Western Blot Protocol

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About This Protocol

This step-by-step protocol provides everything you need to carry out a successful Western Blot. Written by our PhD qualified expert antibody team, this protocol includes advice for planning your western blot, carrying it out safely, analysing your results, as well as recipes for all the solutions you will need, and a troubleshooting guide. You can also view this protocol online here.

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1. Introduction

Western blotting, also known as immunoblotting, is a key technique in molecular biology to investigate changes in protein expression in a range of different tissue types. Proteins are first denatured before being loaded onto an acrylamide gel with an electric current applied. Proteins are separated by their mass before being transferred onto a membrane (either PDVF or nitrocellulose) and probed with antibodies to reveal expression of specific proteins.

2. Key Decisions

Before starting any western blot experiment it is important to consider the following points:

- What percentage acrylamide gel to use in order to get the best separation (see section 2.1)
- What blocking solution to use for the experimental conditions (see section 2.2)
- What loading controls should be included to account for variability in loading and transfer (see section 2.3)

2.1 Choosing an acrylamide gel percentage

2.1.1 Single concentration gels

In a SDS page all proteins are denatured and have a uniform negative charge applied to them therefore migration is due to differences in size. Polyacrylamide gels are a matrix of cross-linked acrylamide monomers with the tightness of the mesh dependent upon the amount of acrylamide and cross-linker present. Different sized proteins therefore require different formulations of acrylamide gel to get optimum separation (figure 1).

Protein size (kDa)	Gel Percentage (%)
4-40	20
12-45	15
10-70	12
15-100	10
25-200	7.5
>200	5

Table 1 Suggested concentrations of acrylamide gel depending upon protein of interest size. Where multiple proteins of differing sizes need to be separated consider using a gradient gel.

Where there is only one protein of interest or the proteins to be separated are of a similar size then a single concentration gel can be used. Generally the larger the protein the larger pore size is needed in the polyacrylamide gel and the smaller the protein the smaller the pore size (table 1). Once the gel concentration needed has been identified these can either be purchased as pre-cast gels or made in the laboratory using the recipe in table 2.

Reagent	Order	Gel concentration (%)					
		20	15	12	10	7.5	5
dH_2O	1	0.93ml	2.34ml	3.28ml	3.98ml	4.78ml	5.61ml
1.5M Tris-HCl pH 8.8	2	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	3	100µl	100µl	100µl	100µl	100µl	100µl
30% Acrylamide/Bis (29.2:0.8)	4	6.7ml	5ml	4ml	3.3ml	2.5ml	1.67ml
10% APS	5	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
TEMED	6	10µl	10µl	10µl	10µl	10µl	10µl

Table 2. Recipe for the construction of polyacrylamide resolving gels. Makes a 10ml gel. Be sure to add reagents in the correct order with APS and TEMED being added last. CAUTION: Acrylamide is a potent neurotoxin therefore gloves should be worn at all times.

2.1.2 Variable concentration gels

If multiple proteins of significantly differing sizes need to be separated then gradient gels can be instead used. These vary the concentration of the gel along the migration path to provide optimal separation. These can be made in the laboratory but are more easily purchased.

2.1.3 Stacking gels

In order to line up proteins before they enter the resolving gel it is important for proteins to pass through a short layer of stacking gel (Figure 1). This allows proteins to enter the resolving gel at the same point and is the same acrylamide concentration regardless of protein size (Table 3).

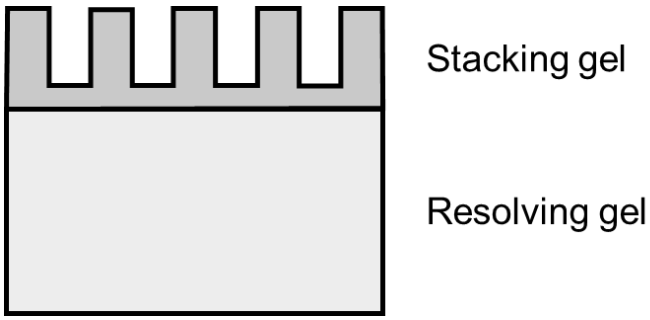

Figure 1. Structure of an acrylamide gel for SDS-PAGE

Reagent	Order	Volume
dH_2O	1	3.05ml
0.5M Tris-HCl pH 6.8	2	1.25ml
10% SDS	3	50µl
30% Acrylamide/Bis (29.2:0.8)	4	650µl
10% APS	5	25µl
TEMED	6	10µl

Table 3 Recipe for acrylamide stacking gel. Makes 5ml suitable for a 10ml resolving gel. Be sure to add reagents in the correct order with APS and TEMED being added last. CAUTION: Acrylamide is a potent neurotoxin therefore gloves should be worn at all times when making or handling the gel.

2.2 Choosing a buffer

Western blots are carried out using either phosphate buffered saline (PBS) or Trizma buffered saline (TBS). While these can often be used interchangeably it is important to consider key differences between the different buffers and make a choice of buffer accordingly (Table 4).

