

A Guide to Protein Blotting

SDS-PAGE

SDS-PAGE stands for sodium dodecyl (lauryl) sulphate-polyacrylamide gel electrophoresis. SDS-PAGE has a number of uses, which include: establishing protein size, protein identification, determining sample purity, identifying disulfide bonds, quantifying proteins and blotting applications. The SDS portion is a detergent the purpose of which is to take the protein from its native shape and open it up into a linear conformation. It's an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform charge, so that they can be separated solely on the basis of their size. The SDS has a high negative charge that overwhelms any charge the protein may have, imparting all proteins with a relatively equal negative charge. The SDS has a hydrophobic tail that interacts strongly with protein (polypeptide) chains. The number of SDS molecules that bind to a protein is proportional to the number of amino acids that make up the protein. Each SDS molecule contributes two negative charges, overwhelming any charge the protein may have. SDS also disrupts the forces that contribute to protein folding (tertiary structure), ensuring that the protein is not only uniformly negatively charged, but linear as well. In electrophoresis, an electric current is used to move the protein molecules across a polyacrylamide gel. The polyacrylamide gel is a cross-linked matrix that functions as a sort of sieve to help "catch" the molecules as they are transported by the electric current. The polyacrylamide gel acts somewhat like a three-dimensional mesh or screen. The negatively charged protein molecules are pulled to the positive end by the current, but they encounter resistance from this polyacrylamide mesh. The smaller molecules are able to navigate the mesh faster than the larger ones, so they make it further down the gel than the larger molecules. This is how SDS-PAGE separates different protein molecules according to their size.

Once an SDS-PAGE gel is run, you need to fix the proteins in the gel so they don't come out when you stain the gel. Acetic acid 25% in water is a good fixative, as it keeps the proteins denatured. The gel is typically stained with Coomassie blue dye R250, and the fixative and dye can be prepared in the same solution using methanol as a solvent. The gel is then destained and dried.

Western blotting (Immunoblotting)

Western blot analysis can detect one protein in a mixture of any number of proteins while giving you information about the size of the protein. This method is, however, dependent on the use of a high-quality antibody directed against the desired protein which you will use as a probe to detect the protein of interest.

Western blotting tells you how much protein has accumulated in cells. If you are interested in the rate of synthesis of a protein, Radio-Immune Precipitation (RIP) may be the best assay for you. Also, if a protein is degraded quickly, Western blotting won't detect it well; you'll need to use RIP.

Recommendations and troubleshooting

Each antibody-antigen interaction has unique characteristics. A protocol giving good results for one antibody-antigen pair might be unsatisfactory for a second antibody even on the same sample. The interaction of antisera with protein epitopes in Western blot detection is dependent on a number of factors all contributing to the final signal/noise ratio. Knowledge about and control of those factors allows for modifications and optimization of the procedure. Polyclonal antibodies usually contain a number of different antibodies interacting independently with different epitopes on the target used for immunization. This number is limited for peptide-antigens but if recombinant or native proteins have been used for immunization the signal obtained has to be regarded as cumulative (caused by several different types of antibodies present in the serum used).

To achieve reproducible results the usage of gloves, forceps, clean and detergent-free laboratory materials, non-expired reagents, freshly prepared buffers with controlled pH, as well as keeping protocol volumes, dilutions, and times is of primary importance in addition to the steps discussed below. In general incubation times should be as short as possible while antibody dilutions should be as high as possible.

Sample

Careful control about the presence of the target protein in the starting material, its preparation and storage under non-degrading/aggregating conditions is essential. If the target protein is degraded or not present in sufficient amounts in the sample loaded it might be impossible to detect. Epitope abundance can be enhanced under altered growth conditions, by selective tissue preparation, or fractionations of complex cellular extracts (i.e. organelle preparation).

Run sample buffer (+ loading buffer) in one lane of your gel to check for contribution of your sample extraction buffer to the background signal.

Alternative preparation methods should be considered if possible.

Include positive and negative controls from the beginning: comparison of samples prepared from material containing no target or higher amounts of the target as well as cellular fractionation (e.g. organelle preparation) will allow to control for specificity of the signals obtained.

