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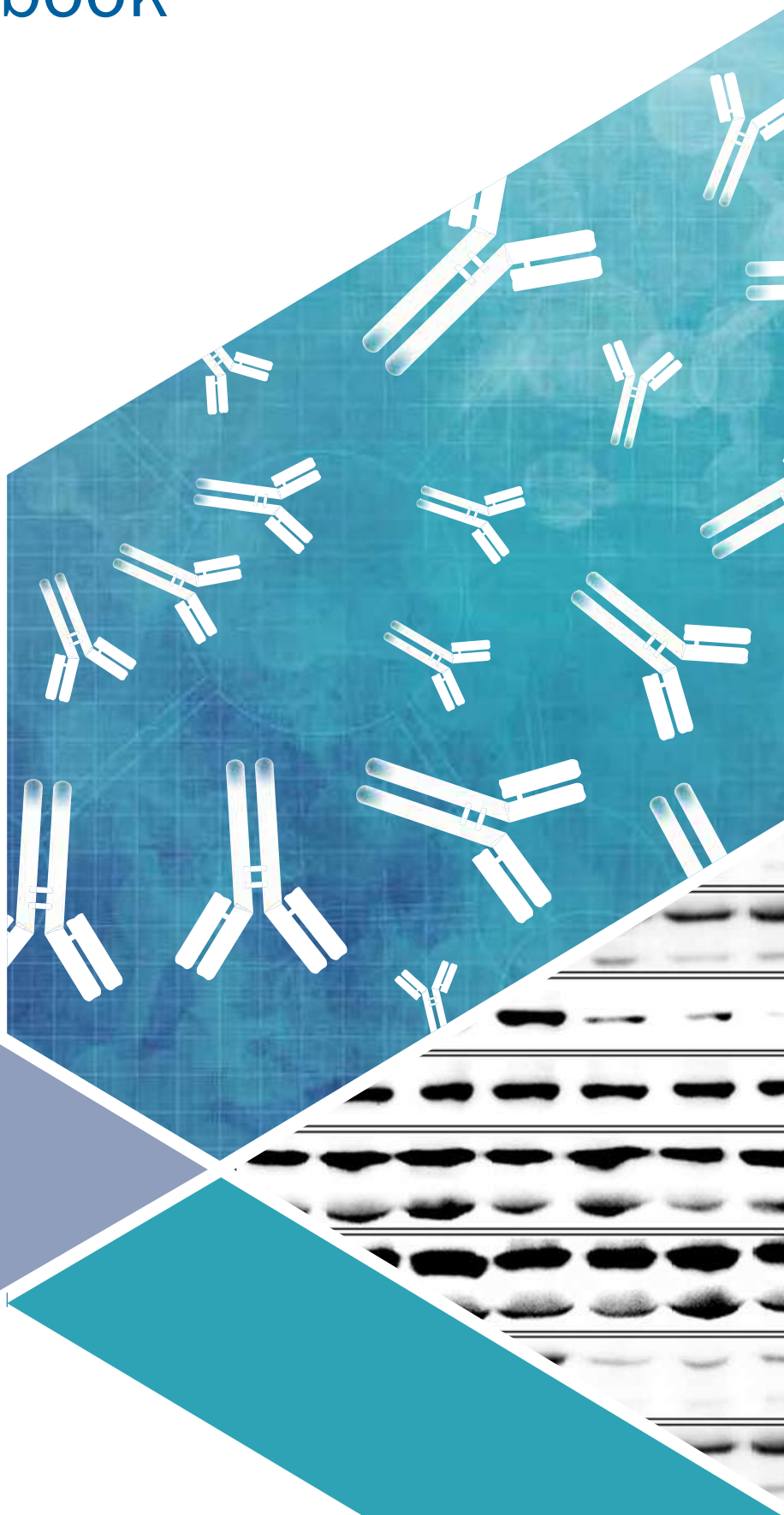
Western Blot Handbook



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INTRODUCTION TO WESTERN BLOTTING

Western blotting uses antibodies to identify individual proteins within a cell or tissue lysate. Antibodies bind to highly specific sequences of amino acids, known as epitopes. Because amino acid sequences vary from protein to protein, western blotting analysis can be used to identify and quantify a single protein in a lysate that contains thousands of different proteins First, proteins are separated from each other based on their size by SDS-PAGE gel electrophoresis. Next, the proteins are transferred from the gel to a membrane by application of an electrical current. The membrane can then be processed with primary antibodies specific for target proteins of interest. Next, secondary antibodies bound to enzymes are applied and finally a substrate that reacts with the secondary antibody-bound enzyme is added for detection of the antibody/protein complex. This step-by-step guide is intended to serve as a starting point for understanding, performing, and troubleshooting a standard western blotting protocol.

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CONTROLS

Western blotting is considered the gold standard for protein detection and quantification in molecular biology research. Proper controls for western blotting are important for determining the source of problems and for validating results. Including appropriate controls from the start can save you time and frustration down the road.

Positive Control Lysate

A positive control lysate is from a cell line or tissue sample that is known to express the protein of interest. This control will yield a positive band on the western blot, even if the test samples are negative for the protein of interest. This control is important to ensure that there were no issues in the western blotting protocol. It will also verify that any negative results are indeed negative. If the positive control lysate does not result in a positive signal, the western blotting protocol requires optimization. See the troubleshooting section for guidance.

Commonly used positive controls:

- Samples from cells exhibiting overexpression of target protein
- Cell line/tissue/experimental condition with proven positive signal
- Purified recombinant protein

Negative Control lysate

A negative control lysate is a lysate from a sample known to not express the target protein. This control is important for determining whether non-specific binding (false positive result) has occurred in the western blotting procedure.

Commonly used negative controls:

- Samples from knockdown or knockout tissue/cell lines
- Samples from RNA interference targeted lines
- Cell line/tissue/experimental condition with proven negative signal

Endogenous control lysate

If testing a sample of recombinant protein, a positive endogenous control lysate known to express the target of interest is recommended. This is suggested because there are several possible difficulties with expression of recombinant proteins that may occur. For example, folding of the recombinant protein might not match that of the endogenous native form. Misfolding may prevent antibody access to the epitope and this is especially common with tagged proteins. Like the positive control lysate, this control will help ensure that the western blotting protocol is working and indicate whether there might be an issue with the recombinant protein.

