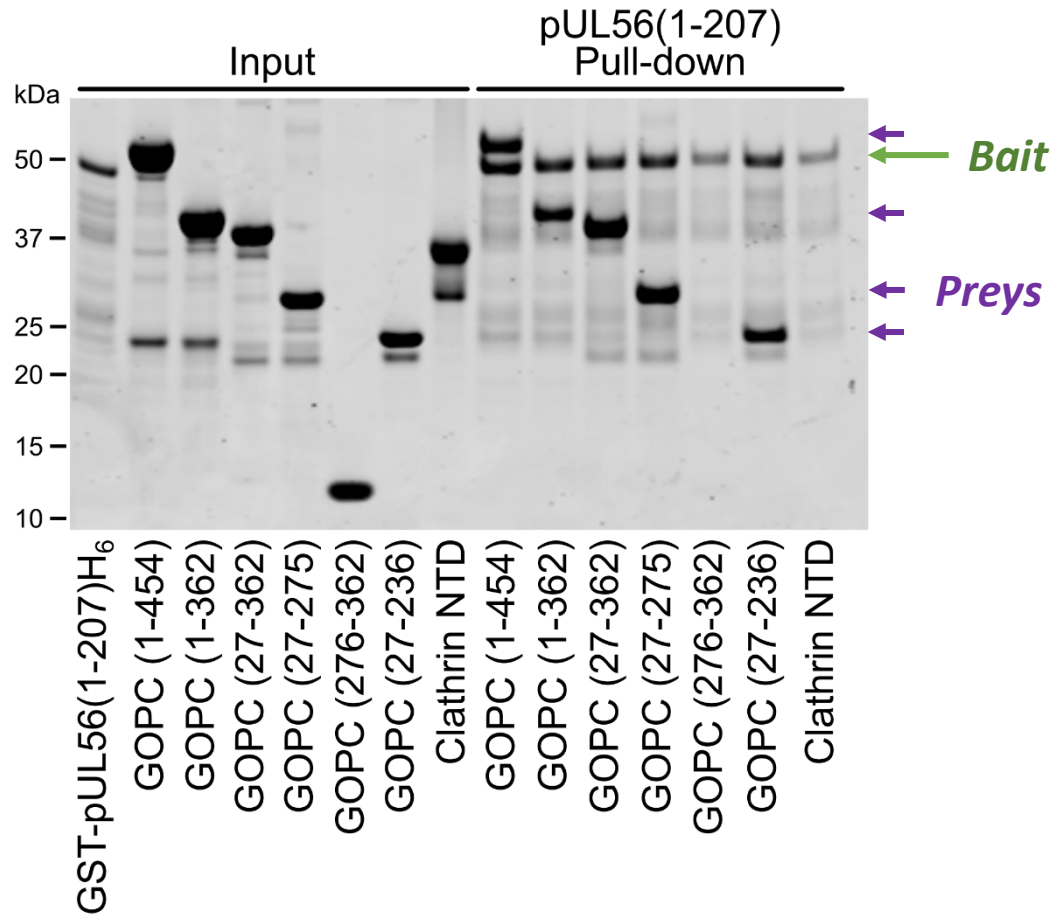

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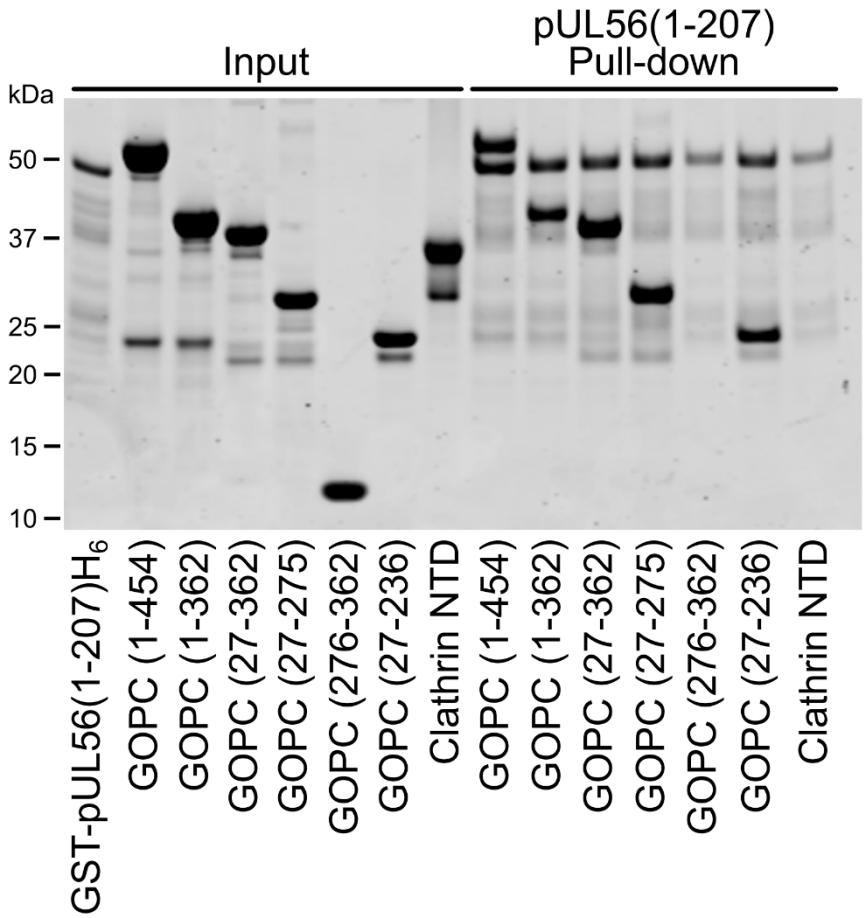
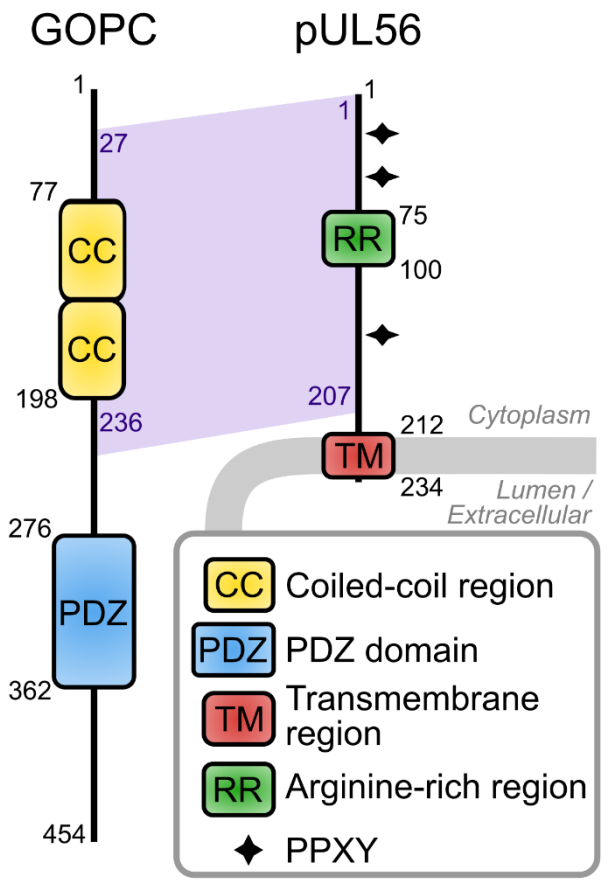
Mapping interaction domains by GST pull-down





Adapted from Soh et al. (2020) *Cell Reports* 33, 108235

Common affinity resins used for pull-downs

Resin	Tag	Pros	Cons
Glutathione (GSH)	GST	Cheap High affinity Low non-specific binding	Large protein tag may disrupt interaction
Ni^{2+} or Co^{2+}	His_6	Cheap Small tag	Low affinity (can be improved by using His_8 or His_{10} tags) High non-specific binding
Amylose	Maltose binding protein (MBP)	Cheap	Very large protein tag Relatively low affinity
Streptavidin	Biotin	Extremely high affinity Highly specific Small tag	Need to biotinylate prey Non-specific binding to endogenously biotinylated proteins
Streptactin-XT	Strep-II	High affinity High specificity Small tag	Expensive

You can use agarose or magnetic resin for pull-downs

Agarose resin	Magnetic resin
To wash, collect beads ***by centrifugation*** (slow)	To wash, collect beads ***using a magnet*** (fast)
Higher binding capacity	Lower binding capacity
More manual handling so less reproducible*	Minimal manual handling so more reproducible
Low non-specific binding	Higher non-specific binding?
Cheap	More expensive

*Top tip: Use gel-loading tips or crushed P10 tips to avoid aspirating beads when washing

