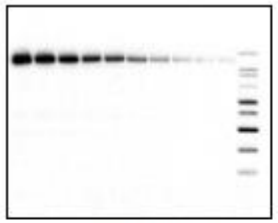
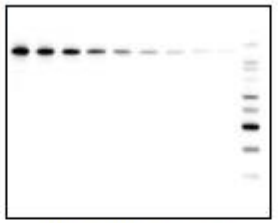
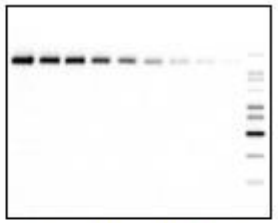
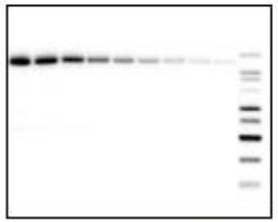
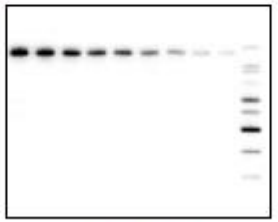

# The 1001 ways to perform western blots

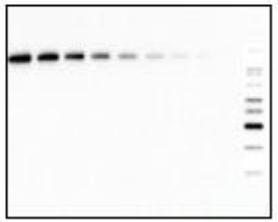

Bolt NC


NuPAGE NC

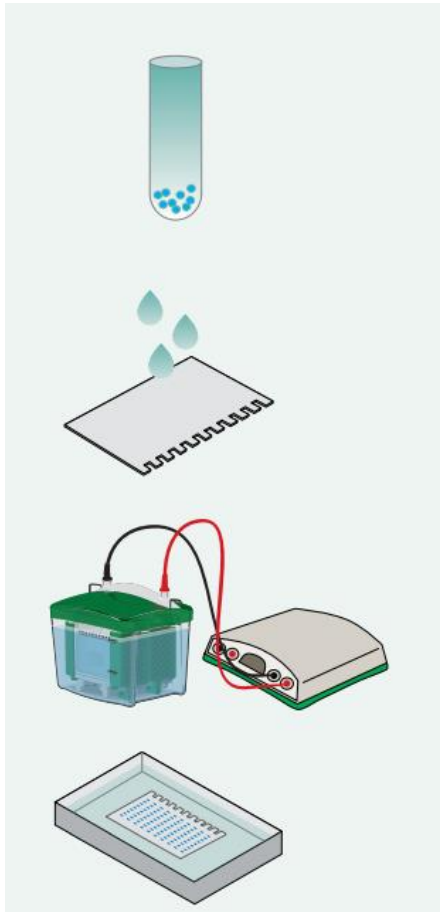

Tris-glycine NC


Bolt PVDF


NuPAGE PVDF

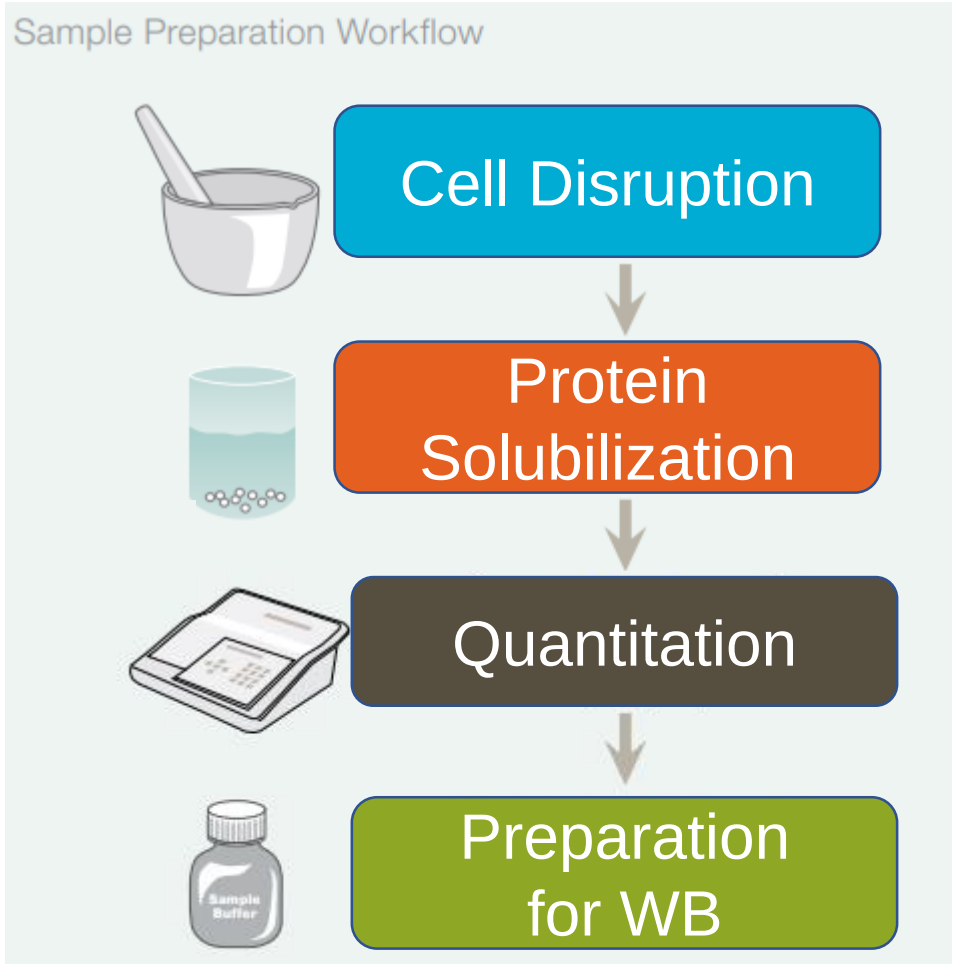

Tris-glycine PVDF

# Western blot workflow



- Sample preparation
- Electrophoresis
- Transfer
- Detection

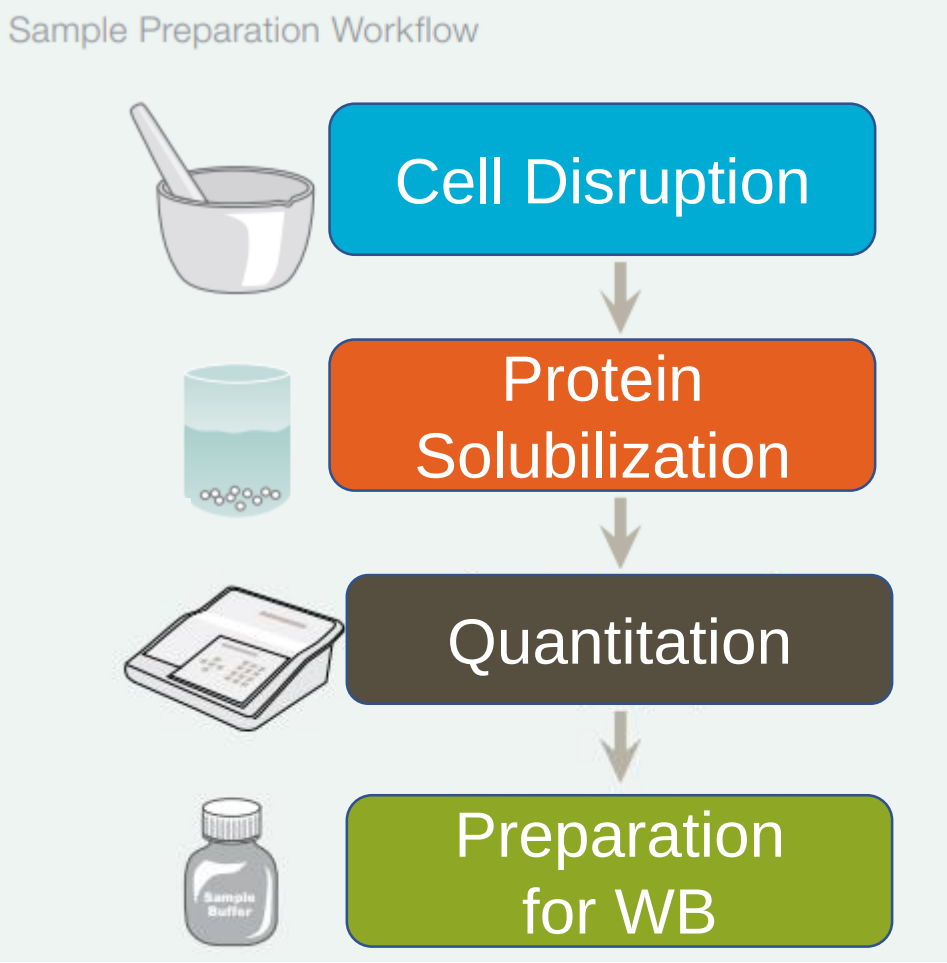
# Sample preparation




**Lysates preparation**

- Often lysis using detergent and soft mechanical disruption. But osmotic lysis is also used (cell fraction preparation)
- Centrifuge all extracts extensively (12 000 x g for 15 min at 15°C) to remove any insoluble material as solid particles may block the pores of the gel
- Add protease inhibitor to the lysis buffer (PMSF, leupeptin, pepstatin, aprotinin). Usually, use of a combination of inhibitors in a protease inhibitor cocktail. If protein phosphorylation is to be studied, include phosphatase inhibitors such as fluoride and vanadate.