Loading control

When performing western blotting, loading controls are required for the semi-quantification of protein levels between wells. Without loading controls, it is impossible to determine that observed alterations in target protein levels are due to experimental manipulations.

What to look for

Expression of loading control protein must be equal across all wells to confirm that observed changes in target protein expression are true. Equal expression of loading controls confirms the samples have been equally loaded and protein has been evenly transferred from gel to membrane.

It is important to consider the following when choosing a loading control:

- **Detection size:** Choose a loading control that can be distinguished in MW from the target protein of interest.
- **Expression level:** Choose a loading control that is highly expressed in your sample. Common loading controls are highly expressed genes required for basic cellular processes and vitality, also known as housekeeping genes.
- **Expression consistency:** Choose a loading control that is ubiquitously and constitutively expressed. The expression should be unchanged throughout an experiment, regardless of experimental treatment, cell type, tissue type, etc.

Common Loading Controls	Target	MW (kDa)
Cytoplasm/Whole Cell	Alpha-tubulin	55
	Beta-actin	43
	GAPDH	37
	Cyclophilin B	21
Mitochondria	HSP60	60
	COX IV	17
Nucleus	Lamin B1	66
	HDAC	60
	PCNA	29
	Histone H3	17
Serum	Transferrin	77

Target Protein	Notes
Beta-actin	Not suitable for nuclear extract as beta-actin is a component of chromatin remodeling complexes. May not be suitable for studies involving subjects with a large age difference.
GAPDH	Not suitable for oxygen-related studies as hypoxia can upregulate GAPDH expression. May not be suitable for studies involving subjects with a large age difference.
Alpha-tubulin	May not be suitable for studies involving subjects with a large age difference. Tubulin expression can be affected by anti-cancer and anti-fungal drugs.
Lamin B1	Not suitable for embryonic stem cells.
PCNA	Not suitable for serving as a loading control for non-proliferating cells.
COX IV	Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if your protein of interest is similar in size to COX IV.
Transferrin	Transferrin levels can be influenced by some disease states and treatments such as retinoic acid.
Histone H3	Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if your protein of interest is similar in size to Histone H3.

SAMPLE PREPARATION

Lysis

The first step in sample preparation is isolating proteins from their source. Usually, protein is isolated from cells or tissues via lysis. Lysis breaks down the cell membrane to separate proteins from the non-soluble parts of the cell. There are various lysis buffers that can be used for sample preparation in western blotting and in general, they vary in the strength of their detergents to release soluble proteins (e.g. SDS, Triton X-100).

Important note: While most antibodies recognize reduced and denatured proteins, some antibodies only recognize proteins in their native, non-denatured form. Usually this is noted on the antibody datasheet. In this case, proteins should not be extracted with denaturing ionic detergents, such as SDS; more mild non-ionic detergents such as Triton X-100 and NP-40 should be chosen.

The location of the protein of interest can generally be used as a starting point to determine the most optimal lysis buffer for obtaining high protein purity and yield. Proteins that are found predominantly or exclusively in a sub-cellular location, such as nucleus or mitochondria, can be enriched by fractionating these specific compartments (see page 20 for available fractionation kits). This is particularly useful for probing weakly expressed proteins. Since every protein is different, lysis buffer and detergent conditions may require optimization for individual western blotting experiments. Refer to the tables below for choosing a lysis buffer and for standard lysis buffer recipes.

Recommended lysis buffers based on cellular location of protein of interest

Subcellular Location	Recommended Buffer
Whole Cell Lysate	NP-40
Nucleus	RIPA or nuclear fractionation for increased protein of interest concentration
Mitochondria	RIPA or mitochondrial fractionation for increased protein of interest concentration
Cytoplasm	Tris-HCl
Membrane-Bound Proteins	RIPA (SDS is generally considered harsh and thus is often well-suited for difficult to solubilize proteins)

Lysis buffer recipes

NP-40	RIPA	Tris-HCl
150 mM NaCl 1% NP-40 or Triton X-100 50 mM Tris pH 8.0	150 mM NaCl 1% NP-40 or Triton X-100 0.5% sodium deoxycholate 0.1% SDS 50 mM Tris, pH 8.0	20 mM Tris-Hcl, pH 7.5

Protease and Phosphatase Inhibitors

Immediately following cell lysis, proteolysis, dephosphorylation, and denaturation begin to occur. This activity should be kept to a minimum by preparing samples on ice or at 4˚C and by adding appropriate protease and phosphatase inhibitors fresh to the lysis buffer.

While there are many commercially available ready-to-use inhibitor cocktails (often proprietary), a homemade mix can be made based on individual needs. The table below lists common protease and phosphatase inhibitors, their targets, and the recommended final concentration in the lysis buffer.

Common protease and phosphatase inhibitors

Inhibitor	Target	Final Concentration
Aprotinin	Trypsin, chymotrypsin, plasmin	2 µg/mL
Leupeptin	Lysosomal, tripsan, papain	1-10 µg/mL
Pepstatin A	Aspartic proteases	1 µg/mL
PMSF	Serine and cysteine proteases	1 mM
EDTA	Mg^{2+} and Mn^{2+} metalloproteases	1-5 mM
EGTA	Ca^{2+} metalloproteases	1 mM
Sodium fluoride	Serine & threonine phosphatases	5-10 mM
Orthovanadate	Tyrosine phosphatases	1 mM
Pyrophosphate	Serine & threonine phosphatases	1-2 mM
B-glycerophosphate	Serine & threonine phosphatases	1-2 mM

Carrying Out Lysis

The cell lysis protocol can vary widely depending on the sample type. For example, lysis of heart or brain tissue from a mouse may require homogenization, which typically involves flash freezing of the sample in liquid nitrogen prior to grinding with a mortar and pestle or an electric homogenizer. Researchers should choose their lysis protocol based on standard protocols in the field for the given tissue. The following is a general example of a protocol for lysing cells grown in culture.

