Buffer Preparation for Western Blot

1.5 M Tris, pH 8.8 (stock buffer for separating gels)
Tris base: 181.65g
ddH2O: 800ml
Dissolve well.
Adjust pH to 8.8 with concentrated HCl.
Bring up the volume to 1 L with ddH2O
(Make sure to let the solution cool down to room temperature before making the final pH adjustment)
Sterilize by autoclaving.

1.5 M Tris, pH 6.8 (stock buffer for stacking gels)
Tris base: 181.65g
ddH2O: 700ml
Adjust pH to 6.8 with concentrated HCl.
Bring up the volume to 1 L with ddH2O
(Make sure to let the solution cool down to room temperature before making the final pH adjustment)
Sterilize by autoclaving.

10X PBS
NaCl: 80g
KCl: 2g
Na2HPO4: 14.4g
KH2PO4: 2.4g
ddH2O: 800ml
Dissolve well.
Adjust pH to 7.4.
Bring up the volume to 1 L with ddH2O
Sterilize by autoclaving.

20X TBS
Tris-base: 48.4g
NaCl: 160g
ddH2O: 800ml
Dissolve well.
Adjust pH to 7.6 with HCl.
Bring up the volume to 1 L with ddH2O
Sterilize by autoclaving.

2X SDS Loading Buffer
1M Tris pH6.8: 2ml
50% Glycerol: 4.6ml
10% SDS: 1.6ml
0.5% Bromophenol Blue: 0.4ml
β-mercaptoethanol: 0.4ml
ddH2O: 1ml

10X SDS Running Buffer
Tris-base: 30g
Glycine: 144g
SDS: 10g
ddH2O: 1 L

10X Transfer Buffer
Tris-base: 30g
Glycine: 144g
ddH2O: 1L

1X Transfer Buffer
10X Transfer Buffer: 100ml
Cold ddH2O: 800ml
Methanol: 100ml

Western Blot General Protocols

Sample Preparation, SDS-PAGE and Transfer

1. Wash cells twice with ice-cold 1X PBS gently. Harvest cells by centrifugation.
2. Resuspend and incubate cell pellets in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Keep on ice for 20 mins. Sonicate for 10-15s to complete cell lysis if necessary.
3. Centrifuge and harvest the supernatant. Perform protein quantification with Bradford assay.
4. Add SDS loading buffer, boil the samples for 5 mins and load 30-50 μg protein into each well of Polyacrylamide gel. Include a prestained protein marker.
5. Run gel at 25mA (2 gels run at 50mA) in 1X SDS-PAGE running buffer until dye front runs towards the end of glass plates.
6. Prepare 1X Transfer buffer. Wet PVDF membrane in methanol, or NC membrane in water. Soak membrane in transfer buffer for 10 mins.
7. Assemble the transfer sandwich and allow protein to be transferred from SDS-PAGE gel to NC or PVDF membrane for 1-2 hours in cold transfer buffer.

Blocking, Blotting and detection of proteins

1. Block blot by soaking in Blocking buffer (5% milk, 1X PBS/0.1% Tween20) for 1 hour in shaking.
2. Incubate blot in primary antibody for 1 hour at RT or 4 ° C overnight with shaking.
3. Wash blot 3-5 times, 5-10 minutes each in Wash buffer (PBST).
4. Incubate blot in secondary antibody for 1 hour at RT with shaking.
5. Wash blot 3-5 times, 5-10 minutes each in Wash buffer (PBST).
6. Detection by freshly prepared ECL.

How Not to Fail a Western Blot Experiment?

1. Include appropriate positive and negative controls.
Always include appropriate positive and negative controls in your western blot experiment. These will help narrow down possible causes just in case the experiment didn' t work out well. Internal controls such as GAPDH, Actin or Tubulin are good candidates for positive controls. Untreated or knocked down cell lysates serve as good negative controls. Refer to our internal control guide at page 4 for more tips and advice!