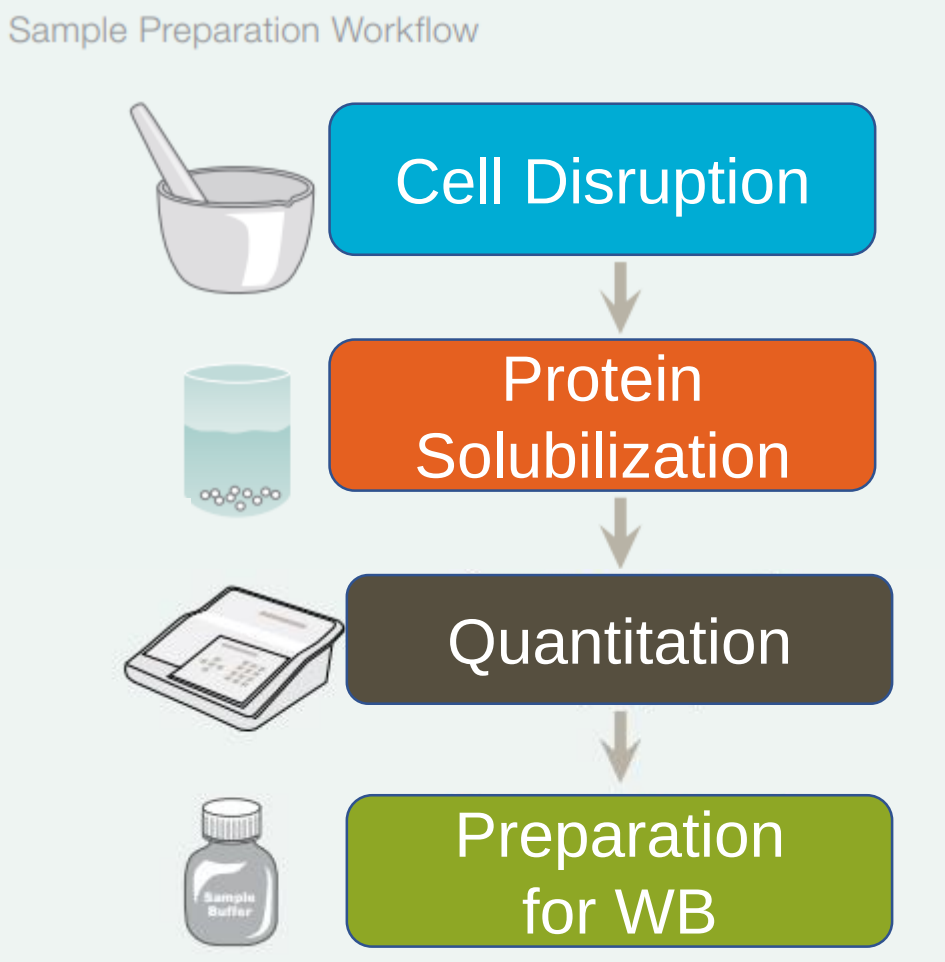
# Sample preparation




**Protein determination**

Determine the amount of total protein in each sample using a protein assay that is compatible with chemicals in your sample buffer (Bradford, BCA, Lowry)

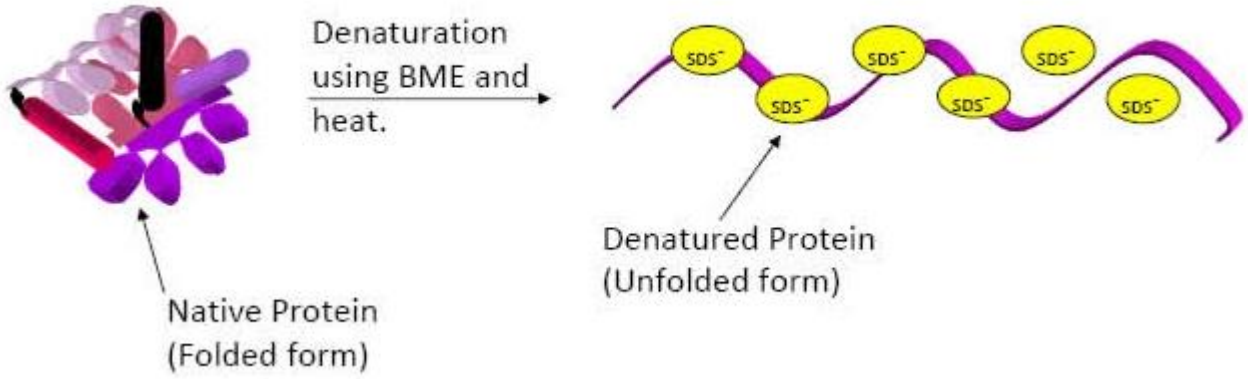
# Sample preparation




**Samples preparation for WB**

-Use reducing agent (2-mercaptoethanol (bME) or dithiothreitol (DTT)) to disrupt intramolecular and intermolecular disulfide bonds and are used to achieve complete protein unfolding and to maintain proteins in their fully reduced states

-Heat your samples (99° 5’ vs 56° 20’)

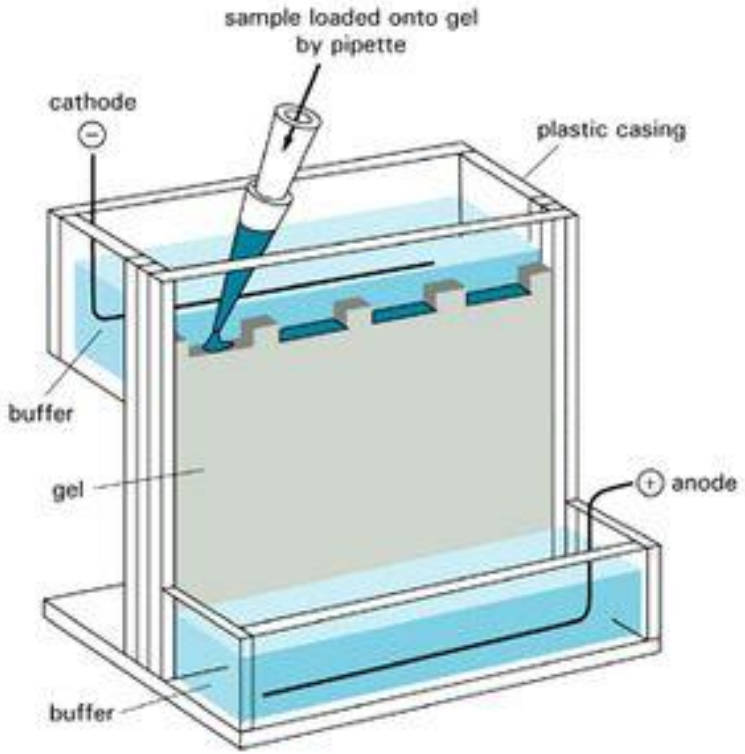


# Electrophoresis – General considerations

No particular gel type or buffer is useful for all proteins, so gel types that offer the highest resolution in the size range of interest is important.

