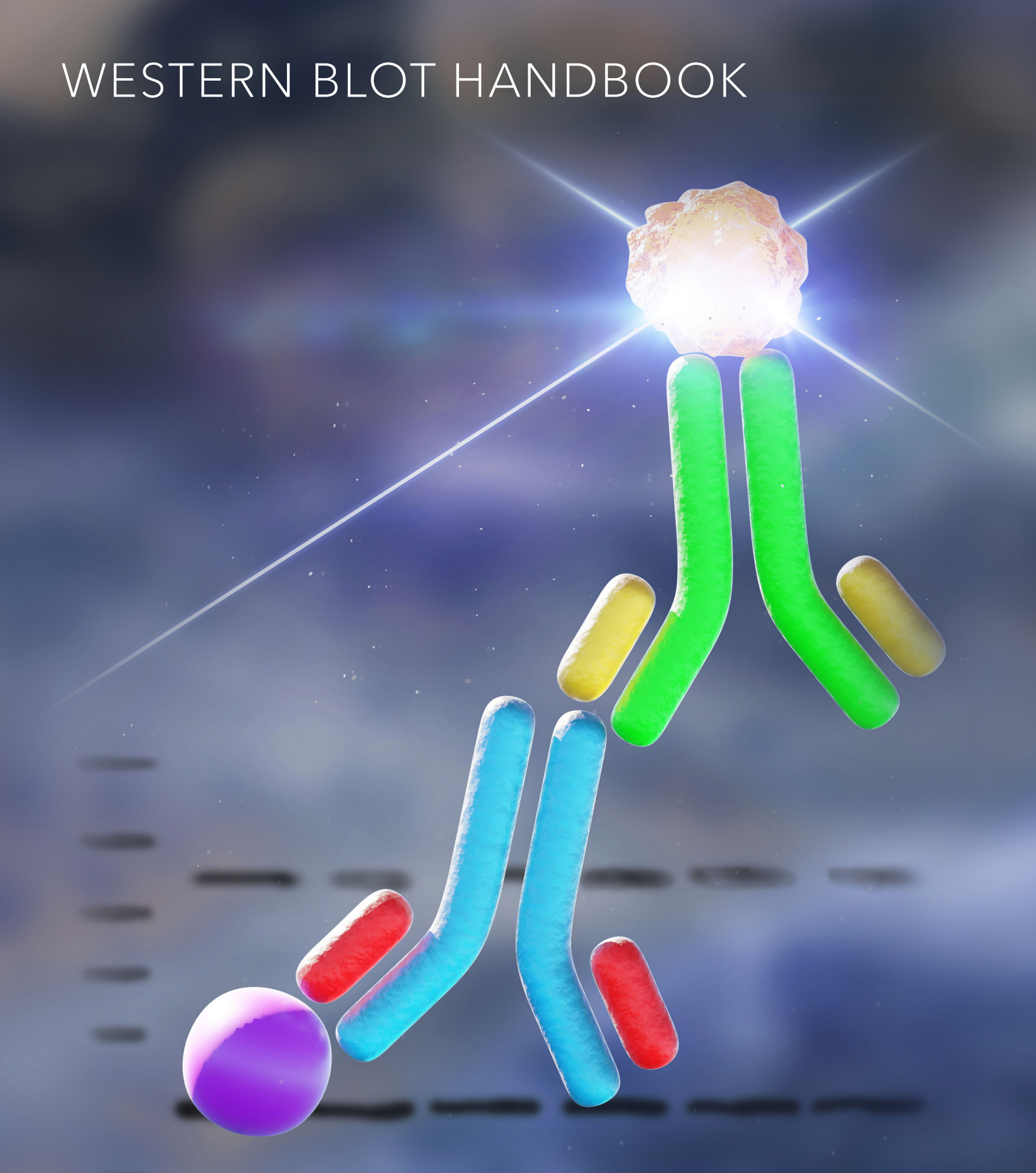
WESTERN BLOT HANDBOOK



bio-techne®

SIMPLE WESTERN: PICK YOUR PERFECT MATCH

JESS™

- Size-based protein analysis
- 13 or 25 samples per run
- Fully analyzed results in 3 hours of run time
- Chemiluminescence and IR/NIR detection
- RePlex assay
- Total protein normalization
- Western blot imaging