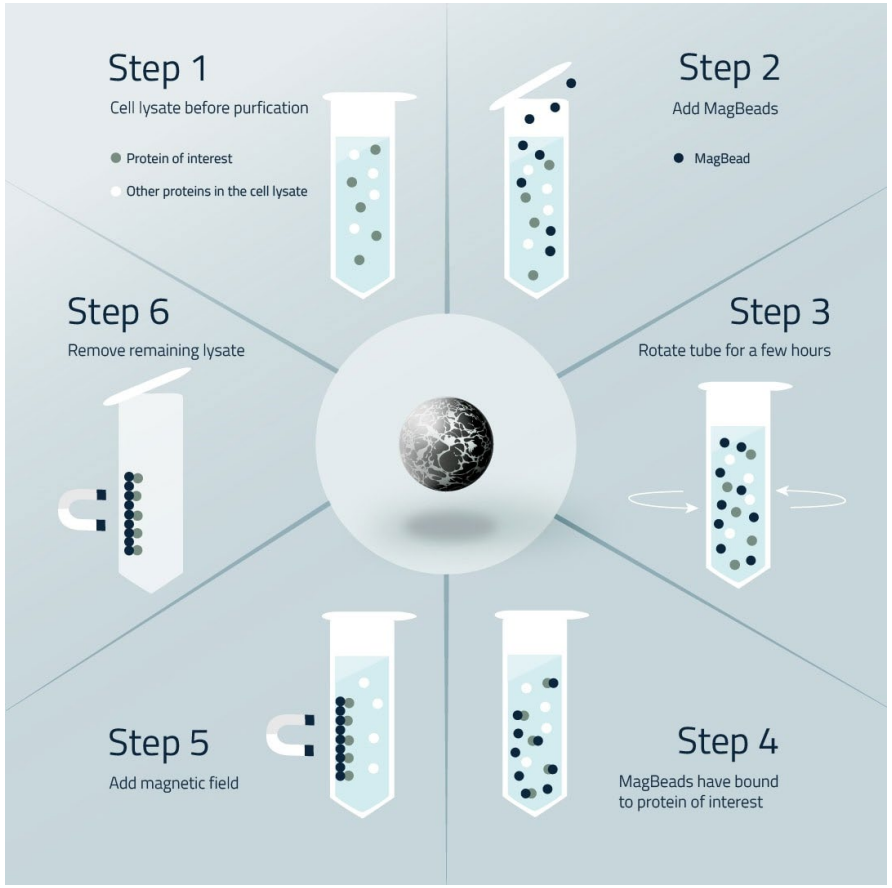


Image: https://cube-biotech.com/knowledge/protein-purification/magnetic-beads/

Immunoprecipitation (IP)

- Pull-down experiments where bait is captured using an antibody
  - Extremely high specificity and high affinity
- Prey proteins are co-immunoprecipitated (coIP'd) with the bait
- Often used with very complex prey mixtures (cell lysates or organ extracts)
- Usually paired with immunoblots (Western blots) to detect bait and prey
  - Allows detection of very low abundance proteins

Immunoprecipitation (IP)

- Incubate antigen-containing mixture with antibody
- Capture antigen:antibody complex, plus associated proteins, on resin (bead support)
  - Can use Protein A or Protein G resin, or a mixture (Protein A/G)
- Wash away unbound protein
- Analyse bound fraction (Western blot, mass spectrometry, etc.)

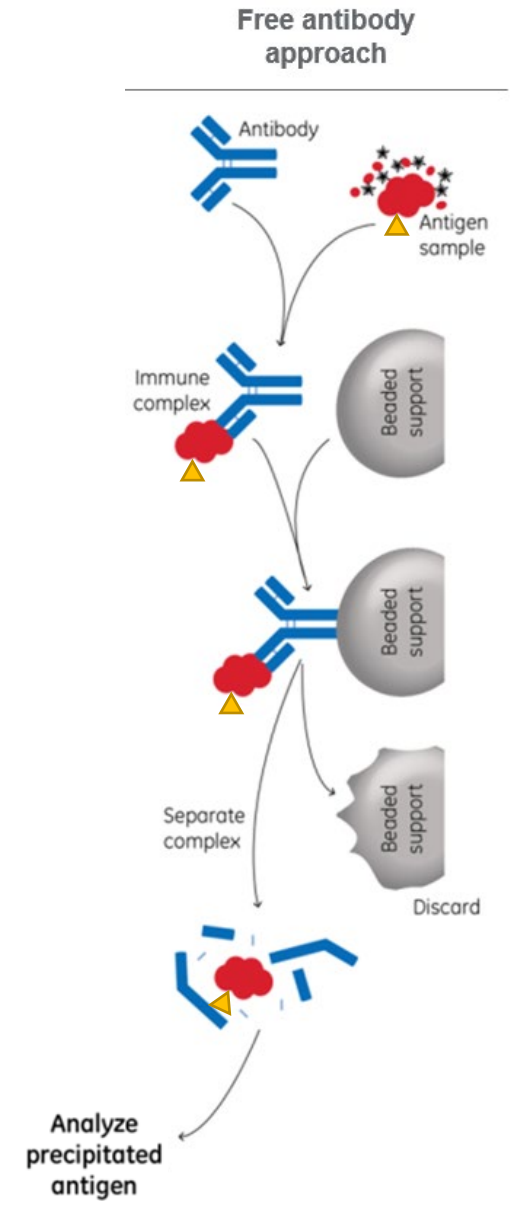
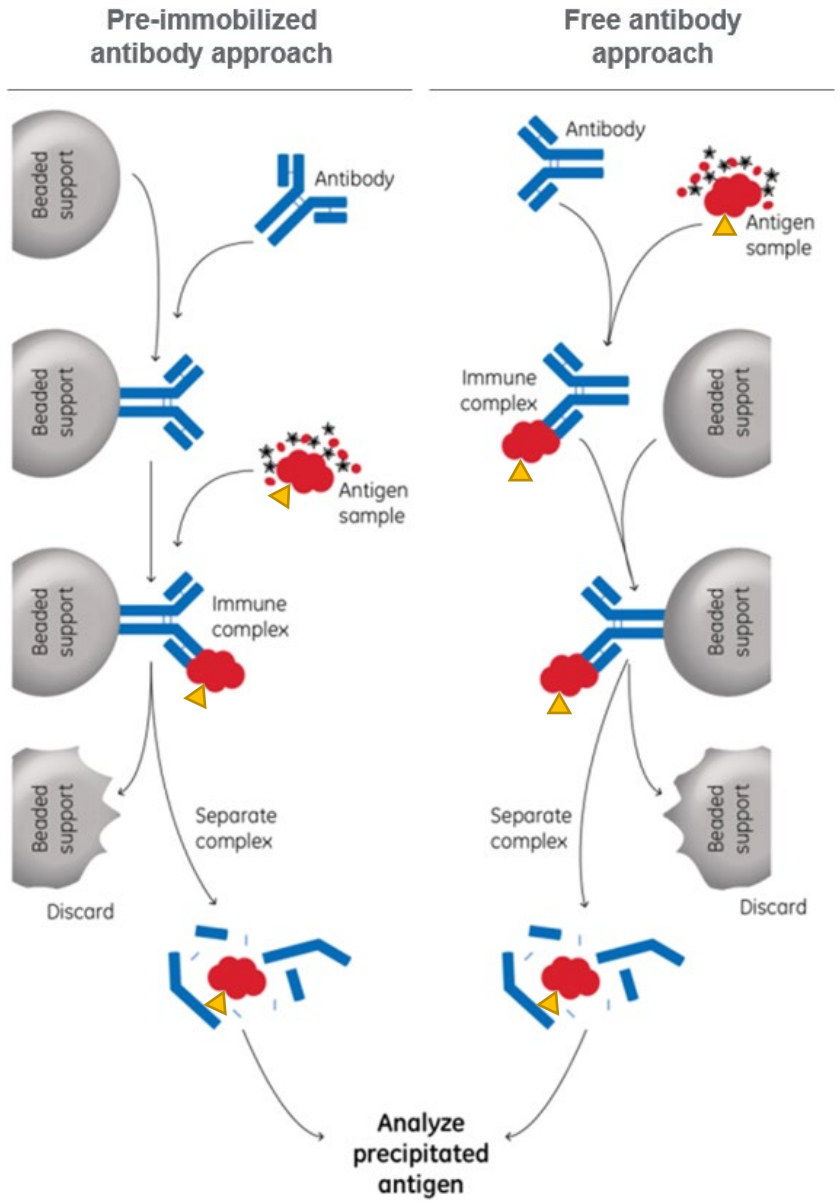


Image adapted from https://www.cytivalifesciences.com/en/us/news-center/tips-for-successful-immunoprecipitation-10001

Alternative IP protocols

- Use resin to pre-clear antigen-containing mixture
  - Reduce non-specific binding
- Capture the antibody with the resin *before* capturing the antigen
- Use an antibody against an *epitope tag* (e.g. myc, HA) or a protein tag (e.g. GFP)
- Use resins with capture antibodies (or nanobodies) covalently bound to the bead support
  - No cross-reactivity with eluted antibody in downstream analysis



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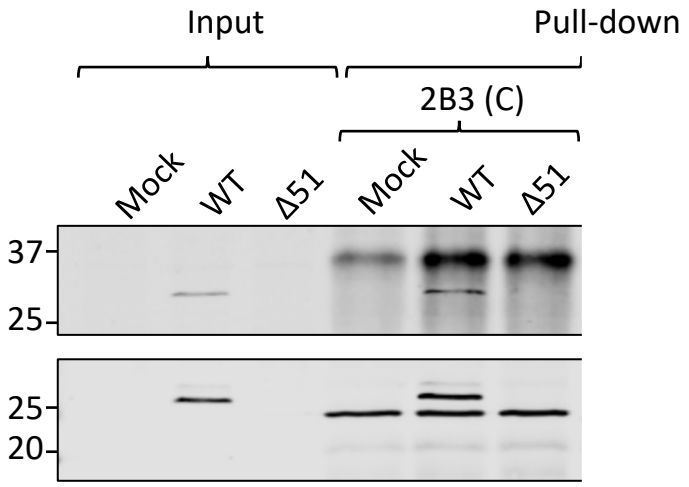
(Some) Caveats of IPs