Factor	PBS	TBS
Temperature pH stability	Relatively stable	pH can be highly temperature dependent
Suitability for use with phosphorylation specific antibodies	Not suitable	Suitable
Suitable for use with alkaline phosphatase labelled antibodies	Not suitable	Suitable
Cost	Lower	Higher
Suitable for use with living cells	Suitable	Not suitable

Table 4 Comparison between PBS and TBS buffers.

2.3 Choosing a blocking solution

It is important to choose the correct blocking solution dependent on what experiment is being carried out and what antibodies are being used. For example BSA and milk contain phosphotyrosine which will interrupt experiments using phosphorylation specific antibodies. Additionally alkaline phosphatase can be inhibited by certain milk preparations. For a summary of the different blocking solutions see table 5.

Blocking solution	Constituents	Advantages	Disadvantages
Milk based	5% non-fat dry milk in PBS-T / TBS-T	- Cheap - Most popular solution used by researchers - Clean background	- Not suitable for phosphorylation specific antibodies - Not suitable for biotin labelled antibodies - Can interfere with alkaline phosphatase labelled antibodies - Needs making up fresh and degrades rapidly
BSA	3% BSA in PBS-T / TBS-T	- Good signal strength - Suitable for all antibodies	- Relatively expensive
Fish skin gelatin	1% fish skin gelatin in PBS-T / TBS-T	- Cheap - Doesn't contain mammalian proteins therefore low background	- Cannot be used with biotin labelled antibodies
Serum	10% serum in PBS-T / TBS-T	- Clean background	- Expensive - Incompatible with some anti-immunoglobulin antibody detection. - Contains immunoglobulins and serum proteins that can cross-react with the primary or secondary antibody

Table 5. Comparison between commonly used blocking buffers in western blotting.

2.2.1 Milk based blocking solutions

The most commonly used blocking solution is a solution of 5% non-fat dry milk in PBS-T which works well for the vast majority of applications at a inexpensive price. Some preparations contain sodium azide as a preservative although it should be noted that azide can inhibit horseradish peroxidase, the most commonly used secondary antibody conjugate in western blots. Due to the rapid growth of microbial contamination milk based solutions should be made up fresh for every application. Milk based blocking solutions should not be used for any experiments involving phosphorylation specific antibodies due to the presence of casein which is a phosphoprotein that can interfere with detection. Alkaline phosphatase antibodies can also be inhibited by some preparations of milk alongside biotin labelled antibodies being interfered with by the milk preparation.

2.3.2 BSA

BSA is also widely used as it is suitable for all detection systems as it does not contain biotin or phosphopeptides. There can however be issues with contamination from native immunoglobulins which can cause issues with cross-reactivity. BSA is normally prepared as a 3% dilution in PBS-T or TBS-T. The main drawback of BSA usage is it's relatively high cost compared to other blocking solutions.

2.3.3 Fish skin gelatin

Fish skin gelatin based blocking solutions offer an excellent alternative to other blocking solutions due to its lack of mammalian proteins which reduces the risk of antibody cross-reactivity with the blocking buffer. Fish skin gelatin is also cheap however it should be noted that it isn't appropriate for use with biotin labelled antibodies due to it containing biotin. Fish skin gelatin is normally used as a 1% solution in PBS-T or TBS-T and has the advantage of remaining liquid at room temperature compared to porcine gelatin.

2.3.4 Serum

Serum is another option when considering which blocking solution to use however is declining in popularity due to its high cost and the risk of cross-reactivity with immunoglobulins present in the serum. It has however been reported to offer a clean background and is commonly used as a 10% dilution in either PBS-T or TBS-T.

2.4 Selection of appropriate loading controls

2.4.1 What are loading controls?

Loading controls are antibodies against a different target to the protein of interest used in immunoblotting (Western blotting). These targets are often highly expressed housekeeping proteins who's expression is stable. Loading controls are essential when the relative expression of proteins is being compared in a gel and are used to:

- Ensure that the loading of proteins is uniform across the whole gel. Where there has been unequal loading the loading control can be used to account for this.

- Ensure that there has been equal transfer of proteins from gel to membrane across the gel

2.4.2 Choosing a loading control

There are several key principles that need to be followed when choosing a loading control for immunoblotting:

- The loading control and target of interest should have different molecular weights to ensure they do not overlap on the gel

- The loading control expression should not be effected by any experimental manipulation between samples.

- The loading control should have high levels of expression in the sample

- The loading control should be in the portion of the gel where there is linear separation otherwise it will not be possible to quantify it.

Use table 6 to help identify a suitable loading control depending on molecular weight of the target protein and the subcellular location of the sample. You can view all the loading controls available from Hello Bio here

Molecular weight (kDa)	Whole cell	Mitochondrial	Nuclear	Membrane	Cytoskeleton	Serum
125	Vinculin					
110				NaK ATPase		
75						Transferrin
66			Lamin B1			
60		HSP60	HDAC1			
55						
50	Alpha tubulin				Alpha tubulin	
	Beta tubulin				Beta tubulin	
45	Actin		YY1		Actin	
40	Beta actin				Beta actin	
35	GAPDH		TBP			
30		VDAC1/Porin	PCNA			
20		Cyclophilin B				
		Cofilin	COX IV		Cofilin	
15			Histone H3			

Table 6. Common loading controls. Molecular weight is of the loading control therefore choose one at a different mass to the target of interest.

