WESTERN BLOTTING

Technical Tips and Troubleshooting

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PRINCIPLES OF WESTERN BLOTTING

- Western Blotting is an **Immunoblotting Technique.**
- Western Blotting is an analytical technique to detect **specific proteins** in a cell extract or tissue homogenate.
- Western Blotting relies on the specific binding between the protein-of-interest and **an antibody raised against this particular protein.**
- Main steps are:
  Sample preparation
  Gel electrophoresis
  Transfer
  Blocking
  Detection
  Analysis

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ANTIGEN OPTIMIZATION

- **Cell lysis is the breaking down of the cell membrane and the separation of proteins from the non-soluble parts of the cell.**
- **Dependent on the location of the protein of interest, a different lysis buffer is needed.**

Location	Lysis Buffer
Nuclear, Mitochondria	RIPA, Fraction protocol
Membrane bound, Whole cell lysate	RIPA or NP-40
Cytoplasma	RIPA, Tris, HCL/Triton

RIPA: 25mM Tris, HCl (pH 7.6) 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS.

NP-40: 50 mM Tris, HCl (pH 8.5) 150 mM NaCl, 1% detergent.

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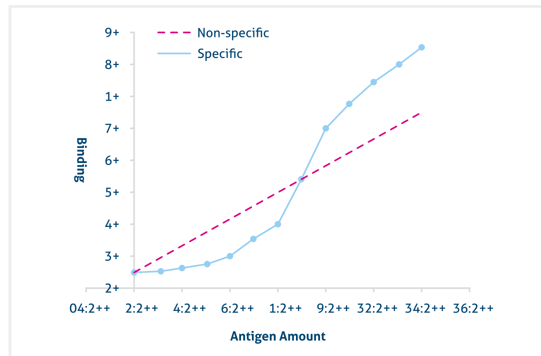
ANTIGEN OPTIMIZATION

- **The non-specific binding is favoured when a low amount of antigen is present**
- **For low abundance proteins the following applies:**

  Insufficient amount of the antigen might lead to non-specific binding.

  Load more protein.



Protein loading amount can help to optimize signal issues.

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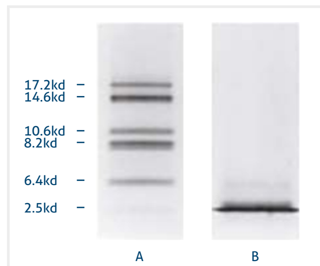
LOW MOLECULAR WEIGHT PROTEINS

Standard Gel:

- Glycin-Tris buffer system
- Detection of 30-250 kDa proteins

Gel for low molecular weight proteins:

- Tris-Tricine buffer
- Detection of <30 kDA proteins



H Schägger and G von Jagow. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem. 1987;166(2):368–79.

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LOW MOLECULAR WEIGHT PROTEINS

Tricine Gel Recipe For Low MW Proteins

Items	Separation	Intermediate	Stacking
Gel Percentage	15%	10%	4%
Gel Volume	6 ml	3 ml	2 ml
38% Glycerol	1.6	—	—
ddH_2O	—	1.2	1.4
30% Acrylamide	2.7	0.8	0.3
3.0 M Tris HCl (pH 8.5)	2.14	1	—
1.0 M Tris HCl (pH 6.8)	—	—	0.3
10% SDS	0.06	0.03	0.02
10% APS	0.06	0.03	0.02
TEMED	0.003	0.003	0.002

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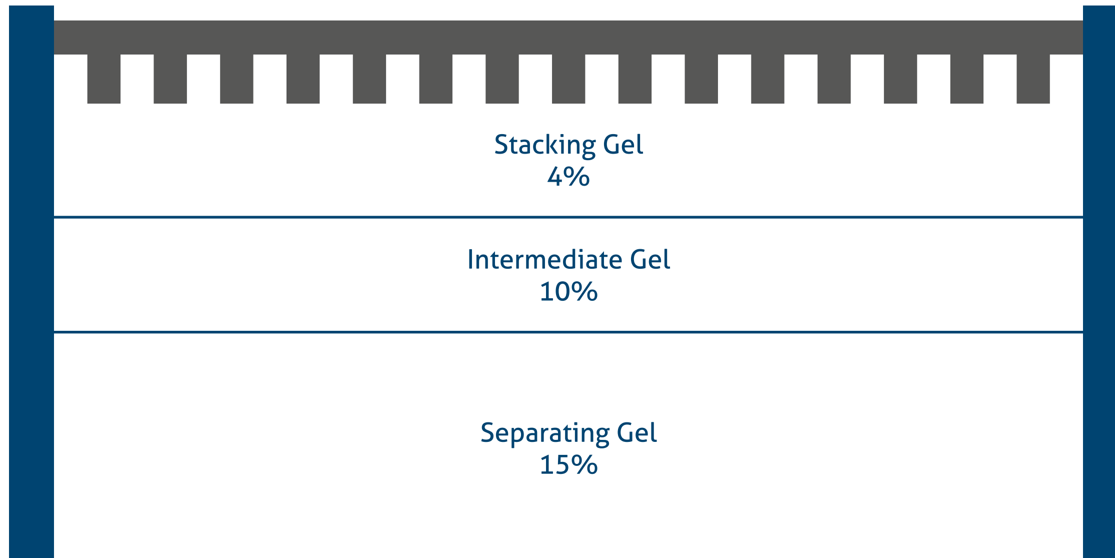
LOW MOLECULAR WEIGHT PROTEINS