- Protein standards — select protein standards that provide maximum resolution in the size range of interest and that offer compatibility and utility for downstream applications such as western blotting
- Gel percentage — choose the percentage that offers the best resolution in the range of interest
- Handcast vs. precast gels — precast gels offer greater convenience and superior quality control and reproducibility than handcast gels; handcast gels provide customized percentages and gradients
- Gel format — select mini- or midi-format gels when throughput is important or sample size is limited; select large-format gels for higher resolution. Select a comb type and gel thickness to accommodate the sample number and volume you are working with
- Buffer system — choose the system that offers the best resolution and compatibility with the protein and application of interest

# Electrophoresis – Loading samples

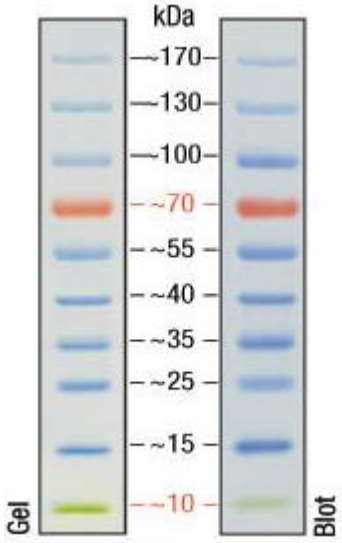



15 to 50µg (depends on the abundance of proteins of interest)

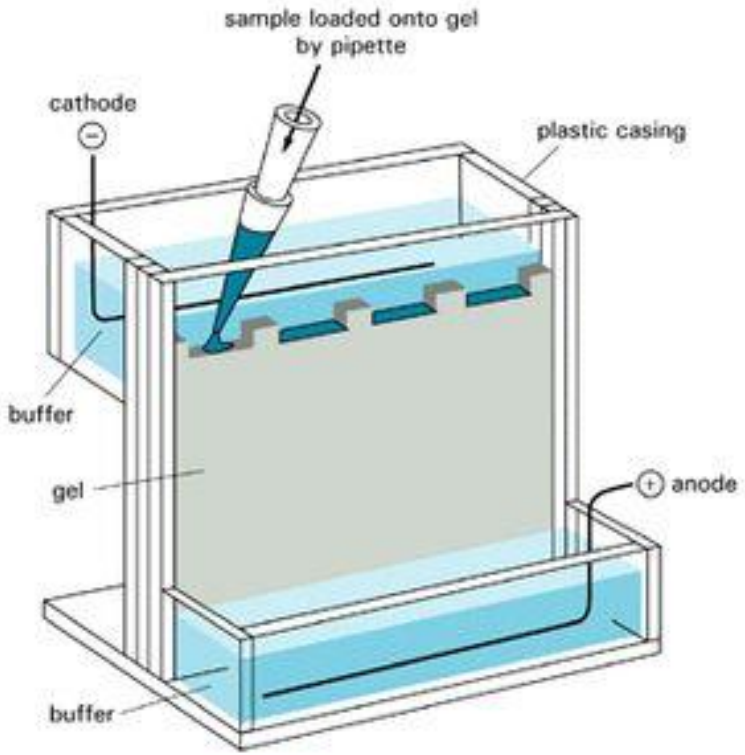
Load a common sample accross gels to be compared

Positive/Negative Control (see antibody validation)

Protein standard 2 to 5µl



# Electrophoresis – Migration




Factors Affecting Electrophoresis

- Alterations to buffer composition; that is, the addition of SDS or changes in ion concentration due to the addition of acid or base to adjust the pH of a buffer
- Gel pH, ionic strength, and percentage of acrylamide
- Number of gels (current increases as the number of gels increases)
- Volume of buffer (current increases when volume increases)
- Transfer temperature (current increases when temperature increases)
- Gel length (increasing gel length demands higher voltage settings to increase field strength accordingly)
- Gel thickness (increasing gel width or thickness at identical gel length leads to higher current; voltage must be kept unchanged)