Proteolytic breakdown of the antigen. This is not uncommon, particularly if samples are stored for prolonged time or if proteins or membranes are fractionated after homogenization of the starting tissue. All additional bands are of lower apparent molecular mass than the full-length protein. Addition of protease inhibitors such as PMSF, pepstatin or leupeptin should be considered.

Too much protein per lane or detection system too sensitive. Overloading of the gel is one of the most common reasons for "ghost bands". Immobilized proteins may provide a concentrated adsorptive surface to which certain IgG may bind non-specifically. Similarly, such non-specific binding may be uncovered when highly sensitive detection systems such as enhanced chemiluminescence are employed. A dilution series of the starting material usually clarifies which of the signals are artefactual.

Concentration of antigen too low. The resolution of SDS-PAGE is limited to 50-100 bands. If the relative concentration of the antigen of interest is too low (less than 0.2% of total protein), it may be difficult to detect. Signal enhancement may then lead to the appearance of artificial bands. Enrichment of the antigen by fractionation or by immunoprecipitation should be considered.

Concentration of proteins may be necessary for example: Mix equal volume of your media with 20% ice cold trichloroacetic acid. Let stand in ice for 15 minutes. Centrifuge at 10000g for 10 minutes. Dry pellet in air and resuspend in 1/100th the volume of 15 mM Tris.HCl pH 7.8 (1/100th of the original media volume).

Separation

The size-related identification of a protein-antibody interaction usually requires gel separation under fully denaturing conditions. Complete reduction of intra- and intermolecular S-S bridges ensures the accessibility of the epitope for interaction with antibodies detecting linear epitopes. In this case, reducing agents have to be added to sufficient final concentrations to both, sample and running buffer. If antibodies recognize non-linear epitopes they require conformational integrity of the target best provided in non-denaturing PAGE systems or Immunohistochemistry applications. Increasing protein loadings might elevate epitope abundance but most often also promotes non-specific cross-reactions, higher background, and impaired separation.

Avoid protein loadings higher than 5-20 µg/lane for standard mini-gel systems.

Check separation with stained marker pattern or by Coomassie/Silver-staining of the gel. Mixing a pre-stained marker with a marker reacting with the secondary antibody (e.g. MagicMark, Invitrogen) will be an advantage for size-determination and can serve as a control for the visualization assay.

Some highly hydrophobic membrane proteins might require 2-8 M urea in the gels and sample buffer to be kept unfold completely during separation.

If DTT is used as reducing agent it should be freshly prepared.

Transfer

The protein transfer on a suitable membrane (blotting) with protein-binding capacity (nitrocellulose, PVDF) is highly dependent on the biochemical properties of the target protein. Higher molecular targets (apparent molecular masses of >100 kDa) require longer blotting times than smaller proteins. At low field strengths (<15 V/cm) the mobility of proteins out of the gel will be decreased and proteins will not be completely transferred from the gel. If field strength is too high (>35 V/cm) proteins might pass through the membrane without binding. The transfer times should be as short as possible to avoid proteins (especially lower molecular weight) from passing through the membrane. This can be monitored by placing 2 membranes on top of each other and comparing signals obtained after incubation with the antibody. In gradient gels the porosity of the gel is matched with the size of the proteins which can be an advantage for efficient transfer.

Check efficiency of a transfer by post-transfer staining of the gel (e.g. Coomassie or Silver) or the filter (Ponceau, reversible).

Drying of a membrane (air dry between clean sheets of filter-paper) can improve immobilization of the protein.

Blotting equipment should be carefully rinsed with distilled water after each use and be kept away from contaminating detergents. Foam pads should be cleaned with (suitable) detergents followed by thoroughly washing. Replace foam pads if they start to acquire colours and/or loose tension.

Do not reuse transfer buffers.

The presence of SDS (0.01 to 0.02 %) in transfer buffer will increase the mobility of proteins (especially large proteins) out of the gel and due to giving a negative charge to the protein will help to maintain it in the soluble state. At the same time, SDS will reduce protein binding to the membrane (especially nitrocellulose) due to decreased hydrophobicity of the protein.

The presence of alcohol in the transfer buffer will decrease protein mobility out of the gel. It will also reduce pore size of the gel, while it will improve binding to nitrocellulose as it remove SDS from proteins

and increases their hydrophobicity. Higher molecular weight proteins might not be transferred completely if methanol is present in the transfer buffer. Try changing the membrane to nitrocellulose, omitting the methanol from transfer buffer, adding SDS and increasing field strength.