3. Equipment and consumables

Assuming a base level of standard laboratory equipment (e.g. pipettes, de-ionised water system, measuring cylinders etc) the specific equipment and consumables you will require for western blotting are detailed by stage within this section.

3.1 SDS Page

Equipment	Consumables
Powerpack	Protein ladder
Gel tank and cassette	Filter papers
Casting plates	Running buffer
Combs (same thickness as casting plates)	Sample buffer
Casting stand and gel holders	Ethanol
Heating block (able to achieve 85ºC)	Resolving gel
Microcentrifuge	Stacking gel
Loading tips (not essential but make gel loading easier)	

3.2 Electrophoretic Transfer

Equipment	Consumables
Powerpack	Transfer buffer
Gel tank and cassettes	Filter papers
Large shallow tray (for preparing transfer cassettes in)	PVDF membrane
Roller (for removing bubbles in transfer sandwich with)	Methanol
Absorbent pads	
Ice pack	
Magnetic stirring plate and stirring bars	
Gel opening tool	

3.3 Immunoblotting

Equipment	Consumables
Rocker	PBS-T
Containers for putting membranes in	Blocking solution
Heat sealer	Primary antibody
Detection system (e.g. film with darkroom or gel imaging system)	Secondary antibody
	Plastic bags for heat sealer
	Enhanced chemiluminescence substrate (ECL substrate)
	Optional: Ponceau stain

4. Protocol

This protocol is written assuming that you have already prepared samples to load. For advice on how to extract proteins from both tissue samples and cell culture please see our protocols.

4.1 Safety

Many of the chemicals used in western blotting have dangerous properties and can cause serious harm if not handled correctly. Always follow local rules and read the full COSHH document for any chemical that you have not used previously. Always wear appropriate PPE such as a lab coat and gloves.

Specifically highlighted hazards:

- Acrylamide is an extremely toxic neurotoxin and carcinogen in monomeric forms. Wipe down benches after use and dispose of gels and any contaminated waste into separated acrylamide waste.
- Beta-mercaptoethanol is toxic and needs disposing separately to normal laboratory waste.

4.2 SDS-PAGE

1. Clean casting stand and gel holders with distilled water. Clean plates with distilled water.

2. Construct gel mould on holder

    a. Place the casting frame upright with the pressure cams in the open position and facing forward on a flat surface.
    b. Select a spacer plate of the desired gel thickness and place a short plate on top of it.
    c. Orient the spacer plate so that the labelling is up. Slide the two glass plates into the casting frame, keeping the short plate facing the front of the frame (side with pressure cams)
    d. When the glass plates are in place, engage the pressure cams to secure the glass cassette sandwich in the casting frame. Check that both plates are flush at the bottom.
    e. Place the casting frame into the casting stand by positioning the casting frame (with the locked pressure cams facing out) onto the casting gasket while engaging the spring-loaded lever of the casting stand onto the spacer plate.
        i. It is often a good idea to fill the gel plates with dH_2O to check for leaks while making the resolving gel. Pour out when ready to pour the resolving gel.

3. Mix resolving gel (adding ammonium persulphate and TEMED last) and pour immediately. The resolving gel should be far enough below the top of the gel plates to allow insertion of the comb + 1cm.

Reagent	Order	Gel concentration (%)					
		20	15	12	10	7.5	5
dH_2O	1	0.93ml	2.60ml	3.59ml	4.26ml	5.09ml	5.93ml
1.5M Tris-HCl pH 8.8	2	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	3	100µl	100 µl	100 µl	100 µl	100 µl	100 µl
30% Acrylamide/Bis (29.2:0.8)	4	6.66ml	5.00ml	4.00ml	3.33ml	2.50ml	1.67ml

10% APS	5	50µl	50µl	50µl	50µl	50µl	50µl
TEMED	6	10µl	10µl	10µl	10µl	10µl	10µl

a. Optional: Degas the mixture using a tap vacuum setup for 10 minutes (make sure to remove bung before turning off tap)

4. Overlay resolving gel gently with water or ethanol to maintain a flat surface on the resolving gel during polymerisation. Leave to set for 15/20 minutes.

a. Tip: Make excess gel and then monitor the polymerisation using this.

5. Mix stacking gel except APS and TEMED. Pour off overlay and use a tissue wick to remove the last remnants. Add APS and TEMED to stacking gel and pour. Add the comb with no air bubbles and leave to set for around 20 minutes.