- IPs do not demonstrate direct binding
  - IPs are performed in ‘crude’ lysates, with lots of other proteins around
  - Indirect binding via membranes or other proteins
- IPs can be ‘fragile’, as minor changes in protocol can dramatically change results:
  - Buffer composition, preclearing, washes, …
- Antibody-binding epitopes and partner-binding epitopes can overlap

Overlapping antibody/antigen binding sites



Adapted from Albecka et al. (2017) *J Virol* 91:e02196-16

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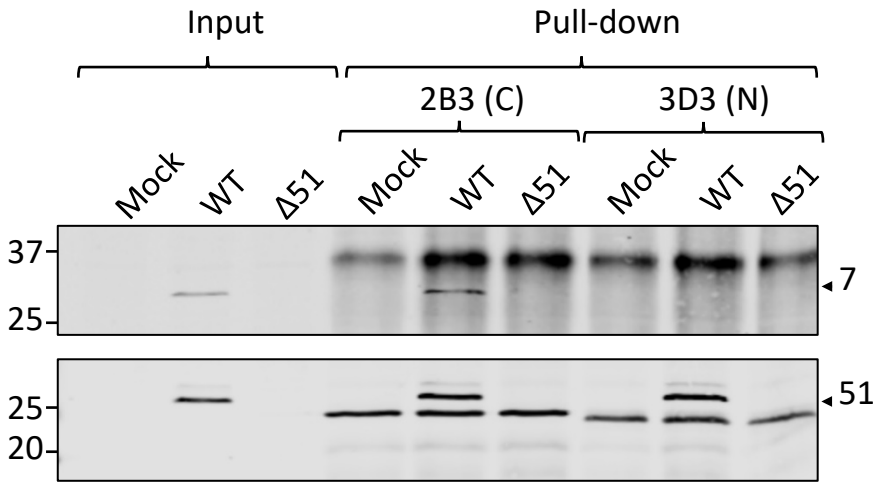

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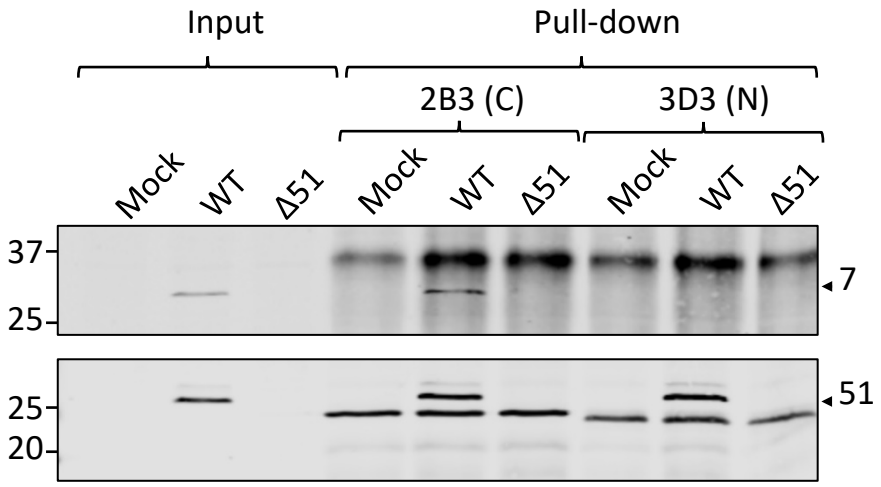

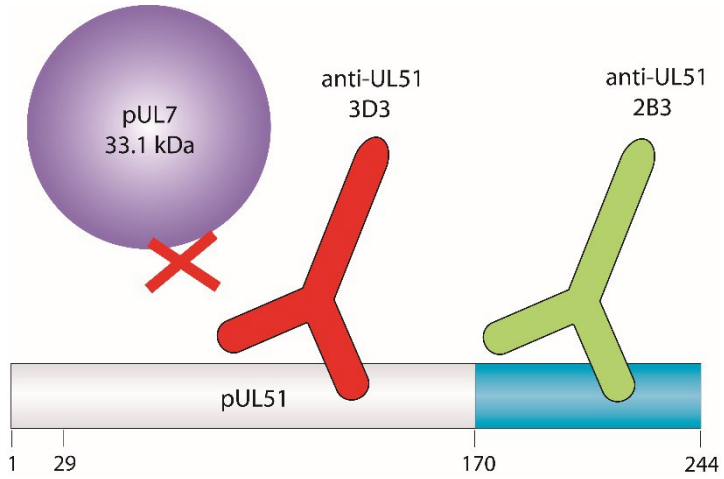

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Western blots

- Electrophoretic separation
  - SDS-PAGE
- Transfer proteins to nitrocellulose or PVDF membrane
- Probe with primary and secondary antibodies
- Visualise by enhanced chemiluminescence or fluorescence

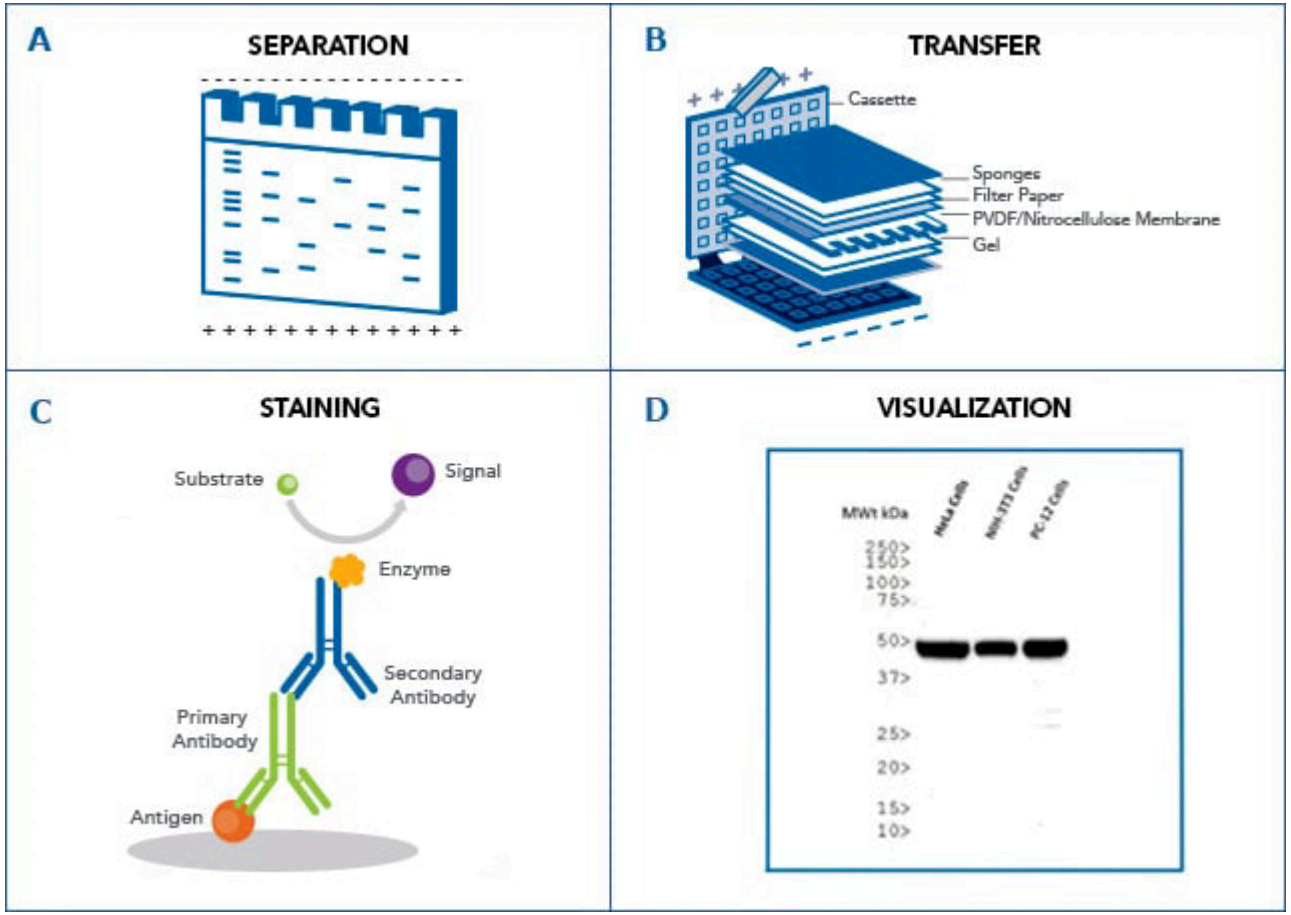
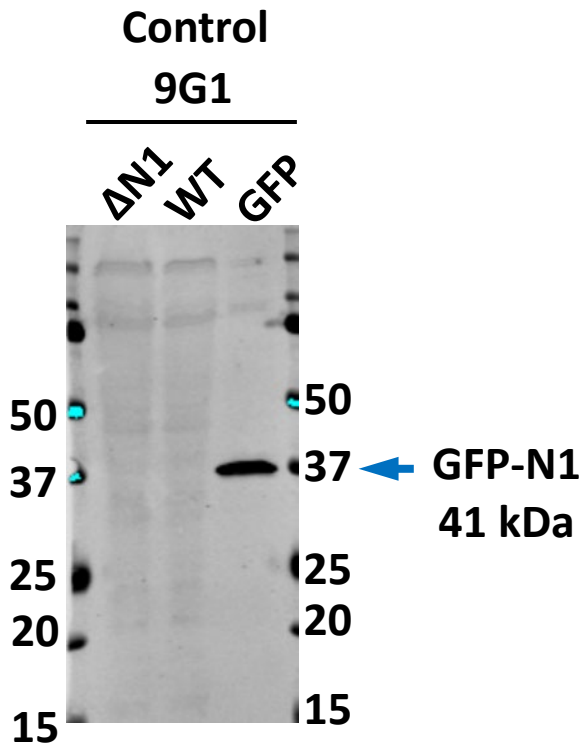


Image: https://www.novusbio.com/application/western-blotting

All of these steps can go wrong!