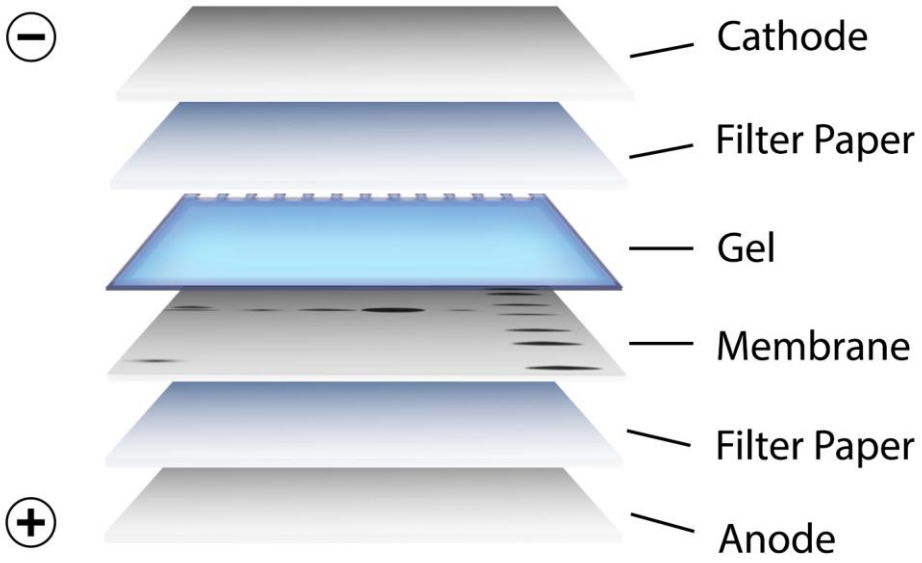
# Electrophoresis – Transfert

Wet transfer

Semi-dry transfer

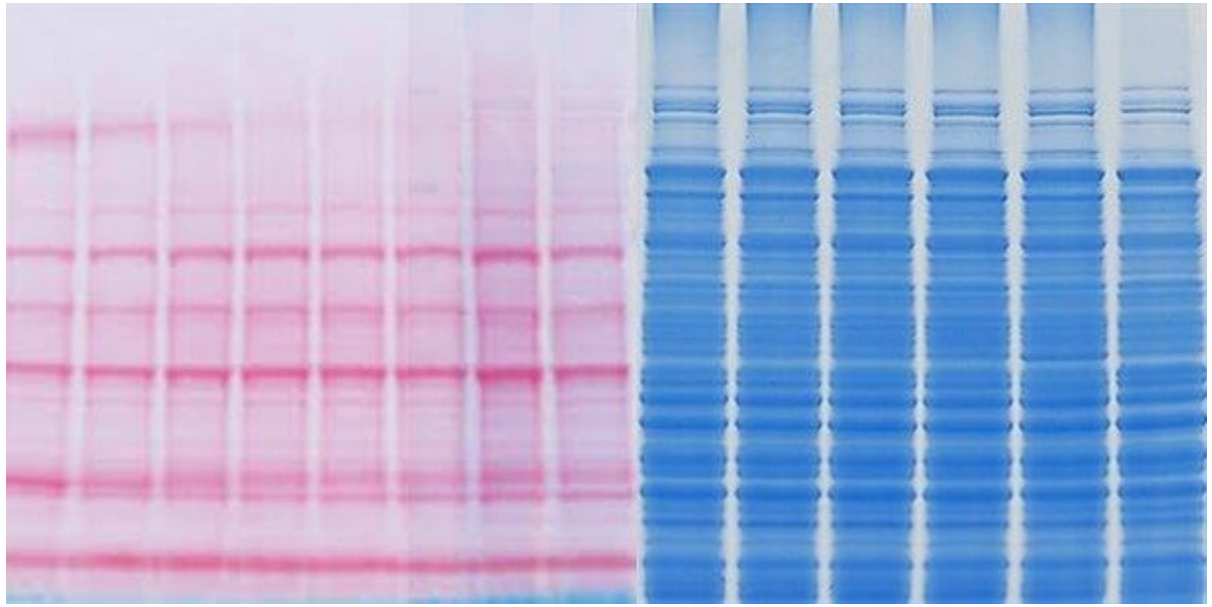

Dry transfer

# Electrophoresis – Transfert




| | PVDF | Nitrocellulose |
|---|---|---|
| **Protein Size** | Better for high MW proteins | Better for mid to low MW proteins |
| **Sensitivity** | Higher protein binding capacity and sensitivity | Strong binding capacity, not as suitable for low expression level targets |
| **Strip & Re-probe** | Performs well | Possible, but can lose sensitivity in the process |
| **Durability** | More durable | Less durable |
| **Background Noise** | Slightly higher signal | Low background signal |
| **Saturation** | Requires methanol or ethanol prior to transfer | No pretreatment required |

# Afterwards… Verifying your transfer



Ponceau staining

Coomassie blue staining

Negatively charged solutions that bind to positively charged amino acid groups and non-polar protein regions.

Ponceau S detects protein levels at 200 ng and higher, is compatible with PVDF, nitrocellulose, or nylon membranes.

Coomassie Blue stains gels (and is only compatible with PVDF membranes) but can detect protein levels at 50 ng and higher

# Membrane saturation

Blocking step helps reduce nonspecific primary antibody binding and reduces background