Reagent	Order	Volume				
		1 gel	2 gels	3 gels	4 gels	5 gels
dH_2O	1	3.05ml	6.10ml	9.15ml	12.2ml	15.25ml
0.5M Tris-HCl pH 6.8	2	1.25ml	2.5ml	3.75ml	5ml	6.25ml
10% SDS	3	50µl	100 µl	150 µl	200 µl	250 µl
30% Acrylamide/Bis (29.2:0.8)	4	650µl	1.3ml	1.95ml	2.6ml	3.25ml
10% APS	5	25µl	50µl	75µl	100µl	125µl
TEMED	6	10µl	20µl	30µl	40µl	50µl

a. Optional: Degas the mixture using a tap vacuum setup for 10 minutes (make sure to remove bung before turning off tap)

6. Heat samples in sample buffer for 10 minutes at 85ºC. Give samples a quick spin before loading to remove debris.

7. Make up 1l of 1X running buffer (diluting 10X buffer as necessary). Construct running assembly making sure seals are tight (use grease if necessary). Add running buffer to top tank (above level of gel, CATHODE). Check for leaks. Add running buffer to bottom tank (ANODE)

8. Remove comb. Gently wash out each well

9. Add samples in sample buffer. Ideally do not use outside wells. Run markers in central well and at side. Any unused wells should be filled with a small volume of 1X sample buffer. Sample loading volumes should be from 5 µL–35 µL per lane (depending on gel).

a. Make sure a consistent quantity of protein is loaded per well ≈ 20µg.
b. Use around 4-5µl of marker, one option is to load 2µl of marker on one side of the gel and 5µl on the other to distinguish sides.
c. Mix marker with sample buffer to make up to the same loading volume as samples

10. Connect up equipment (red to red, black to black)

11. Run at 60V until dye front is in the resolving gel (30 minutes) and then run at 120-150V in gel (45-90 minutes). Vary by experience and do not exceed 200V.

12. Stop when gel front nears bottom.

4.3 Electrophoretic transfer

1. Cut out a rectangle of PDVF membrane using a template to fit the size of the gel. Write the date and name of experiment on the membrane in pen

2. Wash membrane with methanol for 30 seconds (methanol can be reused)

3. Wash membrane with transfer buffer

4. Soak the blotting paper and sponges in transfer buffer

5. Put cassette (red and black) into tank

6. Take gel out of running tank and carefully open the plates; cut off the stacking gel and put in acrylamide waste

7. Soak the gel in transfer buffer
8. Assemble the cassette in the following order making sure to keep everything as wet as possible:

    a. Black side of cassette
    b. Sponge
    c. 3x blotting paper
    d. Gel face down (so the ladder is on the right side of the gel with the red marker nearer the top)
    e. Membrane also face down
    f. 3x blotting paper (after this step you can use a roller to gently get rid of any bubbles between the gel and the membrane)
    g. Sponge
    h. Clear side of cassette

9. Close the cassette and put into the holder making sure the colours align. The proteins will run towards the positive anode (red)

10. Add the ice block and stirring bar

11. Fill up the tank with transfer buffer without it overflowing

12. Turn on stirring plate and put on lid

13. Set the power pack to 400 mA for 90 minutes (check there are bubbles before leaving). **Pause point: The membranes can be left in transfer buffer overnight for processing the next day however this means that the entire protocol will take 3 days as opposed to 2 if the primary antibody addition is achieved on day 1.**

4.4 Immunoblotting

1. Rinse the membrane several times with PBS-T.

2. Add a blocking solution. The choice of blocking will depend upon the target and upon the detection method. E.g. alkaline phosphatase is inhibited by some milk preparations.

3. Incubate with agitation for 2hrs

4. Add the primary antibody solution. All antibodies should be diluted in blocking solution. Incubate overnight with agitation in the cold room.

    a. Add the antibody to strip to a plastic bag then use a heat sealer to seal the pouch. Doing this should mean only around 1ml per bag is needed.

5. Wash the blot with 3 quick changes of PBS-T then 3 times for 5 minutes under agitation.

6. Add the secondary antibody to blocking solution. Incubate for 1-2hrs at room temperature with agitation. For fluorescently labelled secondary antibodies all steps should be done in the dark from now on.

7. Wash the blot with 3 quick changes of PBS-T then 3 times for 5 minutes under agitation.

8. Detect using ECL (for HRP conjugated secondaries). Follow manufacturer instructions and cover membrane with transparent plastic sheet to stop it drying out.

    a. Detection can be either through the use of film and a darkroom or by using a gel imaging system.
        i. Film: Generally the most sensitive but can be expensive and it is more difficult to use for quantitative measurements

ii. Imaging system: Once initial outlay has been paid is much cheaper and allows accurate quantitative measures. However can be less sensitive than film.

4.5 Stripping and Re-probing

Following the completion of an immunoblot it is possible to remove the primary and secondary antibodies then re-probe the membrane for a new target. This is commonly used with a loading control antibody following probing for a protein of interest. The same membrane can be stripped and re-probed multiple times, however each treatment removes protein from the membrane which should be taken into account. This protocol requires a PVDF membrane and detection using ECL.

1. Incubate membrane in two changes of stripping buffer at room temperature for 10 minutes an incubation.

2. Wash membrane in 3 quick changes of PBST

3. Proceed to blocking (step 2 of section 4.4)

If you want to check that stripping has been successful then incubate membrane with ECL detection substrates after step 2 then image. The bands from the previous antibody should have disappeared and there be a clear uniform background.

5. Analysis

5.1 Measuring the molecular weight of a protein

The molecular weight of a protein can be estimated by comparing the migration of proteins of known molecular weight (such as in a protein ladder) and the target protein. A general procedure for doing this is:

1. Run the gel using a molecular weight ladder, transfer to the membrane and then visualise the proteins using a dye such as Coomassie blue or Ponceau.