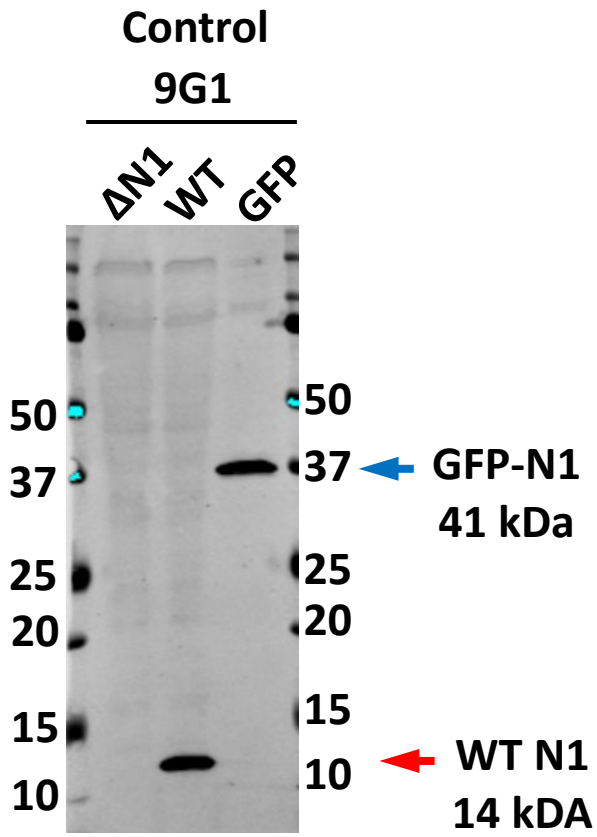
When westerns go bad…

- Separation:
  - Insufficient resolution

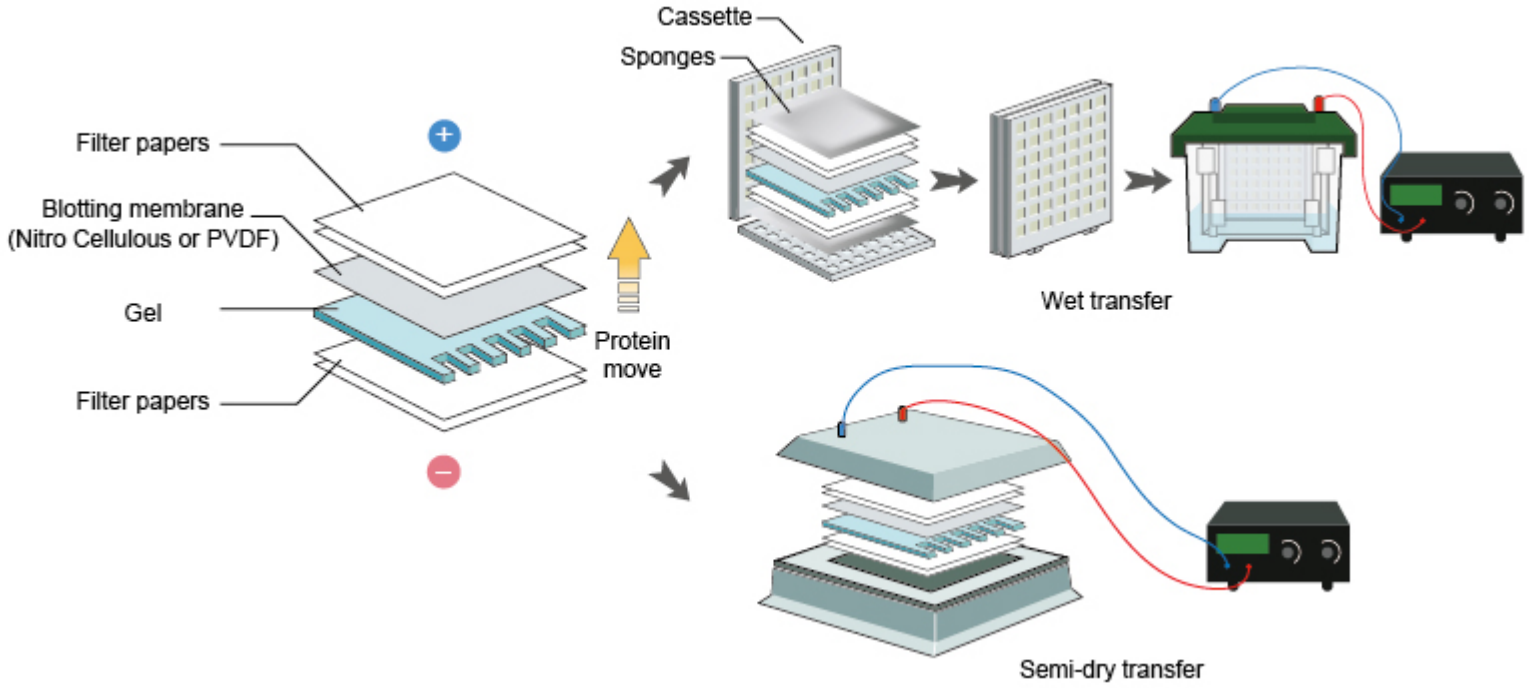


When westerns go bad…

- Separation:
  - Insufficient resolution
  - Running important bands off end of gel
    - Or cutting the wrong strips!



Western blot transfer

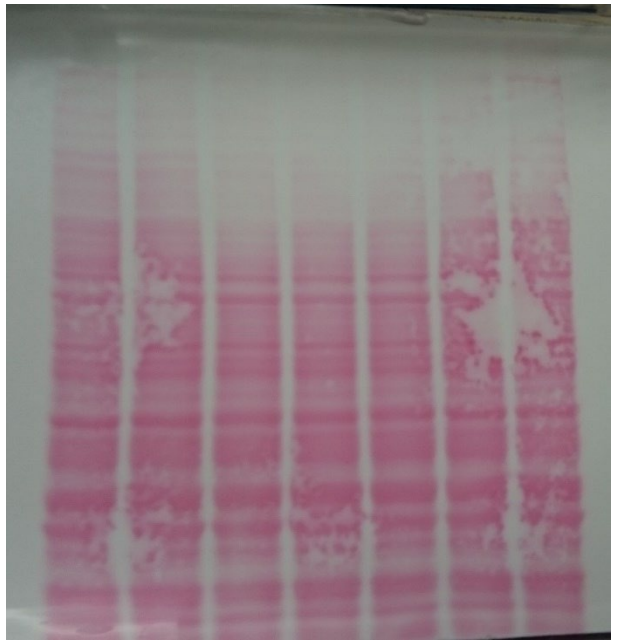


- Wet-tank transfer: slow but better for large proteins
- Semi-dry transfer: fast, but less efficient for large proteins and can ‘over-transfer’ small proteins

Image: https://www.creative-diagnostics.com/Electrophoresis-Protein-Transfer.htm

When westerns go bad…Transfer to the membrane

- Over-transfer of small proteins
  - Esp. with semi-dry blotters?
- Under-transfer of large proteins
- Air bubbles
  - Prevent protein transfer
  - We always use Ponceau S to check transfer



Probe with antibodies

- Commercial primary antibodies are often crap
  - Need extensive validation
- All antibodies can be non-specific
- Secondary antibodies detect heavy/light chains after IP
  - Can interfere with detection of band for protein of interest
  - Avoid by using protein A, biotin/streptavidin or isotype specific Abs