PROTOCOL: Example of Lysate Preparation From Cell Culture

01 Wash cell culture dish on ice with ice-cold PBS.

02 Aspirate PBS and add ice-cold lysis buffer (1mL per confluent 10^7 cells/100mm dish/150 cm^2 flask).

03 Using a cell scraper, scrape adherent cells off the dish and transfer the cell suspension into a microcentrifuge tube.

04 Agitate cells for 30 minutes at 4˚C.

05 Centrifuge cell lysate mixture at 4˚C. The time and centrifugation force vary for each cell type.

06 Transfer the supernatant (lysate) to a fresh tube on ice.

Determining the Protein Concentration

It is important to determine the protein concentration of each lysate to ensure equal loading of the SDS-PAGE gel. This allows protein levels to be properly quantified in western blotting. Protein concentration can be determined by performing a standard Bradford, Lowry, or BCA assay. Protein samples can be frozen at -20˚C or -80˚C for later use or prepared for gel loading for immediate use.

Preparation of Samples for Gel Loading

The epitope usually resides within the 3D conformation of the protein. Thus, it is necessary to unfold or denature the protein to enable access to the antibody. Denaturing is performed by briefly boiling the sample in a loading buffer containing SDS.

The most common loading buffer is **2X Laemmli buffer**. It can also be made at other concentrations such as 4X or 6X, which may be helpful if loading larger volumes of lysates with low protein concentration.

2X Laemmli buffer
4% SDS
10% β-mercaptoethanol or 100 mM DTT
20% glycerol
0.01% bromophenol blue
0.125 M Tris HCl
pH 6.8

Understanding the Components of the Loading Buffer

When **SDS** is added to proteins, they become negatively charged by their attachment to SDS anions. The SDS wrapping around the polypeptide backbone causes protein denaturation. The negative charged conferred by SDS to polypeptide chains is proportional to their length. Because of this, proteins can be separated by SDS-PAGE electrophoresis according to their molecular weight and not by intrinsic electrical charge. Both the loading buffer and the gel running buffer contain SDS to allow this.

DTT and **β–Mercaptoethanol** function as reducing agents. While SDS serves to unfold proteins, DTT or βME further remove tertiary and quaternary structure by reducing intramolecular and intermolecular disulfide bonds. A reducing agent should be added to the loading buffer in most cases. DTT or βME are only excluded from the buffer when an antibody recognizes the non-reduced form of a protein. The antibody datasheet will specify the form of the protein recognized by the primary antibody.

Glycerol is added to sample loading buffer in order to increase sample density so samples can be loaded and sink to the bottom of the well, minimizing sample floating/overflow and promoting even protein loading.

Bromophenol blue is a small anionic dye that is added to enable visualization of protein migration throughout gel electrophoresis. Because it is so small, it migrates faster than the proteins in the samples and thus provides a migration front to monitor the electrophoresis process and prevent sample run-off.

Loading and running buffer conditions

Protein state	Sample loading buffer	Gel running buffer
Reduced and denatured (most common)	SDS + βME or DTT	SDS
Reduced and native	(No SDS) + βME or DTT	No SDS
Oxidized and denatured	SDS (No βME or DTT)	SDS
Oxidized and native	(No SDS and No βME or DTT)	No SDS

PROTOCOL: Sample Preparation

01 Determine the protein concentration of each lysate.

02 Determine how much protein to load (recommended: 10-50 µg/lane) and add an equal volume of **2X Laemmli buffer.**

03 To reduce and denature: Boil lysates in sample buffer at 95-100˚C for 5 minutes.*

*Reducing/denaturing conditions are recommended unless the antibody datasheet indicates otherwise. Occasionally, antibodies only recognize epitopes as they exist on the surface of a protein's native, non-denatured state. Non-denaturing conditions can be generated by leaving SDS out of the sample and migration buffers and not boiling the samples. Further, certain antibodies only recognize proteins in their non-reduced, or oxidized forms. In this case, the reducing-agents such as β-mercaptoethanol (βME) or DTT should not be included in the buffers. Refer to the table below for loading buffer and gel running buffer guidelines. DTT should be made up fresh and stored in the freezer in one-time use aliquots as DTT is known to rapidly lose its potency through oxidization. Some proteins are more effectively reduced with DTT than β-mercaptoethanol, so a change in reducing agent might be required for certain targets.

LOADING & RUNNING THE SDS-PAGE GEL

After sample preparation and loading buffer addition, samples must be loaded onto a gel. Electrophoresis is performed with a negative pole on the well end of the gel and a positive pole on the opposite end of the gel. The negatively charged SDS bound to proteins causes migration of protein complexes towards the positive pole during electrophoresis and proteins can be separated by size. In general, the larger the protein, the slower it moves through the gel. Acrylamide gels can be prepared at different concentrations. As a general rule, low molecular weight proteins are best resolved on high percentage gels, whereas large proteins require lower percentage gels for sufficient resolution.

PROTOCOL: SDS-PAGE

01 Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve your protein of interest based on molecular weight.

Protein Size	Gel Percentage
4-40 kDa	Up to 20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
50-200 kDa	8%
>200 kDa	4-6%

Note: If your protein of interest has multiple isoforms ranging from low to high molecular weight sizes or if you plan to probe a blot for multiple proteins varying in size, gradient gels (acrylamide concentration increases from top to bottom) may be necessary to achieve efficient separation of proteins.

02 Load samples containing equal amounts of protein (10-50 µg protein from cell lysate or 10-100 ng purified protein) prepared in sample buffer into SDS-PAGE wells. Include a molecular weight marker in one of the lanes.

03 Fill the electrophoresis apparatus with 1X running buffer as instructed by the manufacturer.

1X Running buffer
25 mM Tris base
192 mM glycine
0.1% SDS
Adjust to pH 8.3

04 Run the gel as recommended by the manufacturer. Voltage may vary depending on research needs. 1-2 hours at 100V is a standard guideline.