ABBY™

- Size-based protein analysis
- 13 or 25 samples per run
- Fully analyzed results in 3 hours of run time
- Chemiluminescence detection
- RePlex assay
- Total protein normalization

WES™

- Size-based protein analysis
- 13 or 25 samples per run
- Fully analyzed results in 3 hours of run time
- Chemiluminescence detection

SALLY SUE™

- Size-based protein analysis
- 96 samples per run
- Fully analyzed results in 14–19 hours of run time
- Chemiluminescence detection

NANOPRO 1000™

- Charge-based analysis
- 96 samples per run
- Fully analyzed results in 11–19 hours of run time
- Chemiluminescence detection

PEGGY SUE™

- Size-based protein analysis
- Charge-based protein analysis
- 96 samples per run
- Chemiluminescence detection

TABLE OF CONTENTS

WESTERN BLOT HANDBOOK

FALL 2021

bio-techne®

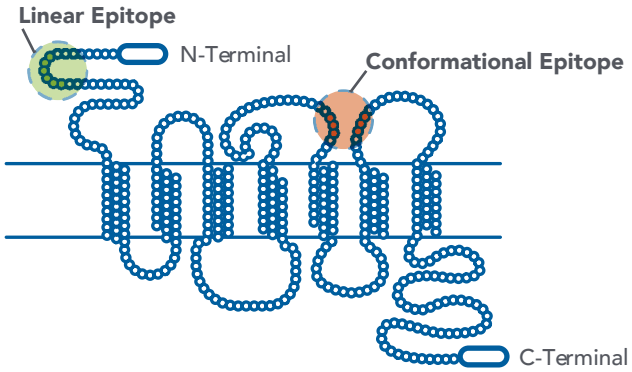
This handbook is intended to serve as a starting point for understanding the principles behind western blotting. Use this guide to learn about the steps required for reliable and reproducible data analysis. Find helpful tools for performing and troubleshooting a standard western blotting protocol.

INTRODUCTION TO WESTERN BLOTTING

Western blotting, also referred to as immunoblotting, is considered the gold standard for protein detection and quantification in molecular biology research. First developed by three separate labs in the 1970s, western blotting started out as a simple, visual assay to test antigen specificity of monoclonal antibodies, enabling rapid screening for clones against retroviral components. This ubiquitous technique is now routinely used to separate proteins by size and identify specific antigens in a complex protein mixture such as cell and tissue lysates.

The western blot technique requires the use of antibodies which bind highly specific sequences of amino acids, known as epitopes, on a solid support. Antibody epitopes are either linear, meaning the amino acids are continuous in the polypeptide chain, or conformational, requiring proteins in their native state, including secondary and tertiary protein structure. Given the protein denaturation step typical in western blotting, antibodies used for this technique generally recognize linear epitopes. For this reason, antibodies that have been validated for western blot are not always compatible with other applications where proteins retain their native conformation, such as flow cytometry, ELISA, and immunohistochemistry (IHC).

Traditional western blot analysis represents the average protein expression across a population of cells, which are often heterogenous. Though this analysis allows for detection of global changes in protein expression, subtle changes in heterogenous cell populations can be lost. This makes it challenging, if not impossible, to characterize specific cell types within diverse populations, such as the tumor microenvironment. Technological advances such as ProteinSimple's Single-Cell Western platform allow for a more thorough analysis of heterogeneous populations and accelerate cancer and immuno-oncology research.



TAKE WESTERN DATA DOWN TO THE SINGLE CELL LEVEL USING MILO™

Milo™ is the world's first automated Single-Cell Western platform. Milo™ measures protein expression in thousands of individual cells in a single run, simplifying the western blot workflow and allowing users to profile heterogeneity in their samples through single-cell analysis. This platform can simultaneously detect multiple proteins in a sample using either spectral or size-based multiplexing strategies.

Benefits of Milo™ Single-Cell Westerns:

- Compatible with western-validated antibodies.
- Measure variation of protein expression across heterogenous cells in a sample.
- Analyze single-cell proteomics to determine the percentage of cells in a sample that are target positive.

Learn more about Milo at www.proteinsimple.com/milo

SIMPLE WESTERN™

Although widely used in protein biochemistry, the traditional western blot workflow presents some challenges. It is time-consuming and labor-intensive, with many hands-on steps. These manual steps can introduce variability that can potentially limit the reproducibility of the results. Furthermore, protein expression data obtained from traditional western blotting is typically considered only semi-quantitative.