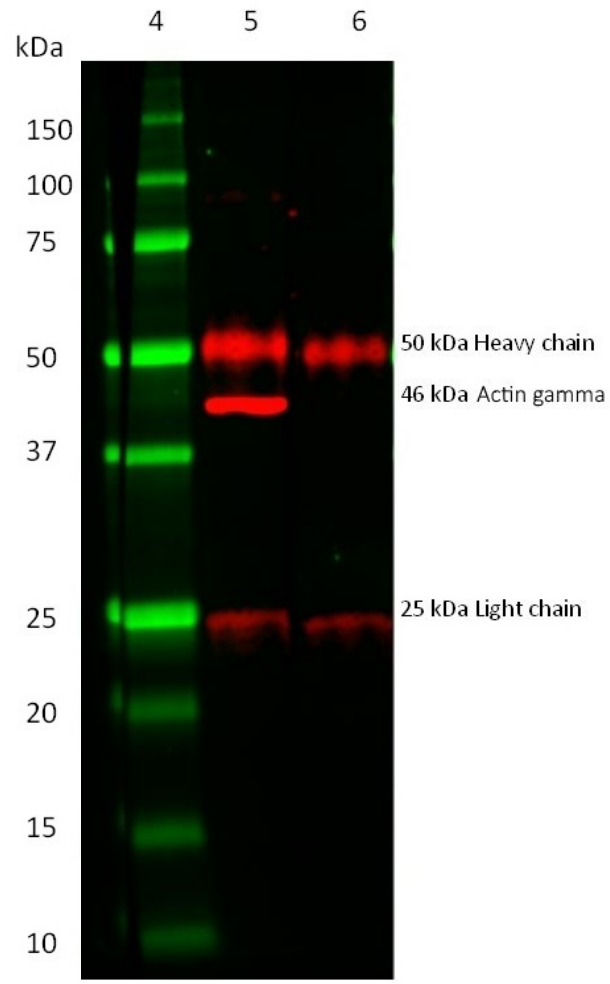
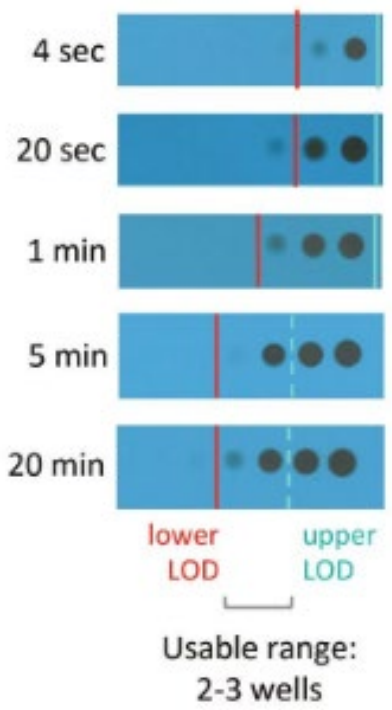


Image adapted from https://www.bio-rad-antibodies.com/reagent/tidyblot-accessory-reagent-star209.html?f=hrp

Visualisation

- Film has a low dynamic range
  - Easy to under-expose (all samples below limit of detection, LOD) or saturate image (all samples above LOD)
- Use digital camera with fluorescent secondary antibodies or chemiluminescence



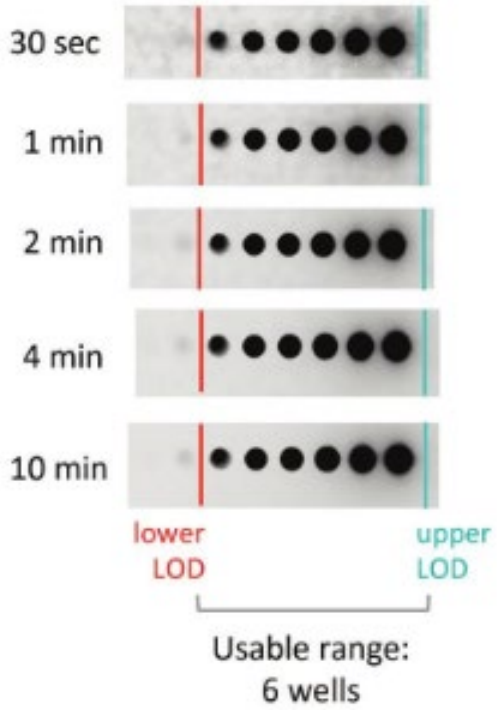
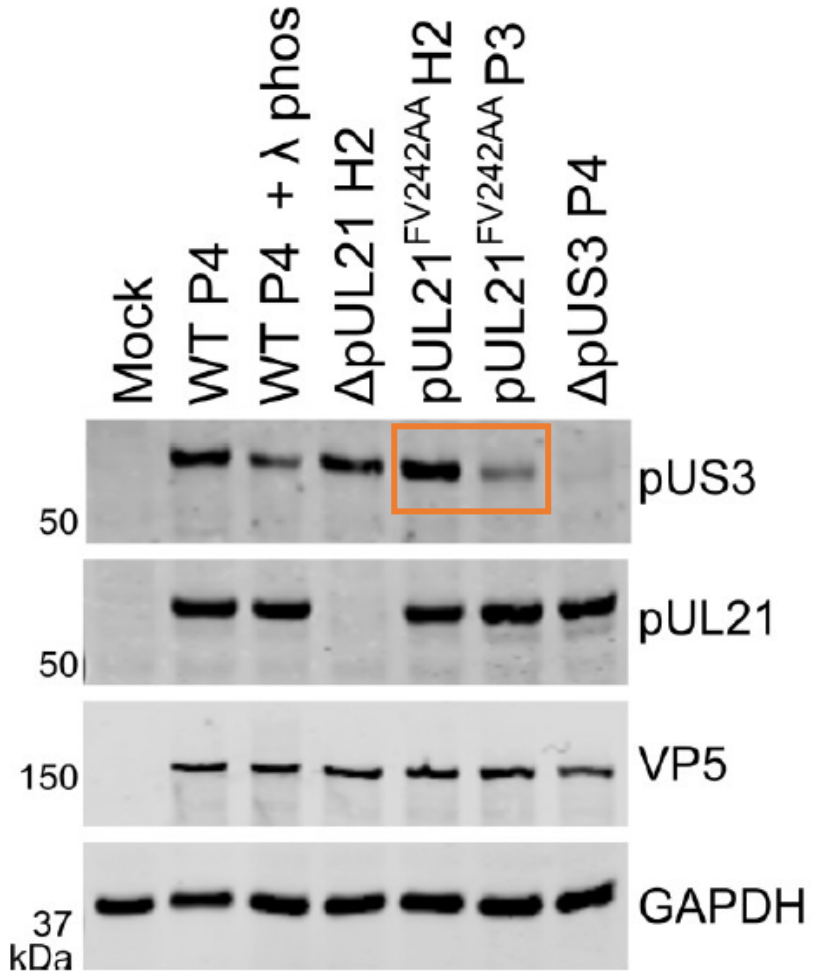


Image: LI-COR Biosciences

Quantifying Western blots

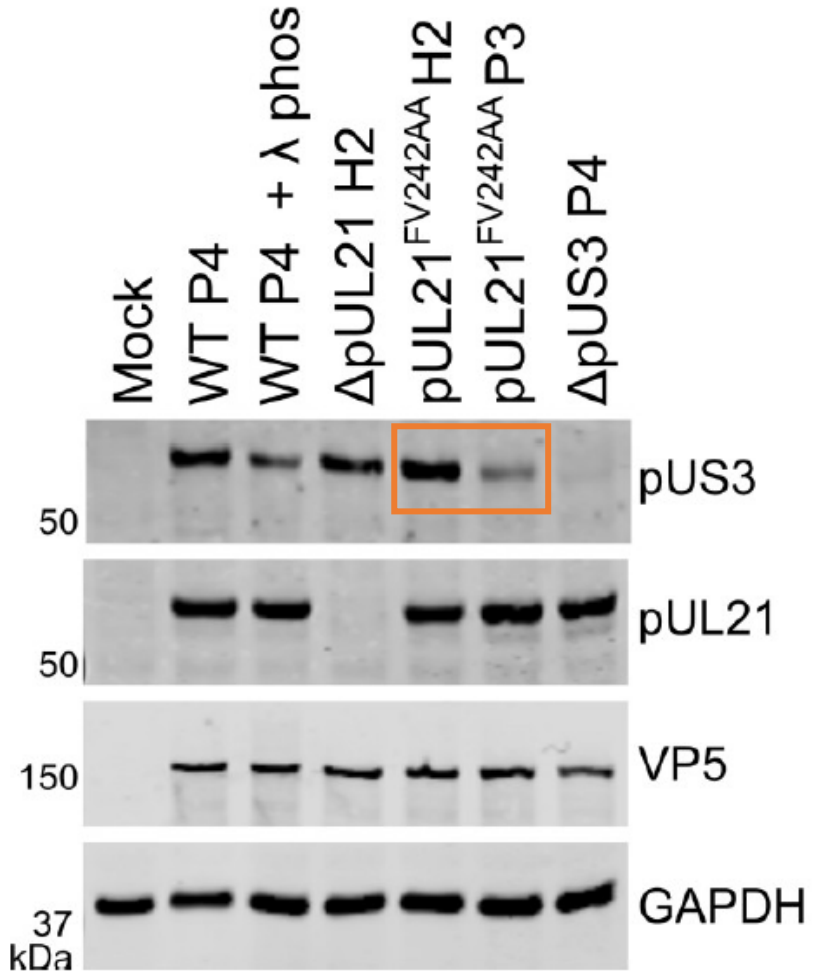
- You can measure the relative intensity of bands on a Western blot to compare abundance



Adapted from Benedyk et al. (2021) *PLoS Pathogens*, 17: e1009824

Quantifying Western blots

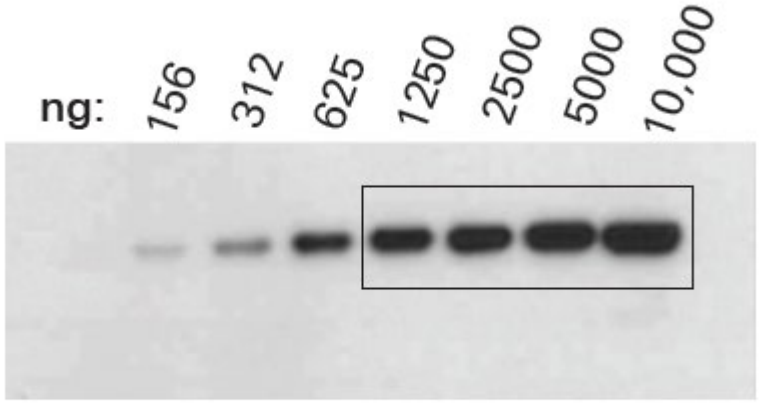
- You can measure the relative intensity of bands on a Western blot to compare abundance
- But only if:
  - Loading between lanes is equivalent
  - All protein in the sample has been transferred onto the membrane
  - The antibody has stained the target in each well evenly
  - The amount of background signal in each well is constant
  - The signal intensity goes up linearly with increasing target protein