Tricine Gel Recipe For Low MW Proteins



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BLOCKING

- As the membrane has the ability to bind proteins, there might be some non-specific bindings.
- Blocking is necessary to optimize the signal-to nose ratio.
- **Bovine Serum Albumin (BSA)** or **non fat milk** with a low percentage of detergent are used for blocking.
- For phospho-proteins BSA is suggested
- Controls to optimize the best blocking reagent:
  blocking buffer + substrate
  blocking buffer + 2.AB + substrate
  blocking buffer + 1.AB + 2.AB + substrate

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TRANSFER

- **Proteins are transferred from the gel to a membrane to make them accessible for antibody detection.**

- **Factors that influence the quality of the protein transfer:**

  Transfer length

  Temperature

  Membrane

  Buffer composition

- **Membrane choice and pore size:**

  Pore size:
  Dependent on MW of target protein.

  Membrane type:
  Dependent on the hydrophilic/ hydrophobic character of the target protein *(see membrane choice)*.

- **Low molecular weight proteins tend to "over transfer":**

  Transfer slowly and reduce voltage and time.

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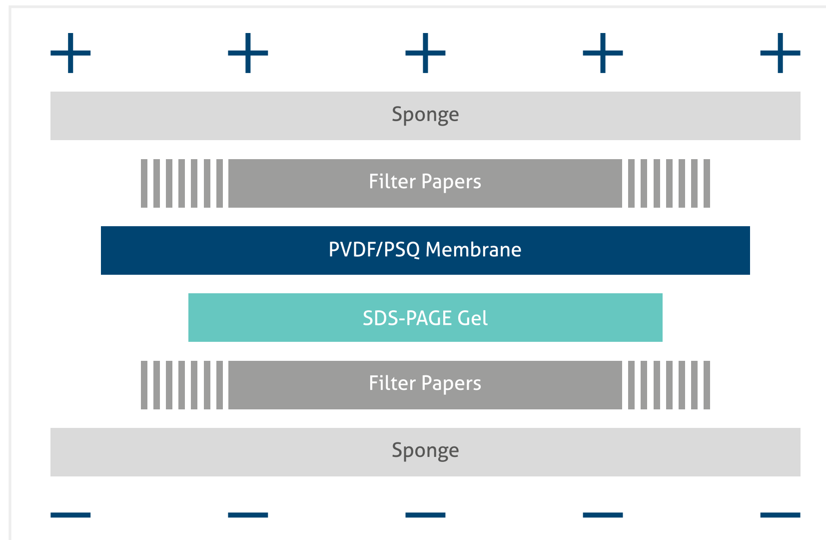
TRANSFER



A representation of the components of a transfer "sandwich." Note the orientation of the gel and membrane: the PVDF membrane is situated nearest to the positive electrode. The binding of SDS to proteins results in the complex having an overall negative charge. Therefore, the SDS-bound proteins travel towards the positive electrode.

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MEMBRANE CHOICE

- Protein binding is based on **hydrophobic interactions and charged interactions.**
- The **PVDF** and the **Nitrocellulose** membrane are the most common used membranes.

PVDF (Polyvinileden Difluoride)	Nitrocellulose
Less fragile.	Fragile.
Soak in ethanol, isopropanol or methanol before use.	Soak in transfer buffer before use.
More expensive.	Less expensive.
Can be stripped and reused a lot of times.	Can't be reused as much.
Preferable for proteins with a hydrophilic character.	Preferable for proteins with a hydrophobic character.