PROTEIN TRANSFER FROM GEL TO MEMBRANE

After electrophoresis is complete, proteins must be transferred from the gel onto a suitable membrane for subsequent immunoassay steps. This is performed by passing an electrical current across the gel to the membrane. The membrane can be either PVDF or nitrocellulose. PVDF is generally better for low molecular weight proteins. This membrane can be purchased in different pore sizes. For proteins less than 30 kDa, the pore size of 0.2 µm PVDF is recommended over the 0.45 µm pore size. Additionally, PVDF membranes have a higher binding capacity (150-160 µg/cm^2) than nitrocellulose (80-100 µg/cm^2).

PROTOCOL: Protein Transfer

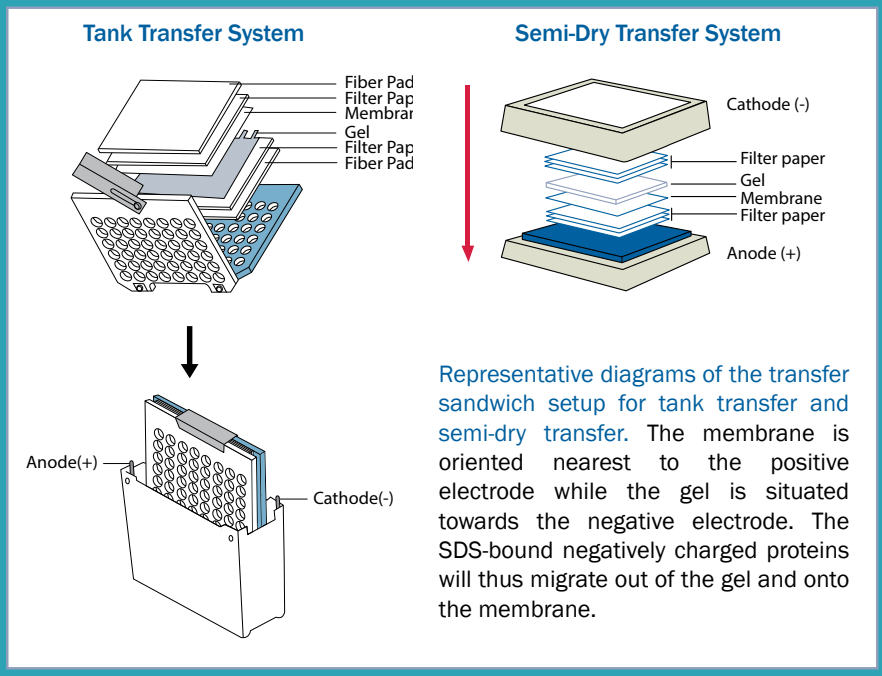
01 Prepare PVDF membrane by wetting it in methanol for 30 seconds and then soaking it briefly in distilled water followed by **1X transfer buffer.** Handle the membrane carefully, ideally with rounded tweezers to avoid scratching or puncturing the surface. **Note:** Do NOT wet nitrocellulose membranes with methanol or the membrane will dissolve.

1X transfer buffer (wet)	1X transfer buffer (semi-dry)
25 mM Tris base	48 mM Tris base
192 mM glycine	39 mM glycine
20% methanol	20% methanol
Adjust to pH to 8.3	Adjust pH to 8.3

02 Soak filter papers and sponges in the transfer buffer for 10 minutes prior to assembly of the transfer "sandwich".

03 After electrophoresis, remove the gel from the electrophoresis apparatus and equilibrate it by soaking in transfer buffer for 10 minutes.

04 Prepare the sandwich according to the illustration below. Sequentially assemble the layers of the sandwich Gently remove any air bubbles with a roller or pipette. Bubbles between the gel and the membrane will inhibit the transfer of proteins to the membrane.



Representative diagrams of the transfer sandwich setup for tank transfer and semi-dry transfer. The membrane is oriented nearest to the positive electrode while the gel is situated towards the negative electrode. The SDS-bound negatively charged proteins will thus migrate out of the gel and onto the membrane.

05 Place the sandwich into a transfer cassette and perform semi-dry or wet transfer according to the manufacturer's instructions of the blotting apparatus.

Semi-dry transfer: generally faster, better suited for larger proteins >100 kDa
Commonly used transfer time: 60 minutes at a constant current (1.25 mA/cm^2)

Wet transfer: recommended for smaller proteins, especially proteins <30 kDa
Commonly used transfer time: 1 hour at 100V at 4˚C.

Tip: Transfer time/voltage may require optimization. Over-transferring (or pulling protein all the way through the membrane) can occur and thus caution must be taken, especially for small proteins.

IMMUNOBLOTTING

Blocking

The first step in immunoblotting is to rinse and block the membrane with non-specific protein, such as milk or BSA. The purpose of this blocking step is to bind non-specific protein to the surface of the membrane where sample protein is not already present. This prevents antibody from binding non-specifically to the membrane, which gives rise to a high background signal. The choice of milk vs. BSA is antibody specific and may require optimization. Often the antibody information sheet will recommend one over the other. For phosphorylated targets, milk should be avoided, as it contains a high amount of phosphorylated proteins

Antibody Binding

Primary antibody: After blocking, the membrane is incubated in a solution containing the primary antibody. As mentioned previously, the primary antibody recognizes the epitope, or the specific amino-acid sequence, of the protein of interest.

Secondary antibody: After washing to remove unbound primary antibody, secondary antibody is added. Secondary antibody recognizes the primary antibody. Secondary antibodies used for western blotting are usually conjugated to an enzyme; the most commonly used enzymes are Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP).

Note: Some primary antibodies are directly conjugated to HRP, eliminating the need for the secondary antibody incubation steps. In this case, it is possible to proceed to detection after the primary antibody incubation and subsequent rinses. Novus offers primary antibodies conjugated to HRP. If elimination of the secondary antibody step is desired, Novus offers Lightning-Link Antibody Labeling Kits, which allow primary antibodies to be labeled with HRP or AP (see page 20 for avalaible labeling kits).

PROTOCOL: Immunoblotting

01 After transfer, rinse the membrane briefly in distilled water or 1X TBST.

1X TBST
20 mM Tris base
150 mM NaCl
0.1% Tween 20

02 Gently mark MW ladder bands with a pencil for size detection. If all blue molecular weight markers were used, this step can be omitted as the bands of all blue markers will be visible after detection when used in conjugation with the Blue Marker Antibody.