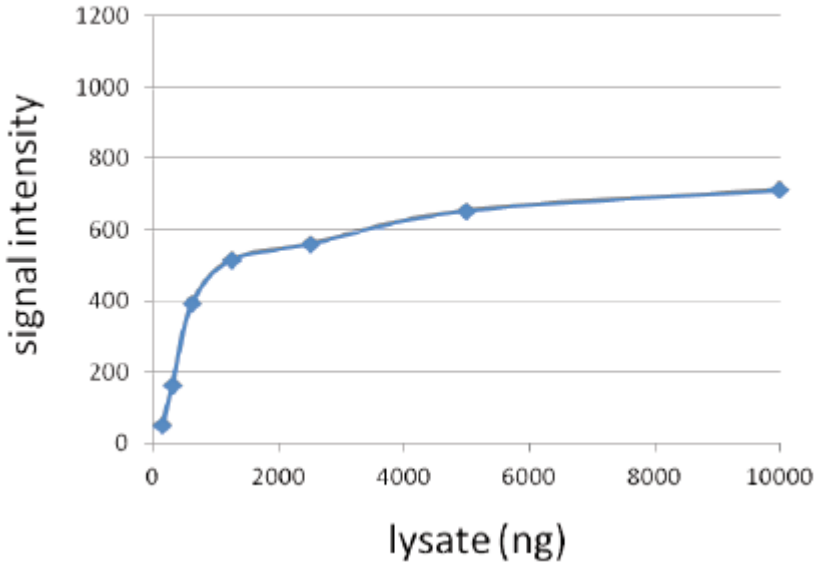


Adapted from Benedyk et al. (2021) *PLoS Pathogens*, 17: e1009824

Reliable Western blot quantitation

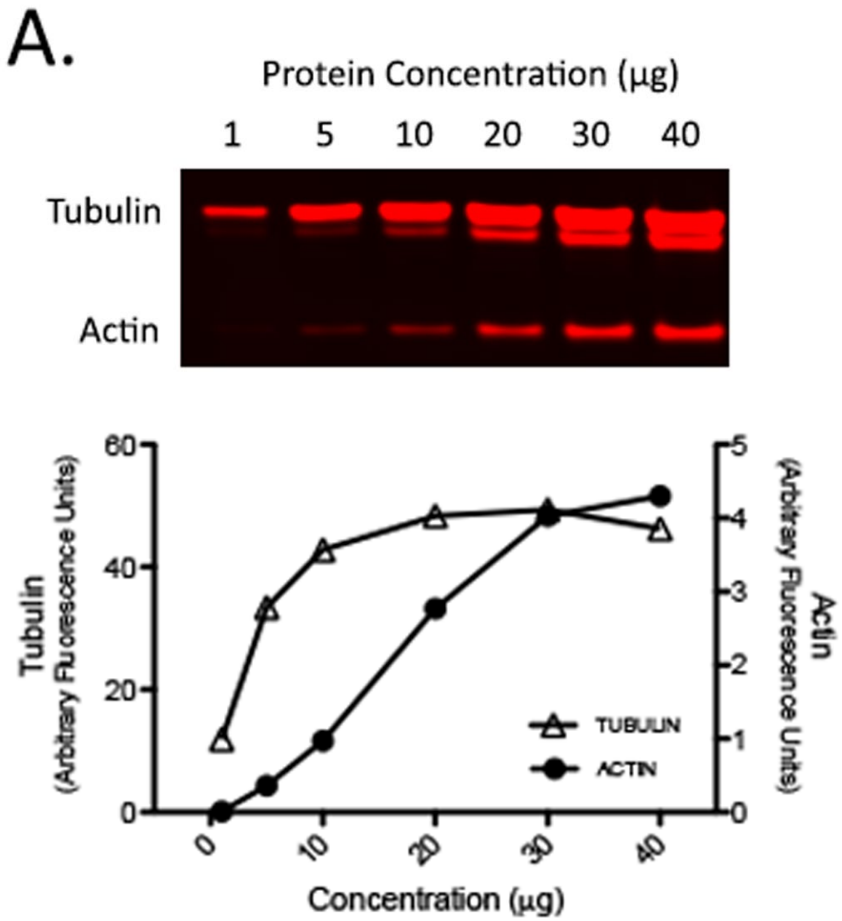
- Do the experiment more than once!
  - Change the order of samples on the gel
- Determine the signal response curve for your antibody
  - Blot a positive control sample at multiple different concentrations and measure response
- Don’t use blotting of a single control protein to normalise the signal between different lanes





Why using a control protein is bad?

- Your treatment might change the abundance of the control protein
- The signal response curve for your control protein may not be linear (in fact it almost definitely isn't)
  - So twice the signal in the control lane doesn't mean twice the amount of sample loaded



Eaton et al. (2013) *PLoS ONE* 8: e72457

Why measuring total protein loading is better

- Quantitation of total protein loading is linear over a much larger scale

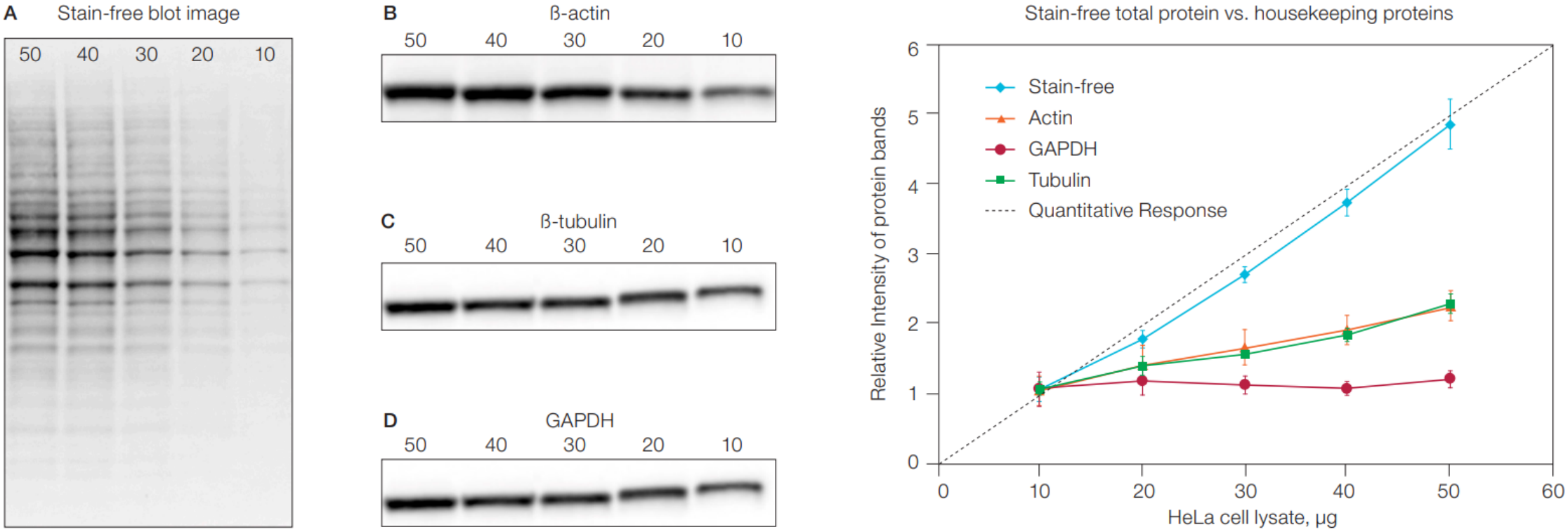


Image: Bio-Rad Laboratories

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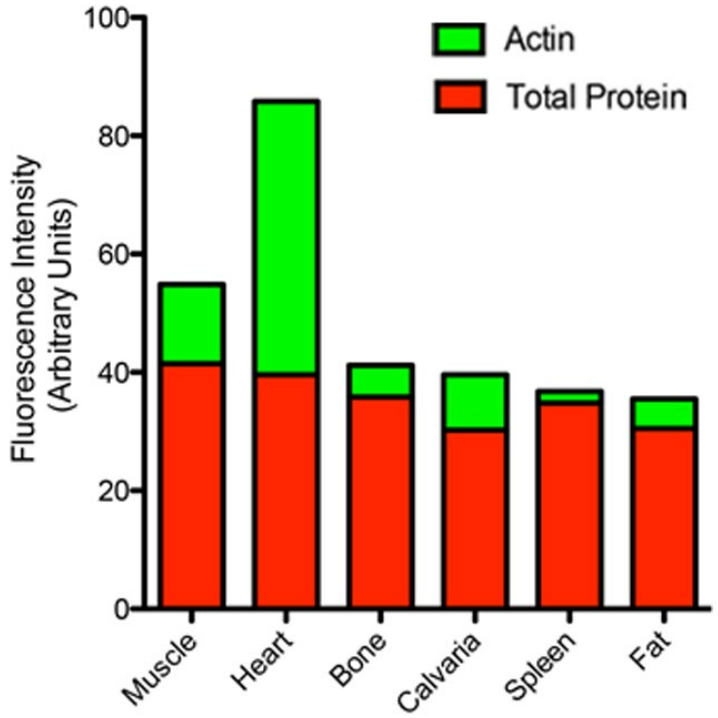
- Quantitation of total protein loading is linear over a much larger scale
- Can directly compare samples from different cells/tissues (where actin or GAPDH may be more/less abundant)