BSA

**vs**

Skimmed milk

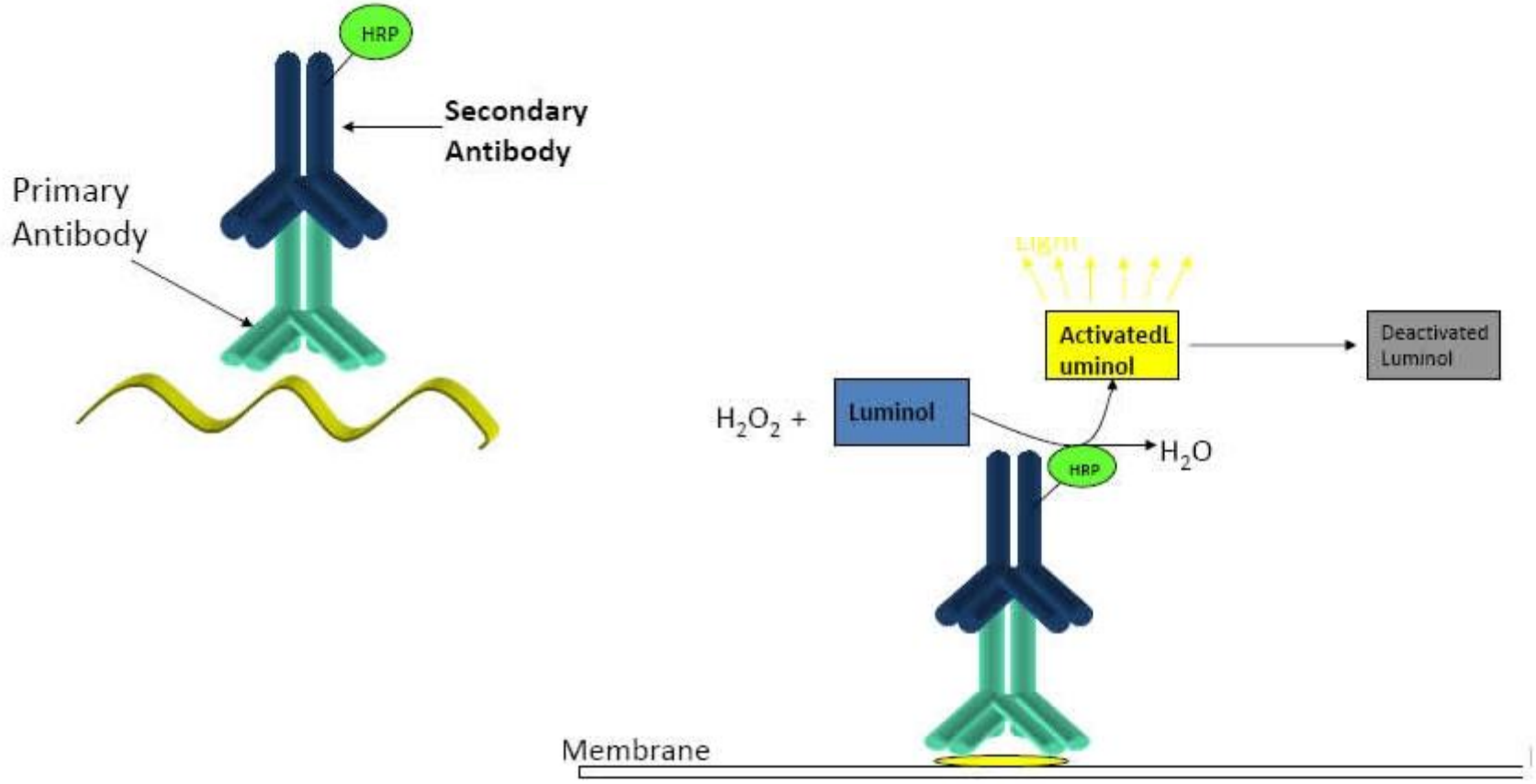
# Primary Antibody

The primary antibody directly binds to the antigen. The variable region of the primary antibody recognizes an epitope on the antigen. It is produced by a host organism that is of a different species than the specimen.
The primary antibody usually does not contain a fluorophore or an enzyme, so the researcher cannot visualize the antigen without further reagents such as a secondary antibody.

Primary antibody buffer:

- BSA-TBS buffer (0.1% to 3% BSA…) – can be stored and reused
- Milk-TBS buffer – short life shelf

# Secondary Antibody and Revelation



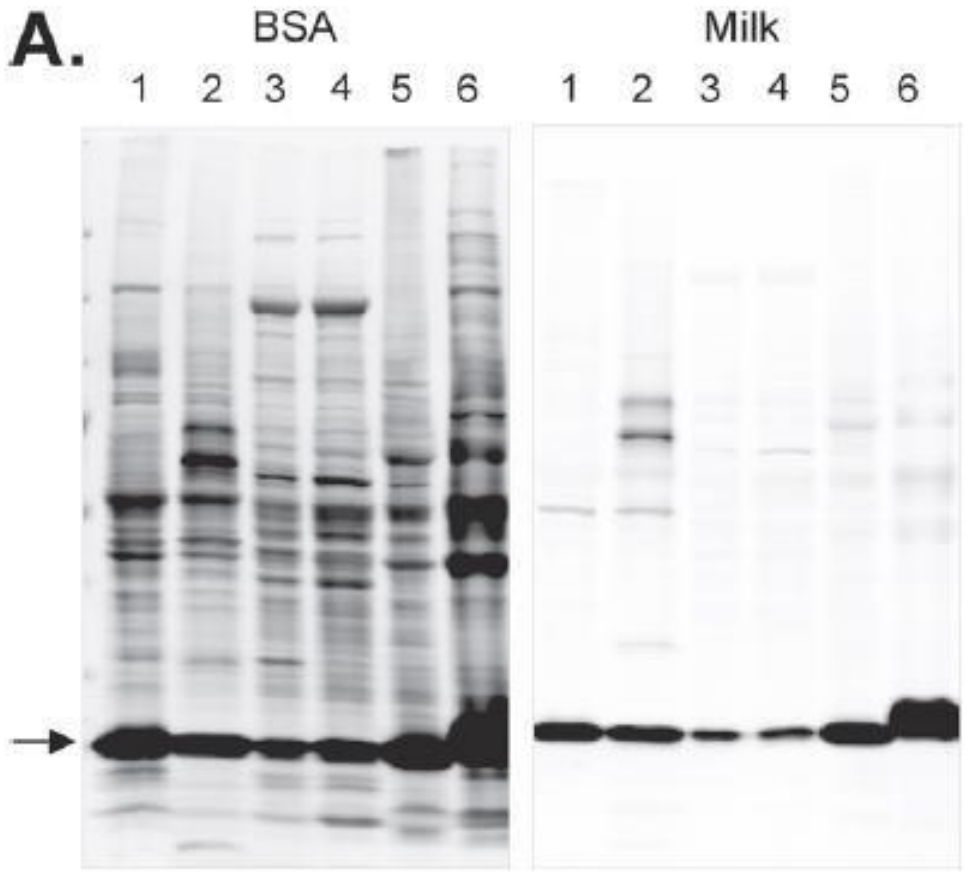
# Primary Antibody validation

Specificity: Controls (Negative/positive)

Selectivity: Antibody dilution and buffers (primary/secondary/blocking)