03 If desired, stain the membrane with Ponceau red (a reversible protein stain) for 30 seconds to visualize protein bands to confirm that protein transfer was successful. Rinsing the membrane briefly with distilled water after Ponceau staining will reveal protein bands. Wash away Ponceau red with several washes in 1X TBST until membrane is clear. Additionally, Coomassie staining of the gel after transfer can help assure that proteins were completely transferred from the gel to the membrane (minimal or no protein staining should be visible on a coomassie-stained gel after successful complete transfer).

PROTOCOL: Immunoblotting Cont'd.

04 Incubate membrane in blocking solution for 1 hour at room temperature or overnight at 4˚C with constant rocking.

Blocking Solution
1X TBS
5% non-fat dry milk OR 5% BSA
0.1% Tween 20

05 Optional step: Rinse the membrane for 5 mins in 1X TBST

06 Dilute the primary antibody to working concentration in 1X TBST with 1% milk or BSA (Whatever was chosen for blocking).

Note: Typical working antibody dilutions range from 1:500 to 1:5000 or greater. Antibody specification sheets often contain suggested dilutions, but optimal dilutions may need to be determined experimentally. See the troubleshooting section of this guide for more detail.

Optional: If using blue molecular weight markers, add 1 µg/mL Blue Marker Antibody to the primary antibody solution to visualize the molecualar weight markers. This antibody does not cross react with protein lysates and will bind specifically to the blue dye of each molecular weight marker.

07 Incubate the membrane in primary antibody solution for 2-3 hours at room temperature or overnight at 4˚C with gentle rocking. Incubation time may require optimization. See antibody datasheet for recommended dilutions.

08 Wash the membrane with 1X TBST three times for 10 minutes each with gentle rocking.

Tip: Increase the number of washes to 5-6 if high background occurs. See the troubleshooting guide.

09 Incubate the membrane in the appropriate diluted secondary antibody (in 1X TBST and may include 1% milk or BSA) for 1 hour at room temperature with gentle rocking.

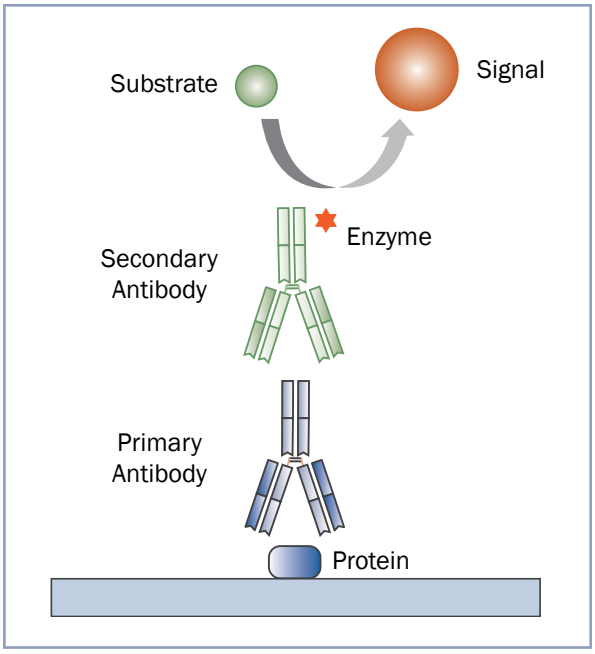
Note: See primary antibody information sheet for proper secondary antibody selection- must recognize host species of the primary antibody. Secondary antibody concentration guidelines are listed in the product information sheet. For more information on secondary antibodies, read Novus' Secondary Antibody Handbook.

10 Wash the membrane in 1X TBST three times for 10 minutes each with gentle rocking.

Tip: Do not let the membrane dry at any point during the blotting process.

DETECTION

In western blotting, the method of detection is dependent on which enzyme is conjugated to the secondary antibody (or in some cases the primary antibody). The most commonly used enzyme is HRP. Once substrate has been added, it reacts with HRP and emits light. The emitted light can be detected using autoradiography film or a chemiluminescence imaging system.



PROTOCOL: Detection

01 Prepare the ECL substrate just prior to use according to the manufacturer's instructions. Novus offers NovaLume, NovaLumePlus, and a highly sensitive substrate PicoTect (see page 20 for available detection reagents).

02 Incubate the membrane in the substrate according to manufacturer's directions. Typical incubation times are 1-5 minutes.

Tip: More sensitive substrates may require shorter incubation times or even dilution to achieve optimal signal and avoid overexposure.

03 Carefully remove the membrane from the detection reagent and sandwich it between layers of plastic (e.g. a sheet protector or plastic wrap).

04 Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system.

Tips: Clip the top right corner of your film as a guide for film orientation in a dark room.
Use multiple exposure lengths to identify the most optimal exposure time.

05 The developed film or image can be lined up in the correct orientation over the blot in order to mark the MW ladder positions if the Blue Marker Antibody is not used.

STRIPPING/RE-PROBING

A single blot can be analyzed sequentially with multiple antibodies by stripping one antibody from the blot and subsequently incubating with an additional antibody. This practice may be useful when sample is limited. Two methods are outlined below. The first method uses heat and detergent to release antibodies and the second uses low pH to inactivate the antigen binding site of the antibody.

Stripping solution #1	Stripping solution #2	1X PBS
100 mM 2-mercaptoethanol	25 mM glycine-HCl	137 mM NaCl
2% SDS	1% SDS	2.7 mM KCl
62.5 mM Tris-HCl	Adjust pH to 2	4.3 mM Na2HPO4
Adjust pH to 6.7		1.47 mM KH2PO4
		Adjust pH to 7.4

PROTOCOL: Stripping With Heat & Detergent

01 In a fume hood, agitate the blot in **stripping solution #1** for 30 minutes at 50˚C

02 Agitate the blot in **1X PBS** for 10 minutes at room temperature. Repeat with fresh buffer.

03 Proceed to the blocking step of the immunoblotting protocol to re-probe the blot with a second antibody.

PROTOCOL: Stripping With Acid pH

01 Agitate the blot in **stripping solution #2** for 30 minutes at room temperature.

02 Agitate the blot in **1X PBS** for 10 minutes at room temperature. Repeat with fresh buffer.

03 Proceed to the blocking step of the immunoblotting protocol to re-probe the blot with a second antibody.

BLOT STORAGE

One of the advantages of PVDF membranes is that PVDF is a chemically resistant polymer that has excellent long term stability. Sometimes it might be desirable to store a blot for future use, such as for stripping and re-probing.