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Why measuring total protein loading is better

- Quantitation of total protein loading is linear over a much larger scale
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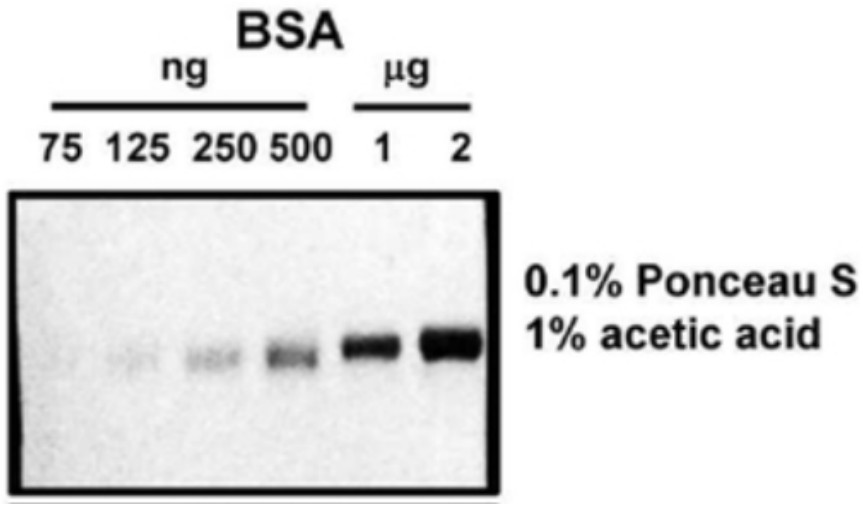
- Can normalise total protein before loading gel (*good*), or measure total loading after transfer (*better*)

Why measuring total protein loading is better

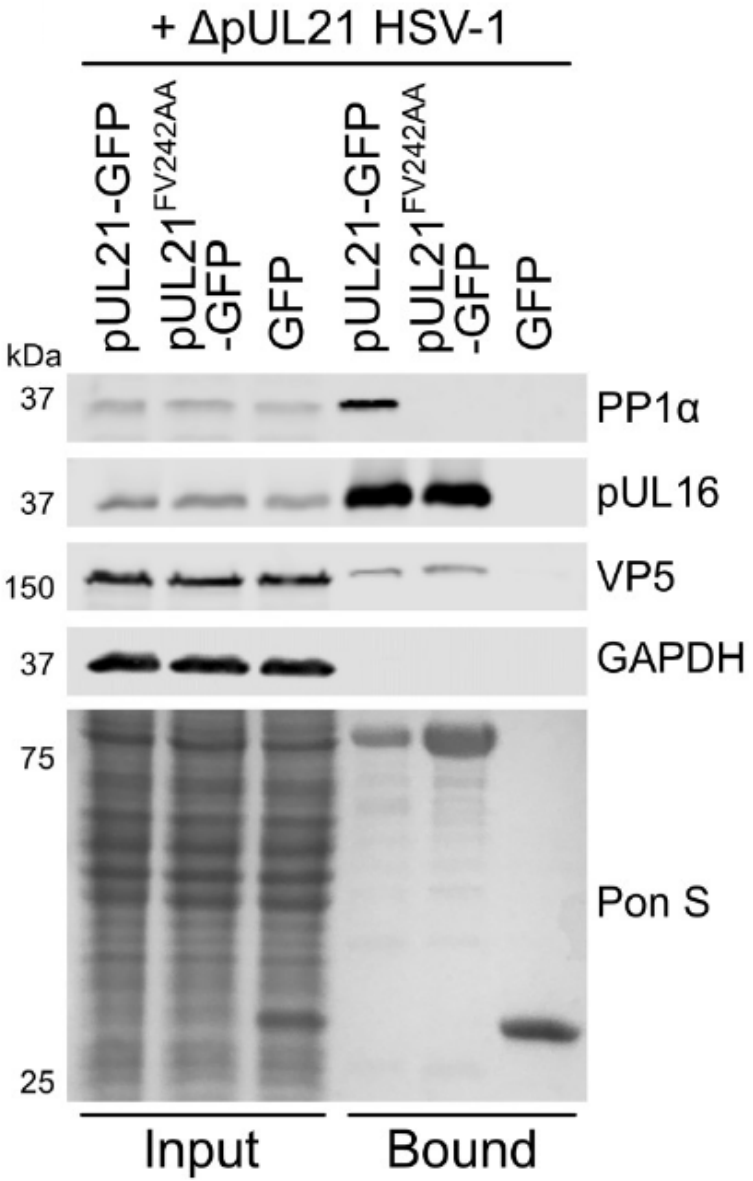
- Quantitation of total protein loading is linear over a much larger scale
- Can directly compare samples from different cells/tissues (where actin or GAPDH may be more/less abundant)
- Can normalise total protein before loading gel (*good*), or measure total loading after transfer (*better*)
- Companies sell expensive total protein stains, but 0.01% Ponceau S in 1% acetic acid works just as well (and is very cheap!)



Sander et al. (2019) *Analytical Biochemistry*, 575: 44-53

What's the take home

- If you think you might want to quantitate your Western blot, Ponceau S stain it and take an image before probing with antibodies
- Ponceau S staining also works well for coIPs (you can detect the bait protein)



Benedyk et al. (2022) *Journal of Biological Chemistry*, 298: 102589

Enzyme-linked immunosorbent assays (ELISAs)

- Use to detect and quantitate the presence of an antigen or antibody in a complex mixture
- Antigen/antibody complex are immobilised on a solid surface (96-well plate) and signal is detected via an enzymatic reaction (usually colourimetric)

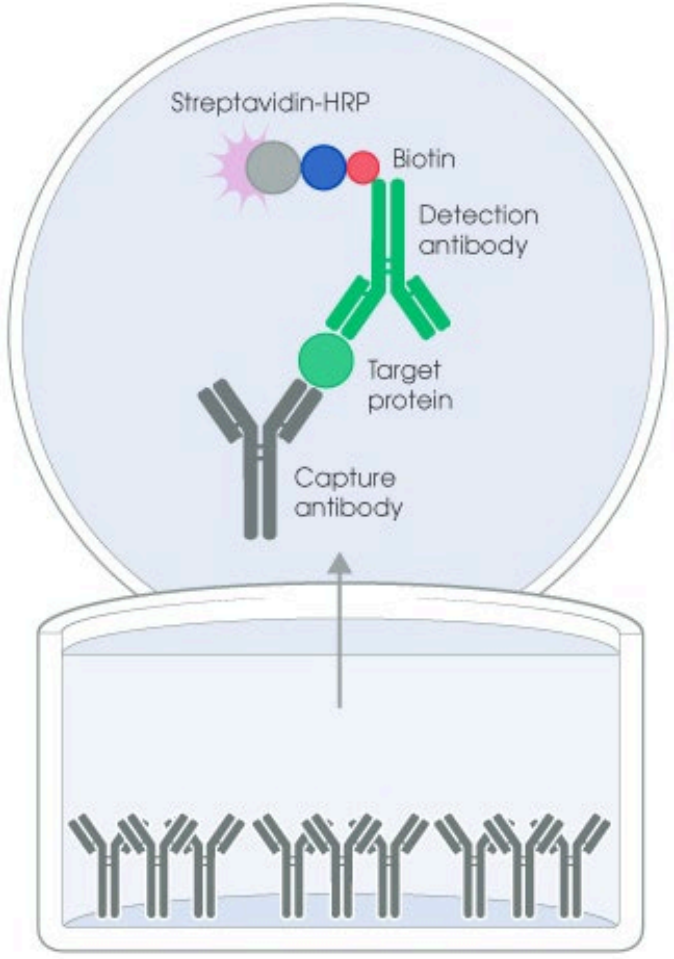


Image: https://www.abcam.com/kits/elisa-principle

Common types of ELISA assay

- **Indirect ELISA** assays:
  - Coat plate with the antigen (Ag)
  - Detect Ag with a primary antibody (1° Ab)
  - Amplify signal using enzyme-conjugated 2° Ab
  - E.g. Measure antibodies in serum to confirm prior exposure to a pathogen

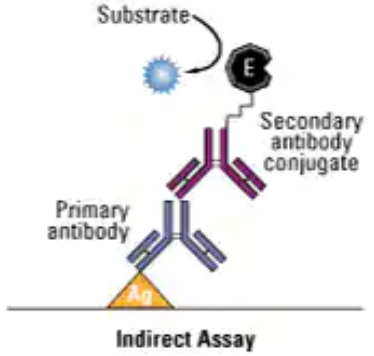


Image: ThermoFisher Scientific

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  - Coat plate with the antigen (Ag)
  - Detect Ag with a primary antibody (1° Ab)
  - Amplify signal using enzyme-conjugated 2° Ab
  - E.g. Measure antibodies in serum to confirm prior exposure to a pathogen
- **Sandwich ELISA** assays:
  - Coat plate with 1° Ab against Ag
  - Incubate with Ag
  - Detect captured Ag with a second 1° Ab
  - Amplify signal using enzyme-conjugated 2° Ab
  - E.g. Detect HCG in pregnancy tests