The thickness of the gel affects protein mobility out of the gel. Thicker gels allow higher loading but lower molecular weight proteins might transfer less efficient.

Many people reuse SDS PAGE running buffer. This may lead to higher background signal as some of the proteins from previous samples may be pulled into the current gel. These extra proteins may be deposited on the membrane by electrophoretic transfer.

Air pockets are not compatible with the passage of electric current. Pressing the gel and membrane 'sandwich' together by gently using a Pasteur pipette as a rolling pin can reduce the occurrence of air pockets between the layers.

Semi-dry transfer vs wet transfer - which transfer method is better and what are their limitations?

Each method has its own pros and cons. Semidry blotting is faster and easier, and requires a lot less buffer volumes than tank blotting. Small Proteins (<20kDa) are transferred with better reproducibility. Big proteins, however, do not transfer well in semidry blotting, and blotting of those is random at best, i.e. in some semidry blots you get big proteins on the membrane, sometimes you don't (same conditions!). Tank blots transfer high molecular weight proteins (>60kDa) better than semi-dry, but more often than not some small proteins are blotted through the membrane and are therefore lost for detection.

If you aren't getting a good wet transfer adjust your methanol from 200 ml/1000 ml to 150 ml/1000 ml without altering your glycine. If your protein is very large e.g. >300kDa then try adding 0.02% SDS.

One thing to be aware of is that proteins bind better to nitrocellulose at a low pH. You may need to go through some trial-and-error to find the optimal pH.

Blocking

To minimize background staining due to non-specific membrane-binding of the antibody tested the membrane has to be saturated ("blocked") with proteins non-reacting with the antibody. Commonly used are low-fat dry milk powder, casein, or BSA, at 2-5% w/v. The shortest possible blocking-time should be determined experimentally for your system. Increasing the blocking-times unnecessarily (e.g. overnight) or incubation at low temperatures might lead to aggregation of blocking protein on the membrane and by this compromising later antibody-epitope interactions. If low background is obtained after 30-60 min of blocking any longer blocking will not improve your results. It should be determined experimentally if adding or omitting the blocking reagent in the subsequent antibody incubation steps is improving the results. Usually the buffer and the detergent concentration used should not abolish interaction of the blocking-protein with the membrane. However, removing excess blocking-protein with a short washing (buffer without blocking-protein) prior to incubation with the primary antibody can increase accessibility of the target protein on the filter for the antibody.

BSA might give lower background than milk powder.

Do not use BSA as blocking agent if BSA-coupled peptides were used for immunization.

As milk contains biotin the use of milk-powder for blocking is incompatible with avidin/streptavidin systems.

If serum is used for blocking it should be considered if it can be excluded that the animal from which blocking serum has been obtained may have been exposed to and developed antibodies to the antigen in question. If this is the case, they may bind to the antigen and prevent the primary antibody from binding.

As a protein-free protein alternative you may block for 1 hr at RT with 0.2-0.5 % Tween-20 in PBS followed by incubation with the primary antibodies diluted in 0.25 % Tween-20 in PBS for 1 hr at RT.

Primary antibody

The primary antibody is the major determinant of the specificity of the target-recognition. The interaction with the primary epitope should be in favour over any cross-reactivity with other similar epitopes present. Common dilutions are between 1:500 to 1:20 000 depending on the reactivity of the antibody used. To check for specificity of the target recognition you can use (1) another antibody against your target which will bind to other epitopes on a target protein, (2) control samples free or depleted of target-proteins, or (3) perform a peptide competition assay (for anti-peptide antibody).

If available, a second antibody known to react with the sample can be used as a control of subsequent assay steps on a parallel filter in the same experiment.

Lyophilized primary antibodies can be incubated for 2-4 h at 4° C after reconstitution prior to use.

To diminish unspecific cross-reactions you may pre-adsorb the primary antibody (overnight, 4°C) with tissue extract which lacks your protein of interest (you can use a transferred membrane where the band/area containing your target protein has been cut out).

Antibodies against carrier protein (KLH, BSA or others) might recognize epitopes of other proteins present in the extract. Those anti-carrier antibodies can be removed by (1) incubating serum sample with a carrier protein in solution (0.1 % (w/v) or spotted on PVDF membrane or (2) passing serum sample through the column with bound carrier protein (use flow through fraction in further experiments).