2. Calculate the relative migration distance (Rf) of each protein standard and the target protein using the equation:

$$Rf = \frac{Protein\ migration\ distance\ from\ bottom\ of\ the\ gel}{Dye\ front\ migration\ distance\ from\ bottom\ of\ the\ gel}$$

This can either be measured using a ruler or within appropriate software.

3. Plot the log(MW) of the protein standards against relative migration distance (Rf) on a graph and generate a curve of best fit. This should be linear if the samples are fully denatured and the gel percentage was adequate for sufficient separation.

4. Use the equation of the best fit line to calculate the mass of the target using its Rf. The general equation is: $MW = 10^{\frac{Rf-c}{m}}$ which in figure 1 simplifies to $MW = 10^{\frac{Rf-1.368}{-0.5053}}$.

5. The actual protein molecular weight as determined by mass spectrometry is likely to differ from the estimated through immunoblotting due to differences in glycosylation status, experimental inaccuracies and some proteins not being amenable to full denaturation by SDS.

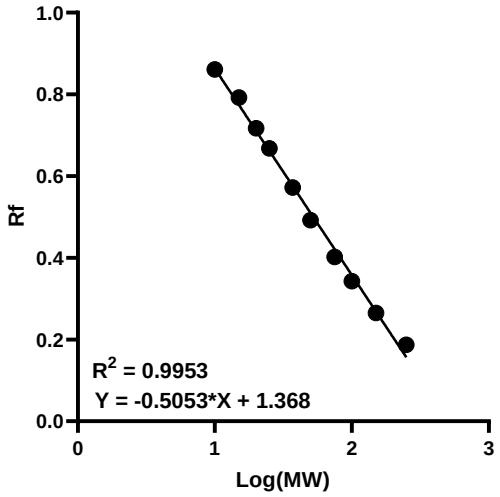

Figure 1. Example standard curve of log(MW) against relative migration (Rf). A high R^2 value indicates increased accuracy in interpretation of the unknown proteins MW.

5.2 Quantifying protein expression from an immunoblot

Protein expression can be quantitatively compared within a immunoblot using densitometry. Crucially this technique can only inform of relative changes in abundance between samples therefore without known standards, cannot be used to give exact concentrations.

Before trying to take any quantitative measurements it is critical to make sure that the signal is not saturated and is in its linear range (figure 2). If the signal is saturated then it isn't possible to accurately compare different protein abundances. However, saturation is a relatively easy problem to solve as by reducing the exposure time this can be avoided. However, where there are large differences in protein abundance between samples then it may be necessary to re-attempt the immunoblot with more similar protein concentrations if it isn't possible to have both

bands properly exposed (i.e. one is always saturated to see the other). Reducing the concentration of primary antibody is also another way of reducing saturation.

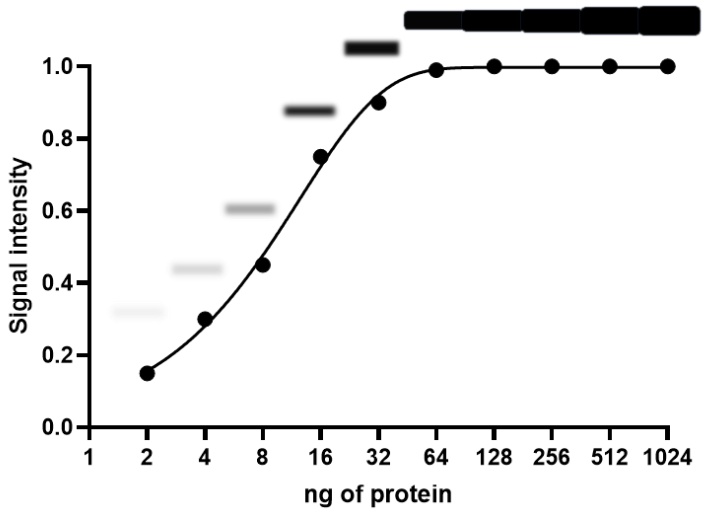

Figure 2: Example curve showing the saturation of western blot signal at high levels of bound protein. Above each datapoint is a representative illustration of the band seen. Adapted from Bell, 2016. BMC Biol; 14:116.

It is next important to subtract any background noise from the image. Differences in background across the blot may not be consistent leading to changes in band darkness that are influenced by background, not signal intensity. This step is easily achieved in both bespoke analysis software and freeware such as ImageJ (https://imagej.nih.gov/).

Next it is critical to normalise the signal intensity for protein loading. Naturally each lane will have a slightly different amount of protein in it therefore by normalising to expression of stably expressed “housekeeping” proteins such as GAPDH or β-actin this can help to ensure that any changes in expression of a target protein are not just due to differences in loading. It is important when designing the experiment to think about whether the manipulation might affect expression of the loading control and another issue is that due to high expression it is often difficult to capture them in their linear range.

Finally it is important to subject results to the appropriate statistical tests for the experimental design. It should be planned before the experiment was carried out what n-number was needed to achieve a sufficient statistical power (often chosen as 0.8).