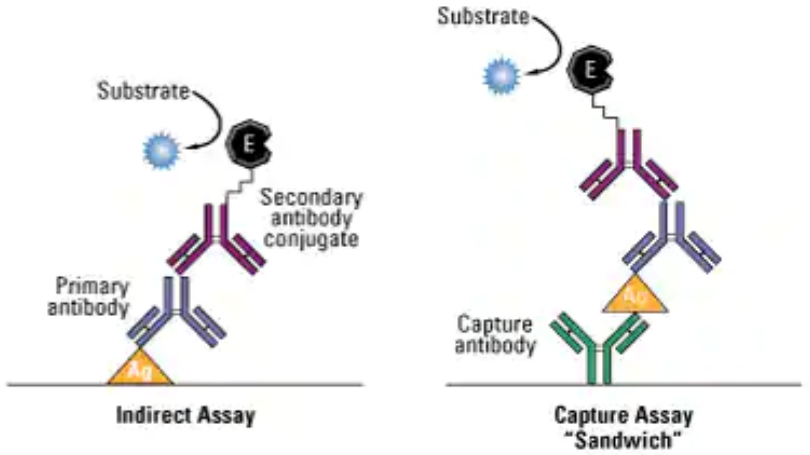

Image: ThermoFisher Scientific

Quantitating an ELISA

- Need to perform a **standard curve:**
  - Measure signal generated by a series of known concentrations of antigen/antibody
  - Plot this (and fit a curve)
  - Use equation of standard curve to determine the concentration of antigen/antibody in the unknown (experimental) samples

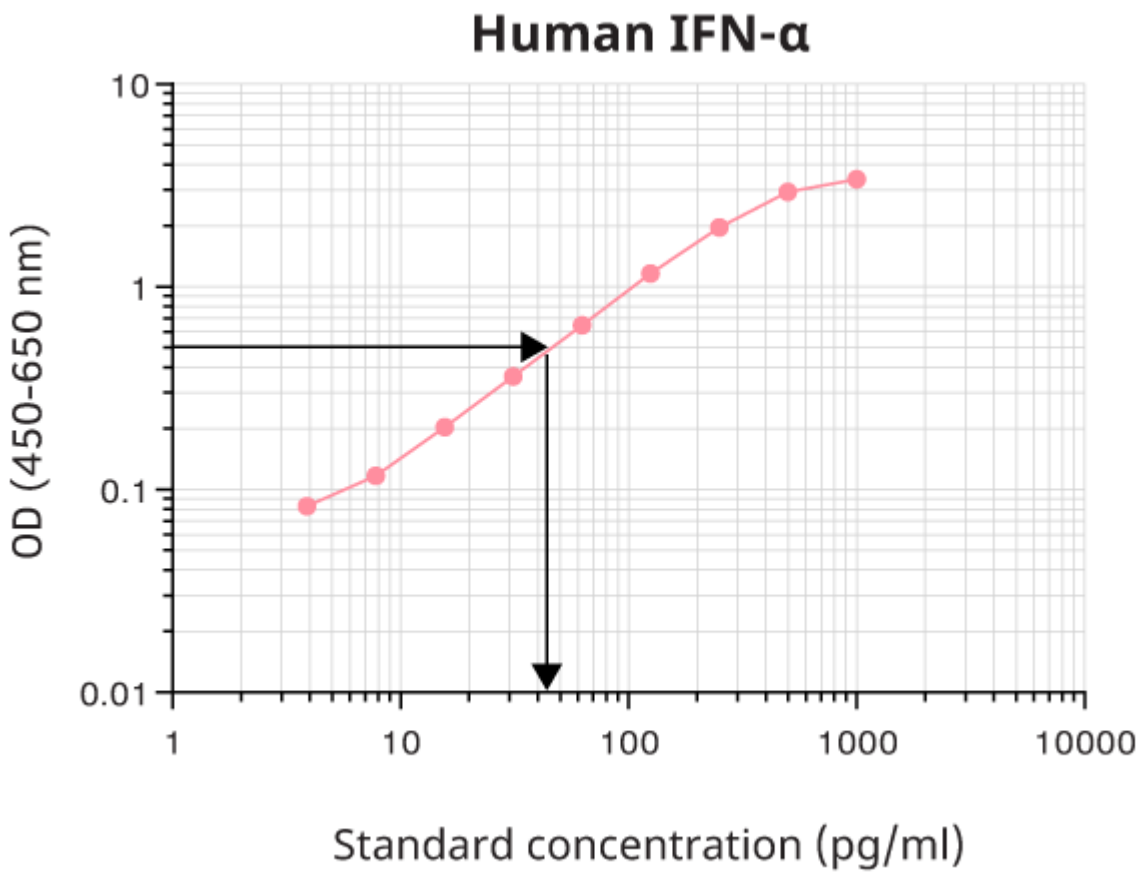

Image: https://www.mabtech.com/knowledge-hub/step-step-guide-elisa

ELISAs

- Pros:
  - Simple, cheap and scalable
- Cons:
  - Need specific purified antibody (or antigen)
  - Needs optimisation for each antigen/antibody pair

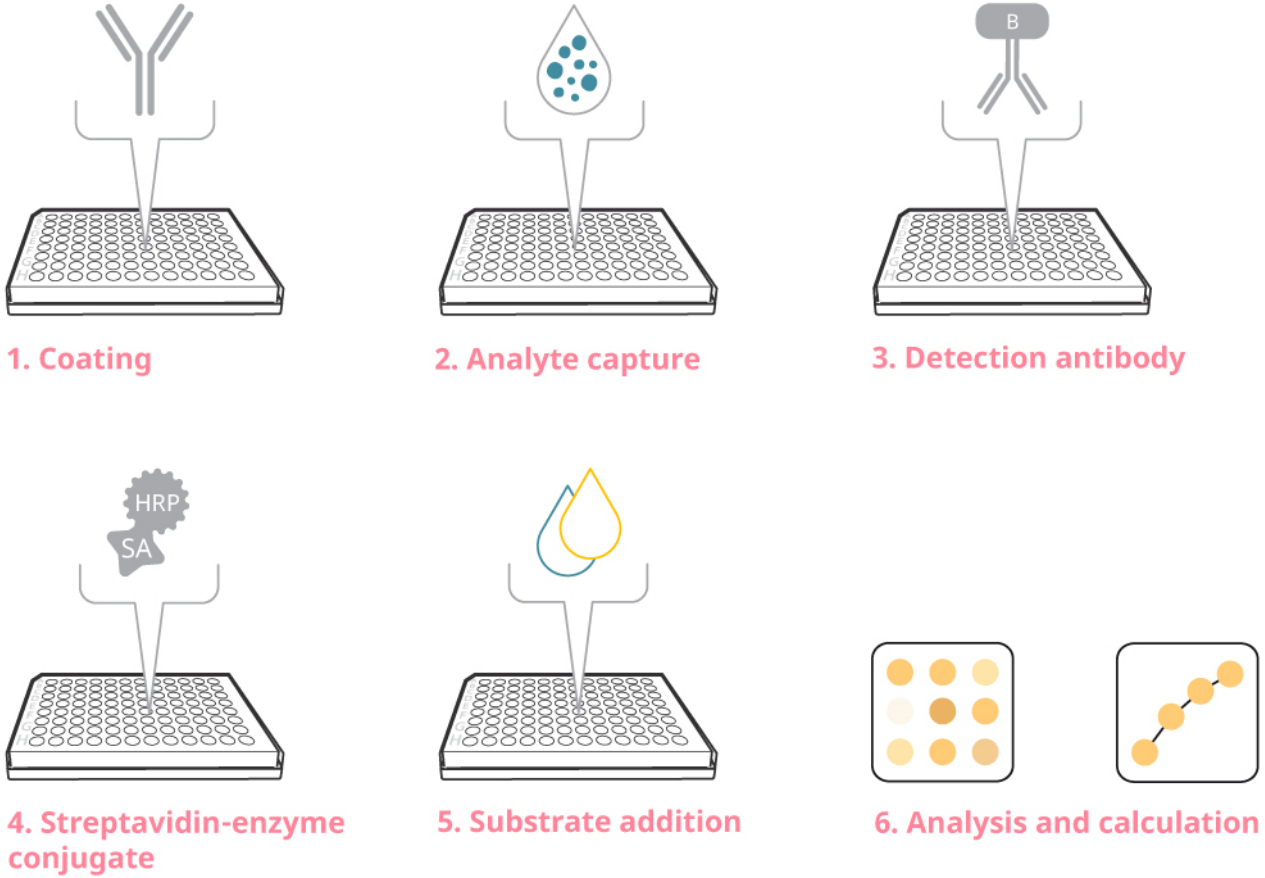

Image: https://www.mabtech.com/knowledge-hub/step-step-guide-elisa

This talk

- Identifying and characterising protein interactions
  - Pull-downs
  - Co-immunoprecipitation
  - Western blots
  - ELISA

Tomorrow: Techniques to quantify the affinity of protein interactions