Storing primary antibodies (at 4°C or -20°C) in solution with blocking reagent for further use might result in decreased primary antibody activity in some cases.

Secondary antibody

The secondary antibody has to be reactive against the primary antibody (e.g. use anti-rabbit to detect primary antibodies raised in rabbit) and usually is coupled to an enzyme or dye that allows for subsequent visualization. Thus, any non-target binding of the secondary antibody will result in background (if bound to the membrane due to insufficient blocking) or false-positive recognition of non-target proteins present on the filter ("cross-reactions"). Usually secondary antibodies are used at dilutions of 1:20 000-1:500 000 depending on the sensitivity of the visualization method (e.g. enhanced chemiluminescence, ECL or alkaline phosphatase, AP). The optimal dilution of the secondary antibody has to be determined experimentally for the detection system used. Different secondary antibodies may even result in different recognition patterns when applied to the same sample.

To check for contribution of the secondary antibody for the result you may cut a suitable area of your filter and run it as a parallel control where the primary antibody is omitted (see example for trouble shooting). If an Ig-reactive marker has been used the signals obtained from the marker can serve as a control of the function of the secondary antibody as well as the subsequent visualization.

Washes

After all steps excess of blocking-protein, primary or secondary antibody has to be diminished by washing. Usually the same buffers as used for the preceding steps are used. In some protocols washing steps include subsequent changes of the washing volumes with omitting the detergent in the last step. For reasons of reproducibility it is recommended to keep volumes and times constant. The intensity of washing steps can be elevated by (a) increased times and volumes, (b) additional changes of buffer, (c) higher detergent concentrations, (4) use of stronger detergents (e.g. SDS instead of Tween-20).

Stripping and re-probing blots

Add 280 µl of 2-beta-mercaptoethanol per 40 mL of stripping buffer (to a final concentration of 100 mM) Wash membrane in TBST (1 X 5 min). Incubate membrane in stripping buffer for 30 min at 50-60°C (use a heating oven for this - and keep the oven door closed due to smell). Wash in TBST (2 X 5 min). Block membrane for 1 h in 5% blocking milk. Follow normal protocol for developing western blots. Membrane Stripping Buffer Recipe: Stripping buffer: 62.5 mM Tris-HCl (pH 6.8), 2% SDS. (add 280 ul of 2-beta mercaptoethanol or a final concentration of 0.1 M 2-Mercaptoethanol).

Optimization

Optimization of western blots is important as this is done to increase the signal to noise ratio of your results, and also decrease non-specific bands. Optimization is carried out usually in a trial and error or a more organized approach by using different blocking, incubation and washing conditions.

Factors in Western Blot Optimization - Several factors are key in optimizing Western Blots:

SDS-PAGE Gel electrophoresis - without a good gel you will not get a great membrane. Selecting the Right Antibodies (both primary and secondary) and using them properly (dilution is important). Blocking conditions. Washing conditions. Development condition.

Tips and Tricks

Problem	Possible cause	Solutions
Streaking of Blots	Excess protein loaded onto gel.	Load less protein.
Smudgy or fuzzy blots	Gel improperly equilibrated and shrinking during blotting	Check gel equilibration times.
Poor staining of blot with Ponceau S	Ineffective transfer	Carefully remove air bubbles when making sandwich. Check that excess temperatures are not reached during electroblotting producing bubbles, or gel/membrane distortion. Check equipment (boxes, tops, power source)
No staining of blot with Ponceau S	No transfer of proteins during Western blotting	Check that the membrane is on the right side of the gel. Check that the gel and membrane make proper contact during blotting
No signal	Protein degradation on storage of	Use fresh blots.