WHAT IS SIMPLE WESTERN™?

Simple Western™ platforms fully automate western blotting workflows, providing data on 24 samples in only 3 hours or 96 samples overnight. Simple Western utilizes a capillary-based electrophoresis technology that provides a gel-free, blot-free process with minimal hands-on time. Proteins can either be separated by size between 2 and 440 kDa or by charge. Simple Western overcomes many of the challenges associated with traditional western blotting, generating highly reproducible and fully quantitative results in less time. Another advantage of Simple Western is that it is capable of providing picogram-level sensitivity on par with traditional westerns, but it only requires very small sample volumes, as little as 3 µL.

For precious samples where there are multiple targets of interest, newer Simple Western instruments like Jess™ and Abby™ that offer RePlex, two sequential immunoassays on the sample in the same capillary can be performed. The automated RePlex process efficiently removes antibodies from the first round of probing for a second round of detection generating more protein expression data per sample. In traditional western blotting, this would require stripping the membrane with harsh treatment conditions that can result in signal loss. With RePlex, the signal is not compromised because the sample is covalently bound to the capillary wall.

TRADITIONAL WESTERN BLOT (CHEMILUMINESCENCE)
1. **Sample Preparation** - Lyse cells with appropriate buffer, determine lysate concentration, add loading buffer, and reduce and denature samples. (~30 min)
2. **Gel Apparatus Assembly** – Add upper and lower chamber buffers. (~5 min)
3. **SDS-PAGE Gel Electrophoresis** – Remove gel comb and load samples into wells, ensuring samples sink neatly to bottom of the well. Run gel to separate proteins by size/ molecular weight. Determine the optimal voltage and avoid overheating the gel. (~1 hr)
4. **Transfer Apparatus Assembly and Protein Transfer** – Proteins are transferred from the gel to a membrane by application of an electrical current via wet transfer or semi-dry transfer setup. (1 hr 15 min)
5. **Membrane Blocking** – Rinse the membrane and block in milk or BSA to prevent antibody from binding non-specifically to the membrane. (1 hr)
6. **Immunoblotting** – The membrane is probed overnight with primary antibodies specific for the target of interest, followed by enzyme-bound secondary antibodies, with membrane washes in between steps. (~14 hrs)
7. **Detection & Imaging** – Substrate is applied to the membrane to react with the secondary antibody-bound enzyme for detection of the antibody/protein complex. (10 min)
8. **Analysis** – Data normalization performed with the help of molecular weight ladders and appropriate controls. - Total time: ~18 hrs

Total time: - 18 hrs

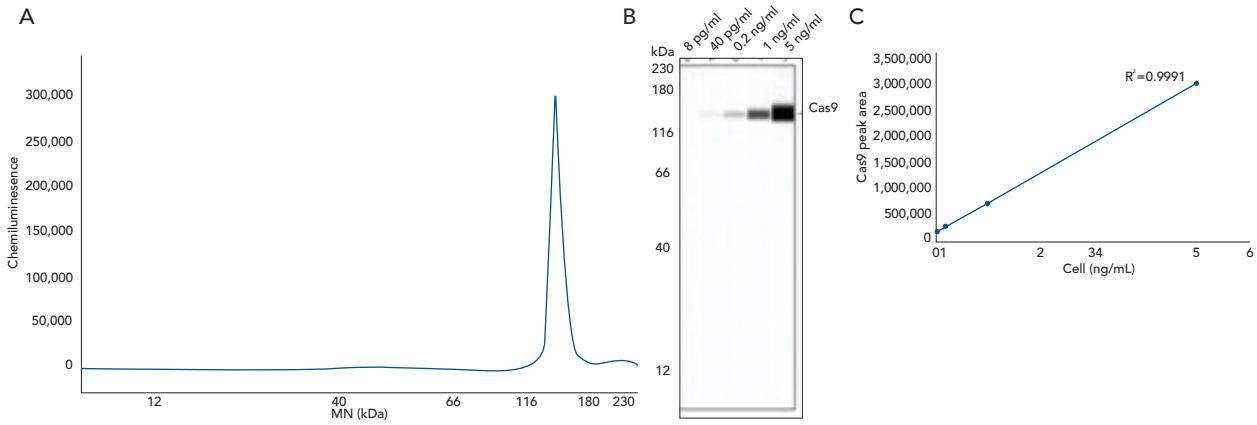
SIMPLE WESTERN
1. **Sample Preparation** – Pipette, mix, and spin reagents. (~30 min)
2. **Load the plate** – Load the reagents and samples into the Simple Western plate. (~5 min)
3. **Start the run** – Place the plate and a capillary cartridge into the instrument and use the built-in software to start the run. (~5 min)
4. **Simple Western Automated System** – Proteins are denatured by SDS/heat, immobilized covalently through UV-sensitive capillary coating, and immunoprobed directly within the capillary by primary and secondary antibodies for either chemiluminescence or fluorescence detection. (~3 hrs)
5. **Automated analysis** – The data is analyzed directly using the integrated Compass Software.