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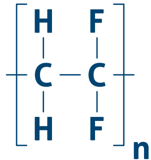
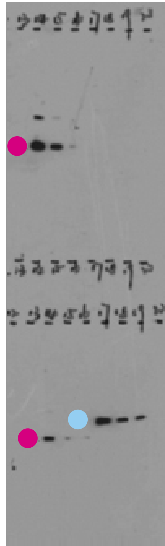
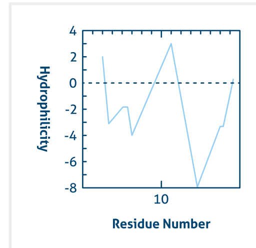
MEMBRANE CHOICE

PVDF







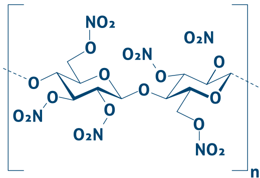
Beta-Catenin

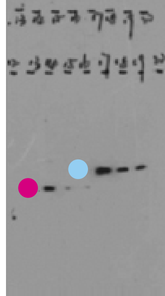
DLMDGLPPGDSNQLAWFDTDL

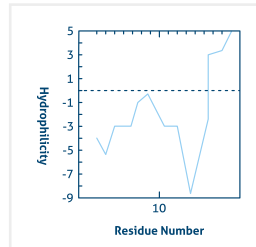
Works on both PVDF & NC

Proteins with a hydrophilic character work better on PVDF.

Nitrocellulose







NALP-3

CLLQNLGLSEMYFNYETKSA

Works better on NC

Proteins with a hydrophobic character work better on PVDF.

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MONOCLONAL OR POLYCLONAL ANTIBODY

Monoclonal

- High specificity.
- Less background.
- Better reproducibility.
- Sensitive to epitope loss/changes.
- Batch consistency.

Polyclonal

- Higher affinity and comparable specificity.
- Better results with non-denatured samples.
- More tolerant of minor changes.
- Species transferable.
- Lot to lot variation.

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CONTROLS

- Control samples on a Western Blot might help to understand the cause of non-specific bands.

Positive Control

- Cells overexpressing the target protein.
- Purified recombinant protein.
- Samples with proven positive signal.

Negative Control

- Samples targeted with RNA interference.
- Knockout samples.
- Samples with proven negative signal.

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LOADING CONTROL ANTIBODIES

- Loading controls are used for semi-quantification of protein levels.
- They ensure that the observed alteration in target protein is due to experimental manipulations.
- Characteristics of a loading control: high expression, constitutive, unchanged expression during experiment.

Selected loading controls for different cell location		
Whole cell/ cytoplasmic	**Nuclear**	**Mitochondrial**
Actin, 42 kDa	Lamin B1,66 kDa	COX-4, 17 kDA
GAPDH, 36 kDa	PCNA,36 kDA	VDCA1/Porin, 31 kDA
Tubulin, 50-55 kDa	TBP, 38 kDA	–

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TROUBLESHOOTING

General Difficulties

Potential cause	Suggested solution
Dark spots.	Antibodies binding to the blocking reagent/filter the blocking buffer.
Sample overloading.	Decrease the total protein loading.
Too much antibody.	Titrate the antibody concentration.
Inverse staining (i.e., white bands on a dark blot).	Too much primary and/or too much secondary antibody.
Uneven bands.	Uneven gel composition/insufficient buffer being added to the tank.
Blank areas/white spots.	Improper/uneven transfer.
“Smiling” bands.	Migration through the gel was too hot or too fast.

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TROUBLESHOOTING

Non-specific Binding

Potential cause	Suggested solution
Sample degradation.	Prepare fresh lysates. Include protease inhibitors.
Interference from other isoforms.	Check literature for known isoforms. Use isoform-specific antibody.
Target protein abundance is lower than threshold of non-specific binding.	Load more protein. Enrich low abundance proteins by IP.
Inefficient SDS-PAGE separation.	Change the gel percentage to suit the target protein's MW.

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TROUBLESHOOTING

Weal/No Signal

Potential cause	Suggested solution
Issues with the primary and/or secondary antibody.	Titrate the antibody. Change incubation time and temperature. Antibody may have lost activity.
Membrane choice.	Select PVDF or NC membranes based on hydrophobicity. Hydrophilicity of the target antigen.
Sodium azide contamination.	The presence of sodium azide inhibits the activity of HRP. Ensure sufficient washing.
Detection reagent not sensitive enough.	Dilute chemiluminescent reagents in high-purity water. Check several exposure times to achieve optimum detection.

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TROUBLESHOOTING

High Background

Potential cause	Suggested solution
Inadequate washing.	Increase washing time and volume.
Dry membrane.	Ensure membrane does not dry out.
Film exposure too long/ detection reagent too sensitive.	Check different types and dilution of the detection reagent.
Insufficient blocking.	Increase the concentration of blocking reagent. Increase blocking time. Add Tween 20 to the blocking buffer.
Antibody concentration too high.	Use higher antibody dilution.

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CONTACT US

Proteintech Group
US Head Office

proteintech@ptglab.com

Proteintech Europe
United Kingdom

europe@ptglab.com

Proteintech
China Office

service@ptglab.com

Support

Available 24 hours via Live Chat and 9–5 (CDT) via phone.

Please visit us at www.ptglab.com for more information about our antibodies and technical tips.