	blots prior to detection	
	Wrong secondary	Check for appropriate secondary
	Exposure time too short	Increase length of exposure
	Insufficient antigen	Load more antigen on gel
	Antigen may have been destroyed	Check that antigenicity is not destroyed by treatment for electrophoresis.
	Detection system	Increase (and optimize) concentration, incubation times, and temperatures of primary antibody. Increase (and optimize) reagent concentration and incubation times, for your specific application. Check that the detection reagents are being stored correctly and used as recommended.
	Low affinity primary antibody	Use solutions without Tween.
	Chemiluminescent substrate has lost activity	Prepare a small amount of working solution (1ml), go into a dark room and add 1 m l of HRP conjugate. Visible blue light should be observed.
	Membrane has been stripped and reprobed	There may be antigen loss during reprobing.
	Insufficient antibody	Antibody may have low affinity to protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
	Antibody may have lost activity.	Perform a Dot Blot.
	Insufficient protein	Increase the amount of total protein loaded on gel. Confirm the presence of protein by another method. Use a positive control.
	Sodium Azide contamination	Make sure buffers do not contain Sodium Azide as this can quench HRP signal.
	Insufficient incubation time with primary antibody	Extend incubation time to overnight at 4°C
Weak signal	Insufficient protein on the gel	Load more protein on the gel. Expose film for an extended period (1-2 hours).
	Poor transfer	Wet PVDF/Immobilon-P membrane in methanol or nitrocellulose membrane in transfer buffer. Ensure that there is good contact between PVDF membrane and gel.
	Incomplete transfer	Optimize transfer time. High MW protein may require more time for transfer. To ensure transfer is complete, stain the membrane with Ponceau S, Amido Black or India Ink. Use prestained MW marker
	Low protein-antibody binding	Reduce the number of washes to minimum. Reduce NaCl concentration in

		Blotting Buffer used for wash steps (recommended range 0.15M - 0.5M).
	Insufficient antibody	Antibody may have low affinity to protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
	Inactive conjugate	Mix enzyme and substrate in a tube. If colour does not develop or, it is weak. Make fresh or purchase new reagents. Switch to ECL.
	Weak/Old ECL	Purchase new ECL reagents.
	Non-fat dry milk may mask some antigen	Decrease milk percentage in Block and Antibody Solutions or substitute with 3% BSA.
Non-specific bands	Antibody (1 ° Ab or 2 ° Ab) concentrations are too high	Reduce antibody concentrations
	SDS can cause non-specific binding to immobilized protein bands	Wash blots well after transfer Do not use SDS during procedure
		Reduce the amount of total protein loaded on gel.
		Use monospecific or antigen affinity purified antibodies (such as R&D Systems "MAB" or "AF" designated antibodies).
	Non-specific binding of secondary antibody	Run a control with the secondary antibody alone (omit primary antibody). If bands develop choose an alternative Secondary Antibody.
		Use monospecific or antigen affinity purified antibodies (such as R&D Systems "BAF" or "HAF" designated secondary antibodies).
	Non-specific binding of primary or secondary antibodies	Add 0.1 - 0.5% Tween® 20 to primary or secondary Antibody Solution.
		Use 2% non-fat dry milk in Blotting Buffer as a starting point to dilute primary and secondary antibodies. Adjust antibody concentration up or down as needed. Increase number of washes. Increase NaCl concentration in Blotting Buffer used for antibody dilution and wash steps (recommended range 0.15M - 0.5M). Increase Tween® 20 concentration in Blotting Buffer used for wash steps (0.1%-0.5%).
	Aggregation of analyte	Increase amount of DTT to ensure complete reducing of disulfide bonds (20 -100mM DTT). Heat in boiling water bath 5-10 minutes before loading onto