Total time: - 3.5 hrs

Note: Simple Western Automated System run-time is based on small box platforms (Jess, Abby, Wes), but higher throughput systems that can run up to 96 samples in one run (Sally Sue, Peggy Sue, NanoPro 1000) require 11-19 hrs run-time.

DATA VISUALIZATION

Data generated by Simple Western instruments automatically appear in the Compass for Simple Western Software once the run has completed. Here, data may be analyzed and exported directly for publication. Simple Western data can be viewed in a variety of ways including in graph view (A), virtual lane view, similar to traditional western blot data (B), and as the direct image of the capillary (not pictured). Simple Western assays also allow for assessment of the linear dynamic range (C), with approximately 1.5-log dynamic range improvement compared to traditional westerns, allowing for detection of proteins across wider concentration range with more accuracy. Among these different ways to view data, the graph view is recommended because it provides the most resolution and quantitative detail. In graph view, data is portrayed as an electropherogram, with size or charge-based migration distance on the X-axis and the chemiluminescent or fluorescent signal intensity on the Y-axis. Simple Western automatically calculates signal height, area, % area, width, signal-to-noise (S/N), and baseline values, and these numbers may easily be exported for further statistical analysis if desired.



Detection of recombinant Cas9 by Wes, a Simple Western™ instrument. (A) Electropherogram of Cas9 detected by a chemiluminescence readout, (B) lane view of Cas9 detection and (C) linearity analysis of Cas9 detection. Cas9 was detected with the CRISPR-Cas9 Monoclonal Antibody (1013816) (R&D Systems Catalog # MAB10252)

Learn more by downloading | Simple Western Produces Publication - Ready Results White Paper

Read more about how scientists utilize Simple Western instruments in their research in our From Your Peers highlight sections

WESTERN BLOT CONTROLS

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 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 experimental samples are negative for the target protein. A positive control is important to ensure that there were no issues in the western blotting protocol. Signal detection in the positive control sample but not in experimental samples confirms negative results for the target protein in the test samples. 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 overexpressing the target protein.
- Cell line/tissue/experimental condition with proven positive signal (endogenous control lysate).
- Purified recombinant protein.*

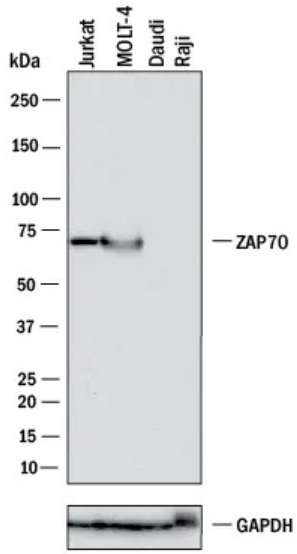
***Note:** Endogenous control lysate known to express the protein of interest are recommended over recombinant proteins. For example, if the control is a tagged recombinant protein the molecular weight will differ from the endogenous version.

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 (a false positive result) has occurred during the western blotting procedure.

COMMONLY USED NEGATIVE CONTROLS:

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



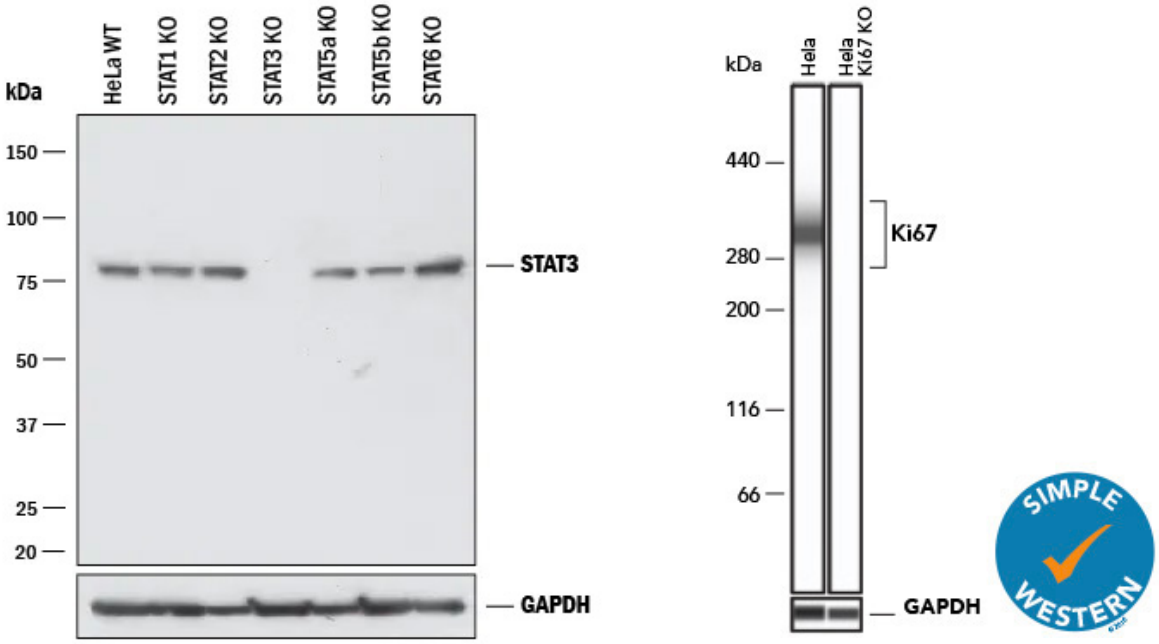
Western blot with positive and negative controls. Western blot shows lysates of Jurkat human acute T cell leukemia cell line (positive control), MOLT 4 human acute lymphoblastic leukemia cell line (positive control), Daudi human Burkitt's lymphoma cell line (negative control), and Raji human Burkitt's lymphoma cell line (negative control). ZAP70 is expressed in T cells but not B cells. Membrane was probed with Goat Anti-ZAP70 Polyclonal Antibody (R&D Systems Catalog # AF3709) followed by HRP-conjugated Anti-Goat IgG Secondary Antibody (R&D Systems Catalog # HAF017). A specific band was detected for ZAP70 at approximately 70 kDa (as indicated). GAPDH is shown as a loading control.

ANTIBODY SPECIFICITY

Antibody specificity refers to the ability of an antibody to recognize and bind to its unique target (antigen). Antibody specificity is a critical component of experimental reproducibility. R&D Systems and Novus Biologicals have employed the 5 Pillars of Validation, in accordance with the recommendations set forth by the International Working Group for Antibody Validation (IWGAV), to verify antibody specificity. Antibody validation methods include genetic validation by knockout or knockdown strategies, biological validation via biological or chemical modulation, and independent validation of two or more different antibodies against the same target.

GENETIC VALIDATION - KNOCKOUT OR KNOCKDOWN

Arguably the most important and rigorous technique for confirming antibody specificity in western blot is through genetic knockdown (KD) or knockout (KO) of the target protein of interest by using CRISPR/Cas9 or small interfering RNA (siRNA)/short hairpin RNA (shRNA) technology. If the antibody is specific for the target, the protein of interest, as indicated by the presence of a band in western blot or Simple Western systems, will be notably absent from the KO line.