PROTOCOL: Storing A Blot

01 Sandwich the dry PVDF blot between two clean sheets of Whatman 3MM paper.

02 Place the sandwich between two sheets of card stock or thin cardboard.

03 Use paperclips to clip the stack together on the edges.

04 Place the stack in a plastic bag and seal the plastic bag closed.

05 Store the blot at 4˚C for up to 2 weeks, -20˚C for up to 2 months, or -70˚C for more than 2 months.

Tip: Thaw frozen blots to room temperature before removal from the plastic bag because frozen blots are prone to breakage.

Tip: Blots can also be stored wet at 4˚C, but sodium azide should be added to prevent bacterial growth. As sodium azide inhibits HRP activity, it should be thoroughly washed out of a blot prior to use.

TROUBLESHOOTING

The following troubleshooting guide is intended to explain causes and possible solutions for common problems with western blotting.

No Signal or Weak Signal

Primary antibody concentration is too low

- Increase the concentration of the primary antibody (titrations may be helpful)
  - Use Novus Antibody Concentration Kits to increase primary antibody concentration
- Increase the incubation time to 4°C overnight
- If re-used too many times, the effective antibody concentration may be too low; use fresh antibody to improve signal

Target protein concentration is too low

- Load more protein per well (titrations might be helpful)
- Use a positive control control lysate known to express the target protein, an overexpression lysate, or a recombinant protein
- Ensure lysis buffer is optimal for localization of target protein
- Use immunoprecipitation or fractionation (i.e. nuclear fractionation) if necessary to increase the concentration of a non-abundant protein
- Include protease inhibitors in the lysis buffer
- Ensure the sample has not degraded

Protein transfer from gel to membrane was unsuccessful

- Confirm that proteins were successfully transferred to the membrane by Ponceau S staining of the membrane
- Confirm that proteins were completely transferred by coomassie staining of the gel
- Confirm equal transfer by analyzing loading control expression

Primary and secondary antibody are not compatible

- Ensure that secondary antibody was raised against the species in which the primary was raised (e.g. if primary was raised in mouse, use an anti-mouse secondary)

Membrane choice was not ideal

- Check the hydrophobicity/hydrophilicity of the antigen sequence
  - PVDF membrane may work better for hydrophilic/polar/charged antigens
  - Nitrocellulose may work better for hydrophobic/non-polar antigens

There are issues with blocking

- Blocking for too long can mask certain epitopes and inhibit antibody binding
- Reduce blocking time
- Reduce concentration of blocking solution
- Reduce percentage or remove blocking reagent from antibody incubation buffers
- Switch to a different blocking solution

Excessive washing of membrane

- Detection reagent issues
- Detection reagents can become inactive over time-
  - Ensure reagents are fresh
  - Test by dot blotting secondary onto membrane and incubating with detection reagent
- Use more sensitive reagents when working with low abundance proteins (titrations may be helpful; if diluting, use high-purity water)

No Signal or Weak Signal Cont'd.

Image exposure was too short
- Increase exposure time (check several times to achieve optimal exposure time)

Antibody only recognizes native proteins
- Do not use reduced, denatured proteins if working with an antibody that only recognizes native proteins

Targets are low molecular weight
- Reduce transfer time to prevent over transfer
  - Use membranes with smaller pore size (0.2 µm vs. 0.45 µm)
  - Wet transfer is recommended for small proteins

Sodium azide contamination has occurred
- Sodium azide (often used to store primary antibodies) inhibits HRP activity
  - Ensure sufficient washing to remove presence of sodium azide
  - Use sodium azide-free buffers

High Uniform Background

Insufficient blocking
- Increase blocking time and/or temperature
- Increase the concentration of blocking reagent (try up to 10%)
- Consider changing the blocking agent (milk vs. BSA)
- Include the optional blocking agents in antibody buffers (can also increase %)

Blocking not compatible
- For phosphorylated protein detection, milk should not be used (milk and casein are phospho-protein rich)
- If your secondary is anti-bovine, anti-goat, or anti-sheep, use 5% serum from the host species of the secondary antibody as a blocking agent

Non-specific binding due to high antibody concentration
- Lower concentration of primary or secondary (titrations may be helpful)
- Include blocking agents in antibody buffers
- Confirm the secondary is specific by omitting the primary and performing a secondary antibody only control blot

Insufficient washing of unbound antibodies
- Increase the number and/or time of washes

Dry membrane
- Make sure the membrane never becomes dry during the western blotting protocol

Film exposure is too long
- Lower the exposure time (may be necessary to test a range of exposure times)

Detection reagents are too sensitive
- Dilute the detection reagent in pure water or use a less sensitive detection reagent

Non-specific bands/wrong size or multiple bands

Target protein is less abundant than the threshold of non-specific binding

- Load more protein in the SDS-PAGE gel
- Enrich low-abundance proteins by immunoprecipitation or fractionation

Sample degradation

- Use fresh lysates
- Keep sample on ice until just before sample buffer addition and boiling
- Always include protease inhibitors and phosphatase inhibitors if detecting phosphorylated target

Other protein isoforms may be present

- Alternative splicing, multimer formation, etc.
- May need an isoform-specific antibody

Post-translational modifications may be present

- Predicted molecular weight can be influenced by many factors such as glycosylation, phosphorylation, protein processing (cleavage from a pro-form to a mature form)
- To confirm specificity, perform positive and negative controls such as recombinant protein or overexpression lysate, downregulated knockdown/knockout lysate