		gel. Perform a brief centrifugation.
	Degradation of Analyte	Minimize freeze/thaw cycles of sample. Add protease inhibitors to sample before storage. Make fresh samples.
	Contamination of reagents	Check buffers for particulate or bacterial contamination. Make fresh reagents.
Diffuse bands	Antibody (1 ° Ab or 2 ° Ab) concentrations are too high	Reduce antibody concentrations
	Too much protein is loaded on the gel	Reduce the amount of protein loaded
Black blots with white bands or signal that decreases quickly	Antibody (1 ° Ab or 2 ° Ab) concentrations are too high	Reduce antibody concentrations, especially the HRP conjugate. Signal that decreases quickly and white bands are an indication that the HRP is "burning out".
Partly developed area or blank areas	Incomplete transfer of proteins from the gel	Air bubbles between the gel and membrane during transfer? Incubate membranes separately to ensure that membrane strips are not covering one another during incubations.
High background uniformly distributed	Antibody (1 ° Ab or 2 ° Ab) concentrations are too high	Decrease antibody dilutions of either primary or secondary antibody or use shorter incubation times.
	Wrong blocking buffer was used	Compare with other blocking buffers
	Insufficient blocking of non-specific sites	Increase the concentration of protein in the blocking buffer. Optimize blocking time and/or temperature Add Tween®-20 to blocking buffer. A concentration of 0.05% Tween®-20 is recommended.
	Cross reactivity of antibody with other proteins in blocking buffer	Use a different blocking buffer. Do not use milk to block membranes when using an avidin-biotin system. Use a freshly prepared solution of blocking agent.
	Insufficient washing	Increase number of washes and the volume of buffer used. Add Tween®-20 to wash buffer if it's not already included. Increase concentration of Tween blocking solution to 0.1% Tween.
High background uniformly distributed	Exposure time is too long	Reduce the time the blot is exposed to film. Expose the film for a minimum period (an initial 15 seconds exposure may be all that is required). If exposure time is too short to be convenient, reduce antibody concentrations. Leave blots in the cassette for 5-10 minutes before re-exposing to film.

	Detection reagents	Rewash blots twice for 10 minutes in wash buffer and repeat detection steps. Excess detection reagents on blots. Drain well by absorbing the excess on blotting paper before placing blots in film cassettes.
	Membrane problems	Make sure membranes are wetted thoroughly and kept wet throughout the procedure. Use agitation during all incubations. Handle membranes carefully - damage to the membrane can cause non-specific binding. Do not handle membrane with bare hands. Always wear clean gloves or use forceps.
	Contamination or growth in buffers	Prepare fresh buffers.
	Contaminated blocking equipment	Clean or replace all equipment.
	Non-specific binding of primary antibody	Use monospecific or antigen affinity-purified antibodies (such as R&D Systems "MAB" or "AF" designated antibodies). Block in 5% milk. Adjust the milk (2-5%) or NaCl (0.15-0.5M) concentrations of primary Antibody Solution. Decrease antibody concentration.
	Non-specific binding of secondary antibody	Run a control with the secondary antibody alone (omit primary antibody). If bands develop choose an alternative Secondary Antibody.
	Insufficient blocking	Start with 5% dry milk with 0.1%-0.5% Tween 20, 0.15 -0.5M NaCl in 25mM Tris (pH 7.4). Incubation time may be extended. Adjust milk concentration up or down as needed. Overnight blocking at 4°C may decrease blocking efficiency since detergents might not be effective at lower temperatures.
	Non-fat dry milk may contain target antigen	Substitute with 3% BSA.
	Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin	Substitute with 3% BSA.
	Insufficient wash	Increase number of washes. Increase Tween 20 concentration in Wash Buffer (0.1%-0.5%). Use stronger detergents: if Tween-20 is used and background is high, a stronger detergent such as NP-40 or SDS may provide a more stringent

		wash.
	Non-Specific Binding of Primary Antibody	Increase NaCl concentration in primary Antibody Solution and Blotting Buffer used for dilution of primary antibody and wash steps (recommended range 0.15M - 0.5M).
	Film overexposed	Reduce exposure time. If target signal is too strong wait 5-10 minutes and re-expose to film.
Blotchy or speckled backgrounds	Antibody (1 ° Ab or 2 ° Ab) concentrations too high	The primary/secondary antibody can cause high background if the concentrations used are too high.
	Aggregate formation in the HRP conjugate can cause speckling	Filter the conjugate through a 0.2µl filter.
	Wrong blocking buffer was used	look for alternatives
	Insufficient blocking of non-specific sites	See under High Background
	Contamination of reagents	Check buffers for particulate or bacterial contaminate. Make fresh reagents.
	Not enough solution during incubation or washing	Make sure membrane is fully immersed during washes and antibody incubations.
		Gently remove any air bubbles. Especially during transfer.
	Uneven agitation during incubations. Air bubble trapped in membrane	Ensure uniform agitation by placing on a rocker/shaker.
	Contaminated equipment	Make sure that the electrophoresis unit is properly washed. Protein or pieces of gel remaining on the unit may stick to the membrane. Wash membrane thoroughly.
	HRP aggregation	Filter conjugate to remove HRP aggregates.
	Long exposure	Reduce exposure time.
Over transfer		Reduce voltage or time of transfer for low molecular weight proteins (< 10 kDa).
Isoelectric point is >9		Use alternative buffer system with higher pH such as CAPS (pH 10.5).
Insufficient transfer of large proteins	Insufficient transfer time Transfer current is too low Improper buffer components The conductivity of the transfer buffer is influenced by its components; Excess methanol in the transfer buffer Air bubbles between the gel and the membrane PVDF membranes that have not been properly hydrated	Too much methanol in the transfer buffer decreases the transfer efficiency of proteins from the gel to the membrane; however methanol aids in protein binding to PVDF or nitrocellulose membranes – a balance is needed
Spots on Film (missing bands)	- Poor Transfer due to air bubbles during transfer to membrane	- use a pasteur pipette as a roller. roll the membrane and gel before transfer to remove