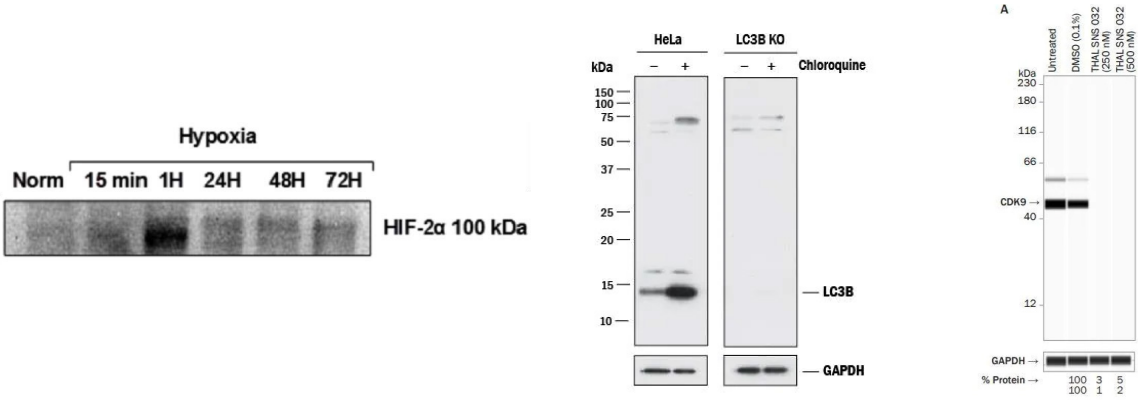


Genetic validation KO examples. (Left) KO validated western blot highlighting lysates of HeLa human cervical epithelial carcinoma parental cell line, STAT1 KO HeLa cell line, STAT2 KO HeLa cell line, STAT3 KO HeLa cell line, STAT5a KO HeLa cell line, STAT5b KO HeLa cell line, and STAT6 KO HeLa cell line. PVDF membrane was probed with Mouse Anti-STAT3 Monoclonal Antibody (232209) (R&D Systems Catalog # MAB1799) followed by HRP-conjugated Anti-Mouse IgG Secondary Antibody (R&D Systems Catalog # HAF018). Specific bands were detected for STAT3 at approximately 80 kDa (as indicated) in the parental HeLa cell line and all KO HeLa cell lines except STAT3 KO. GAPDH (R&D Systems Catalog # AF5718) was used as a loading control. This experiment was conducted under reducing conditions. **(Right)** KO validated Simple Western lane view showing lysates of HeLa parental cell line and Ki67 KO HeLa cell line. A specific band was detected for Ki67/MKI67 at approximately 312 kDa (as indicated) in the parental cell line, but is not detectable in the KO HeLa cell line using Rabbit Anti-Human Ki67/MKI67 Monoclonal Antibody (1297A) (R&D Systems Catalog # MAB7617). GAPDH is shown as a loading control. This experiment was conducted under reducing conditions and using the 66-440 kDa separation system.

BIOLOGICAL VALIDATION – BIOLOGICAL OR CHEMICAL MODULATION

Treating cells with chemical activators and growth factors capable of inducing or inhibiting target protein expression is another method to validate antibody specificity. For example, cobalt chloride ($CoCl_2$) is a cell treatment that can mimic hypoxic conditions and is particularly beneficial in hypoxia inducible factors (HIFs) antibody validation because it stabilizes HIF levels. Besides chemically treating cells to mimic hypoxic environments, cells can also be directly grown in hypoxic conditions and compared to normoxic controls. Other commonly used modulators include autophagy inhibitors such as chloroquine, DNA methylation inhibitors, protein kinase inhibitors, radiation, and cellular activators like lipopolysaccharide (LPS).

An additional method of validation is targeted protein degradation using small-molecule based PROTAC®s (PROteolysis TArgeting Chimeras). PROTAC®s are active degraders made of two small molecules joined by a linker. One binds an E3 ubiquitin ligase and the other binds the target protein of interest, resulting in a ternary complex. Formation of the complex leads to polyubiquitination of the protein of interest and its subsequent degradation by the proteasome.