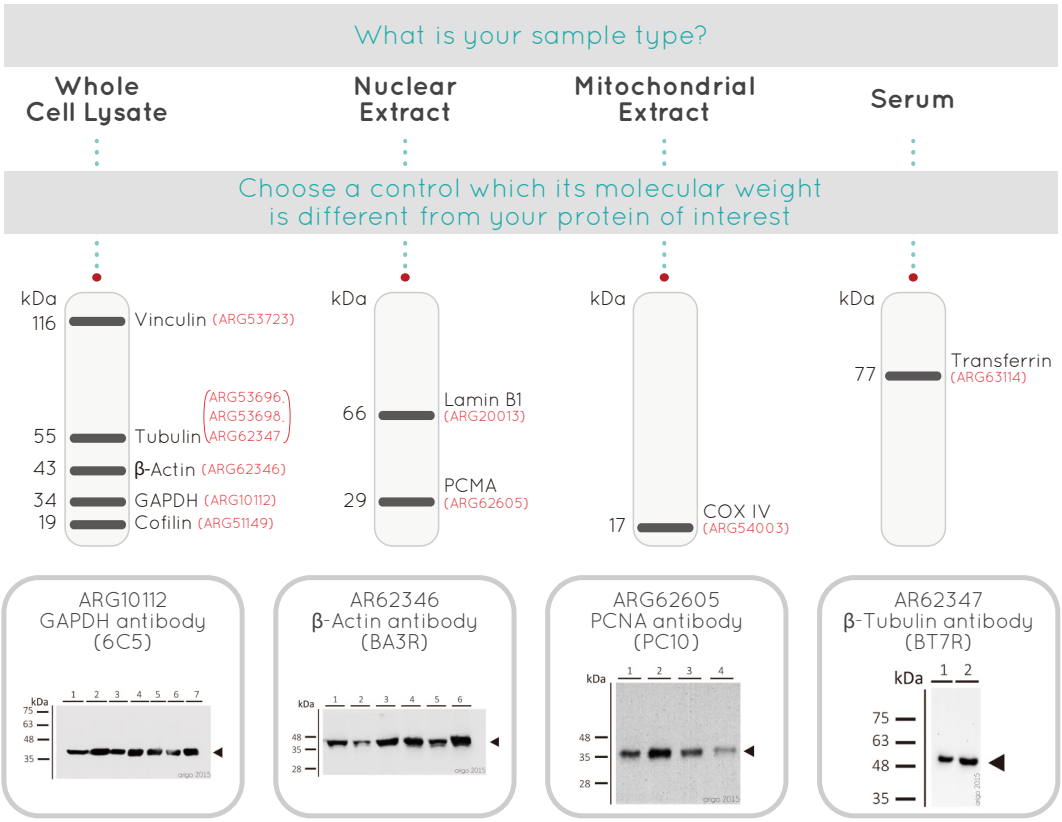
2. Get a reliable antibody source and optimize it!
Make sure that your antibody is WB-validated. Some antibodies do not recognized denatured epitopes, hence not suitable for use in WB experiments. Always optimize the antibody dilution factor as inappropriate dilutions will give you high background or low/weak signals. Refer to the recommended dilution factor indicated in the datasheet and try a few more dilutions to find the optimal condition.

3. Add suitable protease/phosphatase inhibitors for each sample types
Protease inhibitors or phosphatase inhibitors can be added into the freshly prepared cell lysis buffer to prevent proteolytic degradation of target proteins. Each sample type requires different combination of protease inhibitors. Understand what your sample needs and choose the right protease inhibitors to protect them. Need help? Refer to our protease inhibitor guide at page 5 for more details!

4. Keep things "cool" ~
Proteins are sensitive to heat and the whole western blot experiment can be destroyed if high heat is applied during SDS-PAGE, transfer, blocking or antibody incubation steps. If possible, run your SDS-PAGE gel or transfer your blot at 4 ° C. Compared to RT, incubating blot with primary antibodies overnight at 4°C gives better antibody-target binding and a cleaner background.

Internal Control Information

When Western Blot or other experiments are performed, loading controls are required to ensure that (1) the same amount of protein sample is loaded into each lane; (2) protein is transferred from gel to membrane with equal efficiency and (3) antibody incubation and detection is uniform. Loading control must fulfill certain criteria before they are chosen as normalization factor in various samples. Follow our guide below to choose the best internal controls for your experiments!



Protease Inhibitor Information

Within a few minutes, proteases can destroy the proteins you have spent days isolating. Inhibition of proteolytic activity is therefore becoming very important to prevent unwanted degradation of proteins during their isolation and characterization.
See the list below for commonly used inhibitors grouped into different classes of proteolytic enzymes.

Serine Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
Leupeptin	Trypsin, chymotrypsin, pepsin, thrombin, calpain, cathepsinB,H, Papain etc	10-100 μM	Water
PMSF	Broad spectrum serine protease	0.1-1.0 mM	Anhydrous ethanol or isopropanol
Aprotinin	Broad spectrum serine protease but not thrombin or factor Xa	0.3 μM	Water
AEBSF	Broad spectrum serine protease	0.1-1.0 mM	Water

Aspartic Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
Pepstatin A	Renin, Chymosin, Pepsin andother aspartic proteases	1 μM	Methanol or DMSO

Cysteine Protease inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
E-64	Broad spectrum cysteine protease and trypsin	1-10 μM	Water
Chymostatin	Chymase cathepsins A,B,D,G, Papain	10-100 μM	DMSO

Metalloproteinase inhibitors

Inhibitor	Target	Typical working concentrations	Solvent
EDTA	Broad spectrunmetalloproteinase	1-10 mM	Water
Bestatin	Aminopeptidase	1-10 μM	Methanol