	<ul style="list-style-type: none"> - dirty film when adding to the developer - developer rolls are dirty 	<ul style="list-style-type: none"> bubbles. keep membrane and materials wet. - keep film clean before adding to the developer
Too Many Bands in Western Blot	<ul style="list-style-type: none"> - usually due to many non-specific bands - poor primary antibody quality or old antibody - not enough blocking - proteolytic cleavage of antigen - all additional bands are lower MW than your protein, (ie your protein is being detected but was degraded) 	<ul style="list-style-type: none"> - purchase another antibody or replace with a fresh antibody stock - block with 5% dry non-fat milk or BSA prior to adding primary. If you are already blocking, consider blocking during primary and secondary antibody incubations and also increasing blocking milk or BSA concentration. - keep everything on ice, use fresh samples, store in - 80C, and use protease inhibitors such as PMSF
High background; low signal to noise ratio on Western Blot	<ul style="list-style-type: none"> - black or every dark film; film was exposed too long - blocking was insufficient ; non-specific binding of primary antibody and secondary antibodies were not washed completely - washing was insufficient - film glows in dark! - too high secondary or primary dilutions 	<ul style="list-style-type: none"> - expose for less time, decrease exposure time - increase blocking duration of non-fat dry milk 5% incubation or increase the concentration. - try blocking with whole serum of the host animal with (or before) the secondary antibody - increase washing times and volumes to help remove any non-specific signal due to weak antibody binding. - using a stronger detergent to wash - Instead of TBST (Tween-20), use stronger detergents such as NP-40 or SDS which may provide a more stringent wash and reduce background (do this only if you band is strong! - washing also removes some your band of interest!) - very high secondary antibody (or even primary) concentration - perform optimization experiments to determine proper antibody dilutions. dilute the antibodies further.
Low Signal or Weak Signal	<ul style="list-style-type: none"> - due to not enough protein loaded on the gel - primary antibody is old or not good - phospho-proteins usually need overnight primary antibody incubation - not enough secondary antibody - over-blocking - developing reagents are bad / you made a mistake when preparing them 	<ul style="list-style-type: none"> - load more protein sample onto gel - try fresh primary antibody - incubate primary antibody overnight for phosphoproteins at 4 degrees C (cold room shaker) - increase concentration of protein (lyse samples in less lysis buffer) - decrease concentration of blocking agent - check the developing reagents; prepare fresh ECL and re-ECL the membrane - increase the concentration or the length of primary antibody incubation period

		- increase concentration or the length of secondary antibody incubation period
Fuzzy Bands, Bands Smeared, Bands not Sharp	- bands smeared due to hot gel - bands fuzzy due to high voltage	- decrease voltage or current, run in cold room, and prepare new running buffer. (heat causes the gel to lose its rigidity and its resolving power) - pre-soak transfer membrane in the appropriate transfer solution (determined by the membrane manufacturer) for the required length of time. Membrane Stripping and Reprobing Protocol for Western Blotting

This guide is largely taken from information freely available on the world-wide web. As usual though if you have any queries, or indeed comments, please get in touch with the renal scientists through the website.

Dr Julie Williams

Selected Research Articles:

1. The first paper describing the use of electrophoresis to transfer proteins from a gel to a membrane. Towbin H., Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979 Sep;76(9):4350-4
2. The classic paper by UK Laemmli - the most cited article in the journal's history. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.
3. The first paper to use the term western blot. Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. 1981 Apr;112(2):195-203