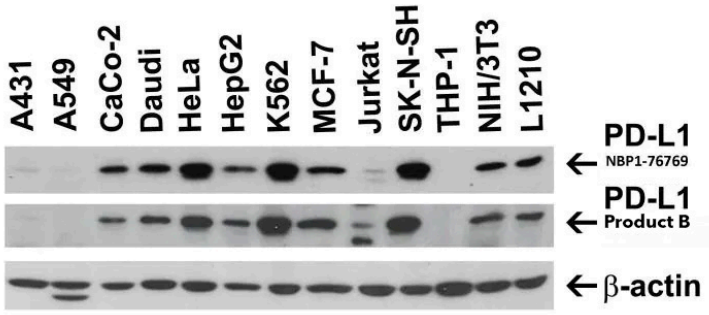


Biological validation examples. (Left) Western blot analysis showing HIF-2 alpha stabilization over time in 791T cells following exposure to hypoxia for various timepoints using HRP-conjugated form of Mouse Anti-HIF-2 alpha/EPAS1 Monoclonal Antibody (ep190b) (Novus Biologicals Catalog #NB100-132H). Image collected and cropped by CiteAb from the following publication (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0065304) licensed under a CC-BY license. **(Middle)** KO and Biologically Validated Western blot showing lysates of HeLa parental cell line and LC3B KO HeLa cell line untreated (-) or treated (+) with 50 µM Chloroquine. Membrane was probed with 2.0 µg/mL of Rabbit Anti LC3B Monoclonal Antibody (1251A) (Novus Biologicals Catalog #NBP2-46892) followed by HRP-conjugated Secondary Antibody (R&D Systems Catalog #HAF008). A specific band was detected for LC3B at a molecular weight of approximately 15 kDa in the parental HeLa cell line but is not detectable in the KO cell line. Chloroquine treatment also increased LC3B expression in the parental HeLa cell line. GAPDH is shown as a loading control, remaining unchanged. **(Right)** PROTAC® in Simple Western. Simple Western lane view from Wes-generated data showing KD of both CDK9 isoforms using Rabbit Anti-CDK9 Monoclonal Antibody (SD204-07) (Novus Biologicals Catalog # NBP2-67811) after THAL SNS 032 (Tocris Catalog # 6532) treatment of MOLT-4 cells (4 h incubation). GAPDH (R&D Systems Catalog # AF5718) was used as a loading control. Protein quantification (relative to DMSO-only control) is shown beneath the corresponding lane.

PROTAC® is a registered trademark of Arvinas Operations, Inc., and is used under license.

INDEPENDENT VALIDATION

Independent validation involves comparing at least two individual antibodies to the same target protein. Ideally, the different antibodies would also target different epitopes. Western blot results should reveal consistency in molecular weight and subcellular localization across the different antibodies, indicating both suitability of the chosen antibody and specificity to the target protein of interest.



Western blot showing Independent Antibody Validation (IAV) via protein expression profile. Loading with 15 µg of lysates per lane and probed with Rabbit Anti- PD-L1 Polyclonal Antibody (Novus Biologicals Catalog # NBP1-76769) (top), and other PD-L1 Antibody (bottom), and loading control beta-actin, followed by HRP-conjugated secondary antibodies, goat anti-rabbit and/or anti-mouse IgG.

NORMALIZATION

Given the errors introduced from loading samples manually and protein transfer variability, normalization of data is a crucial step for correcting experimental variation and for generating reliable, reproducible results. Normalization of protein expression in western blotting involves mathematically comparing the expression of the protein of interest to a standard within the same lane. Normalization enables the user to accurately compare protein expression across multiple samples and to account for lane-to-lane or sample-to-sample variability. There two common methods used for normalization: 1) housekeeping genes used as loading controls, and 2) total protein level measurements. Performing normalization is one important aspect of obtaining quantitative western blot results.

WHAT IS A LOADING CONTROL?

Despite experimental measures to prevent unequal loading, subsequent protocol steps, including uneven transfer from gel to membrane, can affect the final amount of target protein detected. To verify equal loading of the gel and even transfer from gel to membrane, a loading control is required. A loading control is ubiquitously and constitutively expressed, often referred to as a "housekeeping gene". Depending on the subcellular localization of the experimental target, loading controls can be specific for whole cell or subcellular fractions (e.g. nucleus, mitochondria, membrane).

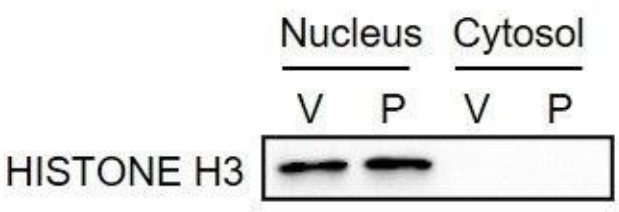
Accurate normalization requires that the protein of interest and loading control protein vary equally with sample concentration. If unequal variation of one protein occurs independent of concentration, then normalization fails to bring target protein expression into proper proportion with the loading control protein. In this case, expression differences between samples can be the result of differences in total protein or loading control abundance and not the result of real differences in target protein expression.