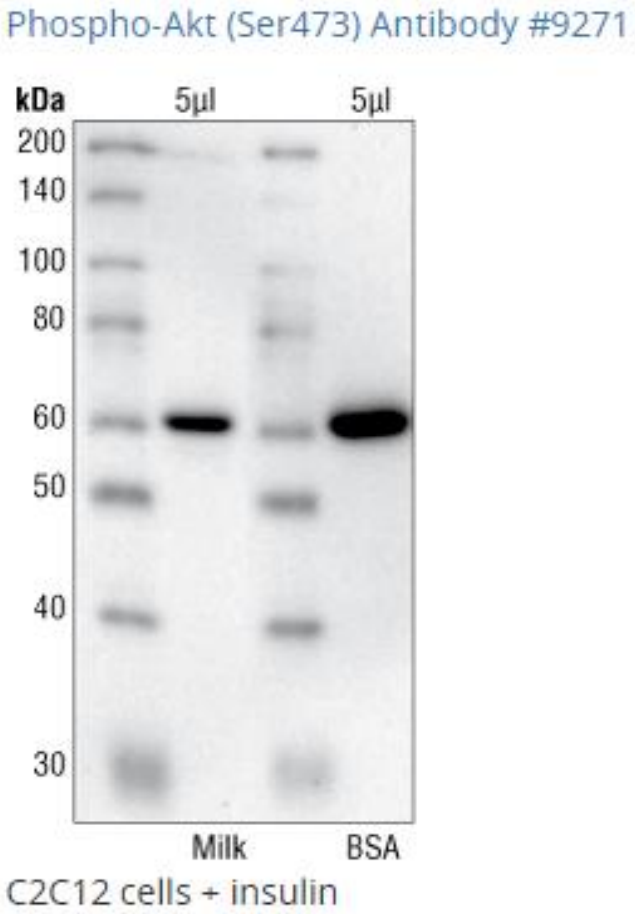
# Primary Antibody validation

Effect of Blocking buffer



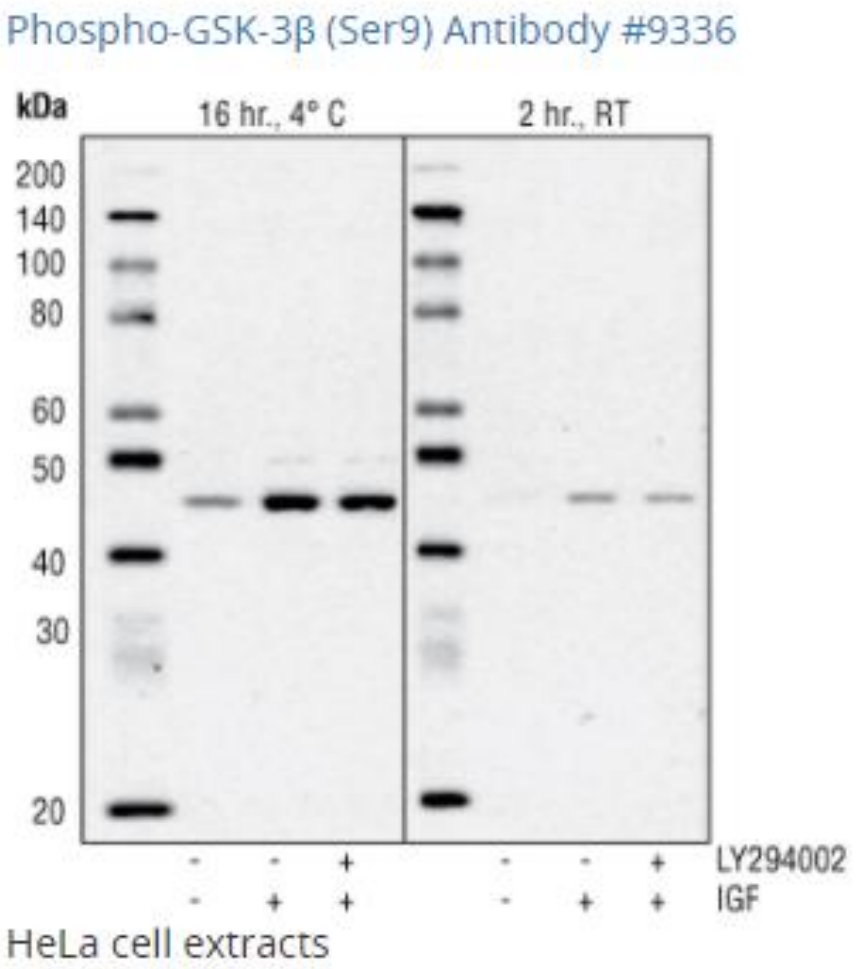
# Primary Antibody validation

Effect of Primary antibody buffer



# Primary Antibody validation

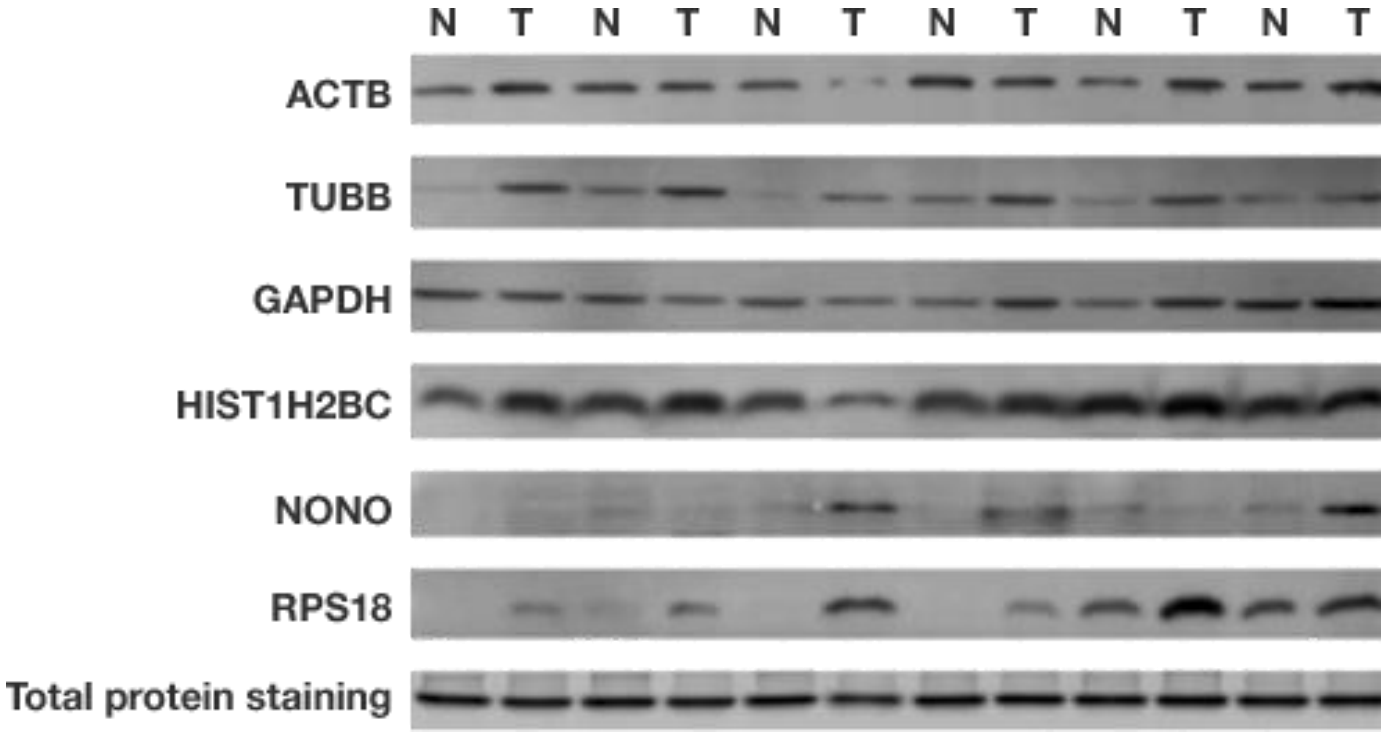
Incubation time: overnight at 4° versus 2h at RT

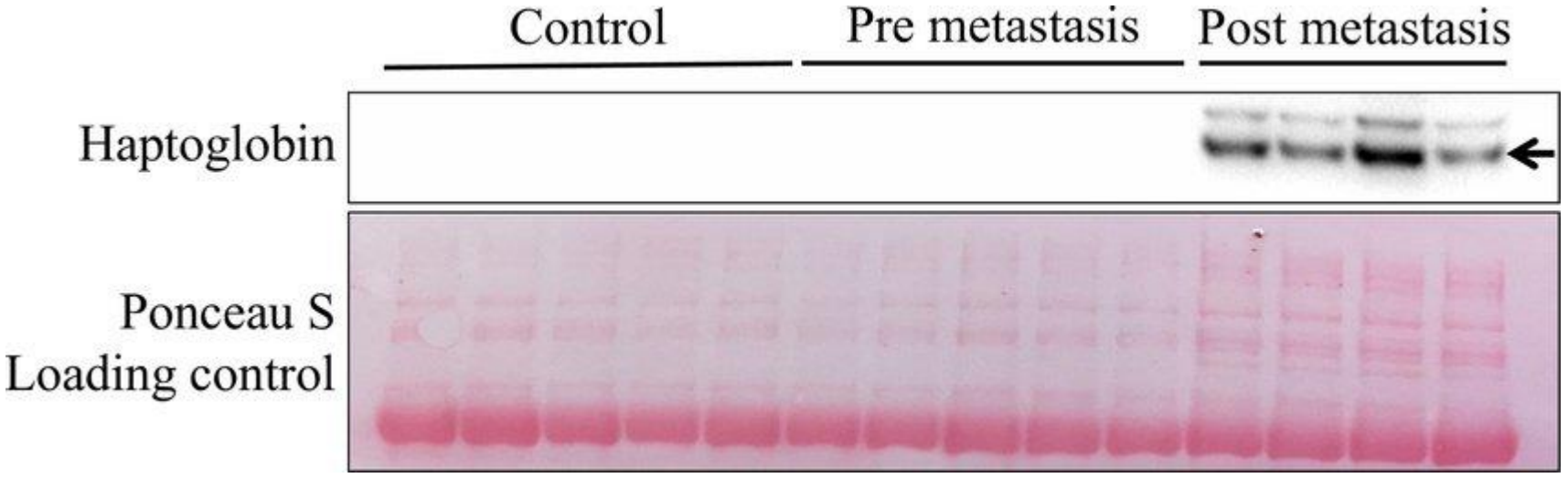


# Loading Controls

| Sample type | protein | MW (kDa) |
|---|---|---|
| whole cell / cytoplasmic proteins | | |
| | beta actin | 43 |
| | alpha actin | 43 |
| | GAPDH | 30-40 |
| | beta-tubulin | 55 |
| | alpha-tubulin | 55 |
| *(high molecular weight)* | vinculin | 116 |
| mitochondria | | |
| | VDCA1/porin | 31 |
| | cytochrome C oxidase | 16 |
| nuclear proteins | | |
| | lamin B1 | 66 |
| | TATA binding protein TBP | 38 |
| | PCNA | 29 |
| | histone H1 | 30 |
| | histone H3 | 18 |
| serum | | |
| | transferrin | 77 |

# Loading Controls - Ponceau




Thank You!