6. Solutions

6.1 Buffers

0.5M Tris-HCl pH 6.8

Note: For longer term storage autoclave then store at 4ºC

Reagent	Amount to add		
	100ml	500ml	1000ml
Tris-base	6.06g	30.29g	60.57g
dH_2O	≈80ml	≈400ml	≈800ml
Conc. HCl	Adjust pH to 6.8		
dH_2O	Make up to final volume required		

1.5M Tris-HCl pH 8.8

Note: For longer term storage autoclave then store at 4ºC

Reagent	Amount to add		
	100ml	500ml	1000ml
Tris-base	18.17g	90.86g	181.71g
dH_2O	≈80ml	≈400ml	≈800ml
Conc. HCl	Adjust pH to 8.8		
dH_2O	Make up to final volume required		

10x running buffer

Notes:

- For 1x buffer dilute with dH_2O in a 1:10 dilution
- SDS powder is a potent respiratory irritant therefore should be weighed out in the fume hood or with breathing protection.
- Store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
Glycine	72g	144g	288g	1.91M
Tris-base	15g	30g	60g	247mM
SDS	5g	10g	20g	35mM
dH_2O	Make up to final volume required			

10x transfer buffer

Notes:

- Store at room temperature
- For 1x buffer dilute 1:10 with dH_2O / 20% methanol

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
Glycine	72g	144g	288g	1.91M
Tris-base	15g	30g	60g	247mM
dH_2O	Make up to final volume required			

10% SDS

Notes:

- Store at room temperature
- Over time SDS may precipitate out of solution. If this occurs remix until all SDS has re-dissolved.

Reagent	Amount to add		
	100ml	200ml	500ml
SDS	10g	20g	50g
dH_2O	Make up to final volume required		

2x Sample loading buffer

Note store in aliquots at -20ºC

Reagent	Amount to add			Final concentration
	5ml	50ml	100ml	
10% SDS	2ml	20ml	40ml	4%
0.2% Bromophenol blue	0.1ml	1ml	2ml	0.004%
Glycerol	1ml	10ml	20ml	20%
0.5M Tris-HCl pH 6.8	1.25ml	12.5ml	25ml	0.125M
ß-mercaptoethanol	0.5ml	5ml	10ml	10%
H_2O	0.15ml	1.5ml	3ml	-

10% APS

Note: store in aliquots at -20ºC

Reagent	Amount to add		
	10ml	50ml	100ml
Ammonium persulphate	1g	5g	10g
dH_2O	Make up to final volume required		

10x PBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
KCl	1g	2g	4g	27mM
Na_2HPO_4	7.2g	14.4g	28.8g	100mM
KH_2PO_4	1.2g	2.4g	4.8g	20mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

10x TBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
Tris-base	12.1g	24.2g	48.5g	200mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

1x PBST / TBST

Notes:

- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.
- The solution will need a good mixing with a stirring bar before being ready to use
- Generally make up fresh and don't keep for longer than a few days.

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
10x TBS / PBS	50ml	100ml	200ml	1x
dH_2O	450ml	900ml	1800ml	-
Tween20	0.5ml	1ml	2ml	0.1%

Stripping buffer

Notes:

- The buffer can sometimes go cloudy over time however this does not appear to impact it's effectiveness.
- Store at room temperature
- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.

Reagent	Amount to add			Final concentration
	100ml	500ml	1000ml	
Glycine	1.5g	7.5g	15g	200mM
SDS	0.1g	0.5g	1g	0.1%
Tween20	1ml	5ml	10ml	1%
dH_2O	≈80ml	≈400ml	≈800ml	-
Conc HCl	Adjust to pH 2.2			-
dH_2O	Make up to final volume required			-

6.2 Gel formulations

Note: Make sure to add reagents in the order indicated. Acrylamide is a potent neurotoxin therefore take appropriate precautious and wear suitable PPE.

5% resolving gel

For proteins larger than 200kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	5.93	11.85	17.78	23.70	29.63	35.55	41.48	47.40	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	1.67	3.33	5.00	6.66	8.33	9.99	11.66	13.32	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

7.5% resolving gel

For proteins sized between 25 and 200 kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	5.09	10.19	15.28	20.37	25.46	30.56	35.65	40.74	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	2.50	5.00	7.49	9.99	12.49	14.99	17.48	19.98	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

10% resolving gel

For proteins sized between 15 and 100kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	4.26	8.52	12.78	17.04	21.30	25.56	29.82	34.08	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	3.33	6.66	9.99	13.32	16.65	19.98	23.31	26.64	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

12% resolving gel

For proteins sized between 10 and 70kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	3.59	7.19	10.78	14.38	17.97	21.56	25.16	28.75	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	4.00	7.99	11.99	15.98	19.98	23.98	27.97	31.97	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

15% resolving gel

For proteins sized between 12 and 45kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	2.60	5.19	7.79	10.38	12.98	15.57	18.17	20.76	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	5.00	9.99	14.99	19.98	24.98	29.97	34.97	39.96	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

20% resolving gel

For proteins between 4 and 40kDa.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	0.93	1.86	2.79	3.72	4.65	5.58	6.51	7.44	ml
1.5M Tris-HCl pH 8.8	2	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	ml
10% SDS	3	100	200	300	400	500	600	700	800	µl
30% Acrylamide/Bis (29.2:0.8)	4	6.66	13.32	19.98	26.64	33.30	39.96	46.62	53.28	ml
10% APS	5	50	100	150	200	250	300	350	400	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

Stacking gel

Note: Make sure to add in the order indicated. Acrylamide is a potent neurotoxin therefore take appropriate precautious and wear suitable PPE.