Speckled or Swirled Background

Membrane mishandling

- Minimize contact with membrane. Use clean tools to handle the membrane

Buffer contamination

- Use fresh buffers

Air bubbles

- Roll out any bubbles between the gel and membrane before transfer

HRP aggregation

- Filter the secondary antibody using a 0.2 µm filter to remove aggregate

Insufficient washing

- Increase the volume of the washing buffer
- Increase the number and/or duration of the washes

Other Issues

White/hollow bands

- Decrease the concentration of primary/secondary antibody or use less protein

Smeared bands/lanes (sample overloading)

- Load less protein into each lane

“Smiling” bands

- Migration was too fast; decrease the voltage while running the gel
- Migration was too hot; run the gel in the cold room

Molecular weight marker lane is black

- The antibody may react with the molecular weight marker
- Add a blank lane between the molecular weight marker and the first sample lane

BENCHTOP WESTERN BLOTTING PROTOCOL

SAMPLE PREPARATION

1 Determine the protein concentration of each cell lysate.

2. Determine how much protein to load (Recommended: 10-50 µg/lane) and add an equal volume 2X Laemmli buffer.
3. Reduce and denature by boiling lysates in sample buffer at 95-100˚C for 5 minutes.

SDS-PAGE

1. Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve your protein of interest based on molecular weight.
2. Load samples containing equal amounts of protein (10-50 µg/lane protein from cell lysate or 10-100 ng/lane purified protein) prepared in sample buffer into SDS-PAGE wells. Include a molecular weight marker in one of the lanes.
3. Fill the electrophoresis apparatus with 1X running buffer as instructed by the manufacturer.
4. Run the gel as recommended by the manufacturer.

PROTEIN TRANSFER

1. Prepare PVDF membrane by wetting it in methanol for 30 seconds and then soaking it briefly in distilled water followed by 1X transfer buffer. Handle the membrane carefully, ideally with rounded tweezers to avoid scratching or puncturing the surface.
2. Soak filter papers and sponges in the transfer buffer for 10 minutes prior to assembly of the transfer “sandwich”.
3. After electrophoresis, remove the gel from the electrophoresis apparatus and equilibrate it by soaking in transfer buffer for 10 minutes.
4. Prepare the sandwich. Sequentially assemble the layers of the sandwich. Gently remove any air bubbles with a roller or pipette. Bubbles between the gel and the membrane will inhibit the transfer of proteins to the membrane.
5. Place the sandwich into a transfer cassette and perform semi-dry or wet transfer according to the manufacturer’s instructions of the blotting apparatus.

IMMUNOBLOTTING

1. After transfer, rinse the membrane briefly in distilled water or 1X TBST.
2. Gently mark molecular weight ladder bands with a pencil for size detection. If all blue molecular weight markers were used, this step can be omitted as the bands of all blue markers will be visible after detection when used in conjugation with the Blue Marker Antibody.
3. If desired, stain the membrane with Ponceau red for 30 seconds to visualize protein bands to confirm that protein transfer was successful. Rinsing the membrane briefly with distilled water after Ponceau staining will reveal protein bands. Wash away Ponceau red with several washes in 1x TBST until membrane is clear. Additionally, coomassie staining of the gel after transfer can help assure that proteins were completely transferred from the gel to the membrane.
4. Incubate membrane in blocking solution for 1 hour at room temperature or overnight at 4˚C with constant rocking
5. **Optional step:** Rinse the membrane for 5 minutes in 1X TBST.
6. Dilute the primary antibody to working concentration in 1X TBST with 1% milk or BSA (whatever was chosen for blocking)

Optional: To visualize the molecular weight markers in addition to the protein of interest, add 1 µg/mL Blue Marker Antibody to the primary antibody solution.

7. Incubate the membrane in primary antibody solution for 1 hour at room temperature or overnight at 4˚C with gentle rocking. This time may require optimization.
8. Wash the membrane with 1X TBST three times for 10 minutes each with gentle rocking.

    Tip: Increase the number of washes to 5-6 if high background occurs. See the troubleshooting guide page 10.

9. Incubate the membrane in the appropriate diluted secondary antibody (in 1X TBST and may include 1% milk or BSA) for 1 hour at room temperature with gentle rocking.
10. Wash the membrane in 1X TBST three times for 10 minutes each with gentle rocking.

    Tip: Do not let the membrane dry at any point during the blotting process.

DETECTION

1. Prepare the ECL substrate just prior to use according to the manufacturer's instructions.
2. Incubate the membrane in the substrate according to manufacturer's directions. Typical incubation times are 1-5 minutes.

    Tip: More sensitive substrates may require shorter incubation times or even dilution to achieve optimal signal and avoid overexposure.

3. Carefully remove the membrane from the detection reagent and sandwich it between layers of plastic (i.e. a sheet protector or plastic wrap).
4. Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system, such as a ChemiDoc.

    Tip: Clip the top right corner of your film as a guide for film orientation in a dark room.

    Tip: Use multiple exposure lengths to identify the most optimal exposure time.

5. The developed film or image can be lined up in the correct orientation over the blot in order to mark the molecular weight ladder positions if the Blue Marker Antibody is not used.

REFERENCE - RECIPES

Lysis buffer recipes

NP-40
150 mM NaCl
1% NP-40 or Triton X-100
50 mM Tris pH 8.0

RIPA
150 mM NaCl
1% NP-40 or Triton X-100
0.5% sodium deoxycholate
0.1% SDS
50 mM Tris, pH 8.0

Tris-HCl
20 mM Tris-Hcl, pH 7.5

Loading Buffer

2X Laemmli buffer
4% SDS
5 % 2-mercaptoethanol
20% glycerol
0.004% bromophenol blue
0.125 M Tris HCl
pH 6.8

Running Buffer

1X Running buffer
25 mM Tris base
192 mM glycine
0.1% SDS
Adjust to pH 8.3

Transfer Buffer

1X transfer buffer (wet)
25 mM Tris base
192 mM glycine
20 % methanol
Adjust to pH to 8.3

1X transfer buffer (semi-dry)
48 mM Tris base
39 mM glycine
20 % methanol
Adjust pH to 8.3