Reagent	Order	Gels								Unit
		1	2	3	4	5	6	7	8	
dH_2O	1	3.05	6.10	9.15	12.2	15.25	18.3	21.35	24.4	ml
0.5M Tris-HCl pH 6.8	2	1.25	2.5	3.75	5	6.25	7.5	8.75	10	ml
10% SDS	3	50	100	150	200	250	300	350	400	µl
30% Acrylamide/ Bis (29.2:0.8)	4	650	1.3	1.95	2.6	3.25	3.9	4.55	5.2	ml
10% APS	5	25	50	75	100	125	150	175	200	µl
TEMED	6	10	20	30	40	50	60	70	80	µl

6.3 Blocking solutions

See section 2.2 for advice on choosing a blocking solution.

Blotto

Note:

- Make up fresh for every application
- For longer term incubations it is possible to add sodium azide to a final concentration of 0.01% to prevent bacterial growth.

Reagent	Amount to add			Final concentration
	10ml	20ml	50ml	
1x PBST/TBST	10ml	20ml	50ml	-
Non-fat dry milk (NFDM)	0.5g	1g	2.5g	5%

BSA

Note:

- Make up fresh for every application
- For longer term incubations it is possible to add sodium azide to a final concentration of 0.01% to prevent bacterial growth.

Reagent	Amount to add			Final concentration
	10ml	20ml	50ml	
1x PBST/TBST	10ml	20ml	50ml	-
Bovine serum albumin (BSA)	0.3g	0.6g	1.5g	3%

Fish skin gelatine

Note:

- Make up fresh for every application
- For longer term incubations it is possible to add sodium azide to a final concentration of 0.01% to prevent bacterial growth.

Reagent	Amount to add			Final concentration
	10ml	20ml	50ml	
1x PBST/TBST	10ml	20ml	50ml	-
Fish skin gelatin	1g	2g	5g	1%

Serum

Note:

- Make up fresh for every application
- For longer term incubations it is possible to add sodium azide to a final concentration of 0.01% to prevent bacterial growth.

Reagent	Amount to add			Final concentration
	10ml	20ml	50ml	
1x PBST	9ml	18ml	45ml	-
Serum from same species as secondary antibody	1ml	2ml	5ml	10%

7. Troubleshooting

Western blotting is a long multi-step process with many different factors that need to be considered. At some point it is inevitable that something will go wrong or not be optimal. Below are compiled some of the most common pitfalls that can cause western blots to not work.

Problem	Potential cause	Suggested solutions
No bands or faint signal	Incomplete transfer of proteins from the acrylamide gel to PVDF membrane	- Use a reversible stain such as Ponceau to check that transfer was successful. - Alternatively use Coomassie stain on the gel following transfer to make sure no to little protein remains. - Increase the transfer time if short - Check that the transfer sandwich was assembled in the correct order so that proteins weren't transferred away from the membrane
	Over-transfer of proteins through PVDF membrane if transfer is for too long with a low molecular weight target	- Reduce either the transfer time or transfer current - Choose a PVDF membrane optimised to small proteins
	Reagents may have deteriorated due to meeting use by dates or improper storage conditions	- Make sure all reagents are stored following manufacturer suggestions and are within date
	Incorrect species of secondary antibody	- Ensure that the secondary antibody used is specific for the species the primary is raised in
	Buffers are contaminated by bacteria	- Make sure all solutions are free from contamination. An easy way to check is to ensue that there is no cloudiness visible when solutions are swirled.
	Exposure time too short	- Try increasing the exposure time
	Antibody concentration is too low	- Increase the primary antibody concentration and incubate for a longer amount of time

Problem	Potential cause	Suggested solutions
No bands or faint signal	The protein of interest isn't present in the samples	- Make sure that the protein is expressed in the sample through literature searches - Consider using a positive control.
	Too low an amount of protein is loaded	- Increase the amount of protein loaded to at least 20µg per lane. - If the amount of protein in a sample hasn't been determined use an assay such as the Bradford assay to determine.
	Antibody re-use	- Antibody re-use reduces the activity and specificity of antibodies therefore should be avoided.
	Buffer components may inhibit detection method	- Sodium azide can inhibit HRP detection systems therefore make sure all buffers are azide free.
	Protein precipitated in the gel due to low solubility or aggregation	- Try including SDS in the transfer buffer and reducing the methanol concentration.
	Detection reagents inactive	- Make sure the detection reagents are within their use by dates. - Try new detection reagents
White spots on blot	Air bubbles in transfer sandwich assembly	- Make sure to roll bubbles out of the sandwich during assembly with a roller before transfer - Check that the transfer sandwich assembly is tightly held within the cassette and change sponges if it isn't
	Gel overheated during transfer causing bubbles	- Ensure that adequate cooling is present during transfer through either an ice pack or performing the transfer in a cold room. - Reduce the current of the transfer - Pre-cool buffers before use
	Improperly prepared PVDF	- Ensure that PVDF is properly wetted in methanol before being equilibrated in transfer buffer
High background	Wrong blocking solution	- Ensure blocking solution is appropriate to experimental conditions (see section 2.3)
	Incomplete blocking of membrane	- Increase blocking incubation time - Increase the concentration of blocking buffer

Problem	Potential cause	Suggested solutions
High background	Contaminated blocking buffer	- Do not re-use blocking buffers, make up fresh each time
	Too high antibody concentration	- Reduce primary and secondary antibody concentrations
	Insufficient washing	- Increase the number and duration of washing steps after the secondary antibody incubation
	Too long exposure	- Reduce exposure
	Antibody has lost specificity due to improper storage	- Use fresh antibody or freshly thawed aliquots that have been stored at -80°C
	Buffers are contaminated by bacteria	- Make sure all solutions are free from contamination. An easy way to check is to ensue that there is no cloudiness visible when solutions are swirled.
Smears on blot	Blot contamination from touching membrane	- Only handle membrane as little as possible using the edges, use forceps where able.
	Uneven incubations	- Make sure a rocker or shaker is used for all incubations to ensure that the membrane surface is thoroughly covered in solution.
	Parts of membrane dried out	- Ensure that there is always an excess of liquid at all stages in the process.
Black spots on blot	Antibodies binding to clumped blocking reagent or blocking reagent clumped to membrane	- Make sure blocking buffers are thoroughly vortexed before use - Use fresh blocking buffers
	Aggregated secondary antibody	- Centrifuge secondary antibody to remove aggregates before use.
	Acrylamide gel stuck to membrane	- Ensure all gel contamination is removed after transfer before further processing.
Smile shaped bands	Electrophoresis voltage was too high	- Reduce voltage - Run gel using chilled buffers, in cold room or using an ice pack
Unevenly run bands	Poor acrylamide polymerisation	- Ensure that gel is fully polymerised following recommended recipe before sample loading.
Blurry bands	Electrophoresis voltage was too high	- Reduce voltage - Start at a lower voltage then turn up after 30 minutes

Problem	Potential cause	Suggested solutions
White centred bands (ECL detection)	Rate of reaction was too high and ECL has been depleted	- Reduce primary antibody concentration - Reduce exposure - Reduce secondary antibody concentration
Extra bands	Target protein has multiple splice variants or isoforms	- Check in literature for previously reported splice variants. - See if a splice variant specific antibody is available.
	Target protein has been subject to proteolytic cleavage	- Try including protease inhibitors to the lysis buffer when preparing samples. - Make sure samples are prepared on ice - Try using a fresh sample
	Antibody epitope is present in another protein	- Ensure a negative control sample has been included. - Try a different antibody using a different epitope - Decide if the extra band is an issue for the planned experiment.
	Secondary antibody concentration too high	- Reduce secondary antibody concentration - Try a secondary only blot to check non-specific binding of the secondary
	Native IgGs present within samples detected by secondary	- Use pairs of antibodies with no relation to the species the samples come from - Decide if this is an impediment to the designed experiment
	Contaminant IgGs within primary antibody reacted with non-specific proteins	- Ensure primary antibodies have been purified in an appropriate way. For polyclonal antibodies these should be immune-affinity purified to the immunogen whereas monoclonal antibodies can be purified with protein A/G.
	Incomplete blocking of membrane	- Increase blocking incubation time - Increase the concentration of blocking buffer
Target is at lower MW than expected	Target protein has been subject to proteolytic cleavage	- Try including protease inhibitors to the lysis buffer when preparing samples. - Make sure samples are prepared on ice - Try using a fresh sample

Problem	Potential cause	Suggested solutions
Target is at lower MW than expected	Target protein has been subject to proteolytic cleavage	- Try including protease inhibitors to the lysis buffer when preparing samples. - Make sure samples are prepared on ice - Try using a fresh sample
	Antibody epitope is present in another protein	- Ensure a negative control sample has been included. - Try a different antibody using a different epitope - Decide if the extra band is an issue for the planned experiment.
Target is at a higher MW than expected	Target protein may have been subject to post-translational modifications such as glycosylation	- Check literature for previous reports of post translational modification in the target - Attempt to remove the post-translational modification using enzymes such as deglycosylases.
	Target protein may have formed dimers or multimers	- Try increasing the concentration of β-mercaptoethanol in the sample buffer - Try using stronger reducing agents

8. Further reading

Bell, 2016. Quantifying western blots: none more black. BMC Biology; 114: 116

Gassmann et al, 2009. Quantifying Western blots: Pitfalls of densitometry. Electrophoresis 2009; 30: 1845-1855

Pillai-Kastoori et al, 2020. A systematic approach to quantitative Western blot analysis. Analytical Biochemistry; 593: 113608