TBST

1X TBST
20 mM Tris base
150 mM NaCl
0.1 % Tween 20

Blocking Buffer

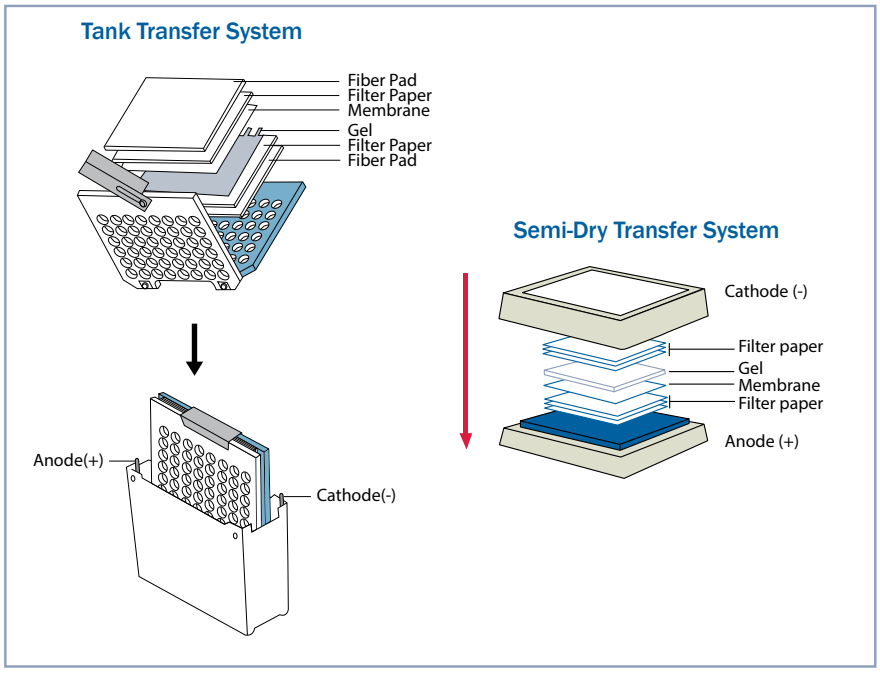
Blocking solution
1X TBST
5% non-fat dry milk OR 5% BSA

Stripping Buffer

Stripping solution #1
100 mM 2-mercaptoethanol
2% SDS
62.5 mM Tris-HCl
Adjust pH to 6.7

Stripping solution #2
25 mM glycine-HCl
1% SDS
Adjust pH to 2

Transfer Sandwich Assembly



REAGENTS AVAILABLE FROM NOVUS BIOLOGICALS

SAMPLE PREPARATION

Enrichment Kit

Fractionation Kits to Enrich Protein and Isolate Organelles
Total Protein Extraction Kit #NBP2-37853
Nuclear Fractionation Kit #NBP2-29447
Mitochondria Fractionation Kit #NBP2-29448
Endoplasmic Reticulum Fractionation Kit #NBP2-29482
Nuclear/Cytoplasm/Membrane/Cytoskeleton Fractionation Kit #NBP2-47659

IMMUNOBLOTTING REAGENTS

Secondary Antibodies

Goat Secondary Antibodies
Anti-Mouse IgG (H+L), HRP conjugated #NBP1-75144
Anti-Rabbit IgG (H+L), HRP conjugated #NBP1-75297
Anti-Rat IgG IgG (H+L), HRP conjugated #NBP1-75388
Anti-Mouse IgG (H+L), AP conjugated #NBP1-75127
Anti-Rabbit IgG (H+L), AP conjugated #NBP1-75294
Anti-Rat IgG (H+L), AP conjugated #NBP1-75385
Anti-Mouse IgG (H+L), Biotin conjugated #NBP1-75128
Anti-Rabbit IgG (H+L), Biotin conjugated #NBP1-75295
Anti-Rat IgG (H+L), Biotin conjugated #NBP1-75386
Anti-Mouse IgG (H+L), DyLight 680 #NBP1-72882
Anti-Rabbit IgG (H+L), DyLight 680 #NBP1-72951
Anti-Rat IgG (H+L), DyLight 680 #NBP1-72979
Anti-Rabbit IgG (H+L), DyLight 800 #NBP1-72954

Molecular Weight Ladder Detection

Blue Marker Antibody for Chemiluminescent Detection of Blue Prestained Ladders
Blue Marker Antibody #NBP2-33376
Blue Marker Antibody, HRP conjugated #NBP2-33376H

Antibody Labeling Kits

Kits to conjugate unlabeled primary antibodies
Lightning-Link™ HRP Antibody Labeling Kit #701-0010
Lightning-Link™ AP Antibody Labeling Kit #702-0010
Lightning-Link™ Biotin Antibody Labeling Kit #370-0010
Lightning-Link™ DyLight 680 Antibody Labeling Kit #327-0010
Lightning-Link™ DyLight 800 Antibody Labeling Kit #329-0010

CONTROLS

Loading Control Antibodies

Loading Control Antibodies for Western Blot
Sampler Kit: Loading Control Antibody Pack #NBP2-25090
Whole Cell: Alpha Tubulin Loading Control Antibody #NB100-690
Whole Cell: Beta-Actin Loading Control Antibody #NB600-501
Whole Cell: GAPDH Loading Control Antibody #NB300-221
Mitochondria: HSP60 Loading Control Antibody #NBP1-77397
Mitochondria: Cox IV Loading Control Antibody #NB110-39115
Nucleus: Lamin B1 Loading Control Antibody #NB100-56403
Nucleus: HDAC Loading Control Antibody #NB100-56340
Serum: Transferrin Loading Control Antibody #NB500-418

DETECTION REAGENTS

Chemiluminescent Substrate

Secondary Antibodies for Chemiluminescent Western Blots
PicoTect Chemiluminescent Substrate #NBP2-29912
NovaLume Chemiluminescent Substrate #LUM-500

R&D SYSTEMS NOVUS BIOLOGICALS TOCRIS proteinsimple

bio-techne®
Global info@bio-techne.com bio-techne.com/find-us/distributors TEL +1 612 379 2956
North America TEL 800 343 7475 **Europe | Middle East | Africa** TEL +44 (0)1235 529449
China info.cn@bio-techne.com TEL +86 (21) 52380373
bio-techne.com

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