COMMON LOADING CONTROLS

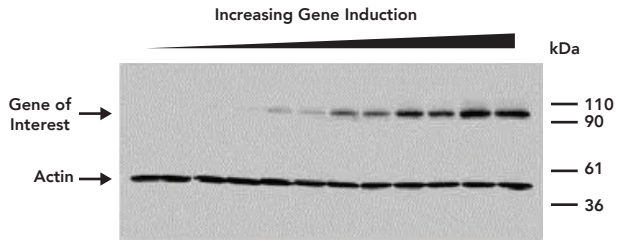
LOCALIZATION	LOADING CONTROLS	MOLECULAR WEIGHT (kDa)
Cytoplasm/ Whole Cell	Vinculin alpha Tubulin beta Tubulin Actin beta-Actin GAPDH Cyclophilin B Cofilin	116 55 55 45 43 37 21 18
Membrane	Sodium Potassium ATPase Alpha 1 beta-Catenin CD44	112 86 82
Mitochondria	HSP60 VDAC1/porin COX4	60 31 17
Nucleus	Lamin B1 HDAC1 PCNA Histone H3	66 60 28 17
Serum	Transferrin	77

WHAT SHOULD I CONSIDER WHEN CHOOSING A LOADING CONTROL FOR MY EXPERIMENT?

- **Detection size:** Choose a loading control that can be distinguished in molecular weight from the target protein of interest
  - If probing for the expression of the autophagosome marker, LC3B (~14-17 kDa), avoid using loading controls such as Cofilin or Cyclophilin B (~20 kDa), since their molecular weight is similar to the protein of interest.
- **Expression level:** Choose a loading control that is highly expressed in the sample. Common loading controls are highly expressed genes required for basic cellular processes.
- **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.
- **Linear Range:** The linear range is the span of signal intensities that result in a linear relationship between the amount of protein and signal detected. For accurate normalization of western blot data, both the protein of interest and loading control protein should fall within the linear range of detection.



Western blot highlighting Histone H3 expresion and subcellular localization to the nucleus. Western blot showing MDA-MB-231 cells were treated with vehicle (V) or paclitaxel (P). Cytosolic and nuclear lysates were prepared and immunoblot assay was performed with Rabbit Anti-Histone H3 Polyclonal Antibody (Novus Biologicals Catalog # NB500-171). WB image submitted by a verified customer review.



Expression consistency of beta-Actin loading control. Western blot showing Mouse Anti-beta-Actin Monoclonal Antibody (AC-15) (Novus Biologicals Catalog # NB600-501) used as a loading control and MCDK cells induced with $C_{22}H_{24}N_2O_8$ to control the expression of the gene of interest. Beta-Actin staining confirms the increase in expression of the gene of interest by showing an equal amount of lysate was loaded in each lane.

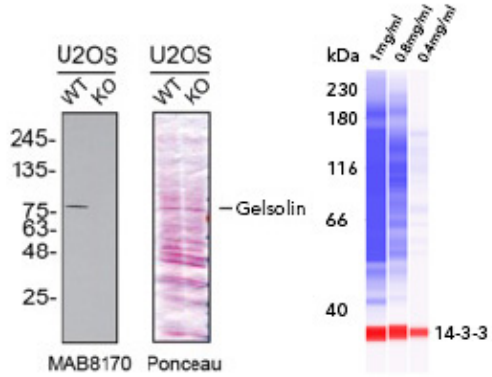
The expression level of the loading control protein changes in response to experimental treatment. If large changes in loading control expression are observed despite loading equal amounts of sample on the gel, then a different loading control target should be considered. This is exemplified in the image shown above where treatments 1, 2, and 3 result in varying levels of expression of the loading control. Changes in loading control expression due to experimental conditions will affect normalization and result in inaccurate data analysis. Choose a loading control protein whose expression remains constant across all experimental conditions.

LOADING CONTROL TIPS AND CONSIDERATIONS

TARGET PROTEIN	NOTE
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.
HSP60	Should not be used as a control in cellular stress studies as cellular stress has been shown to upregulate HSP60 expression.
COX4	Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if the protein of interest is similar in size to COX4.
Transferrin	Transferrin levels can be influenced by some disease states and treatments such as retinoic acid.
PCNA	PCNA is a nuclear loading control that is expressed during DNA S phase. Thus, it is not recommended as a control for non-proliferating cells as its expression is downregulated in non-proliferating cells.
Histone H3	Many proteins run at around 15~17 kDa; hence, it may be necessary to consider an alternative control antibody if the protein of interest is similar in size to Histone H3.

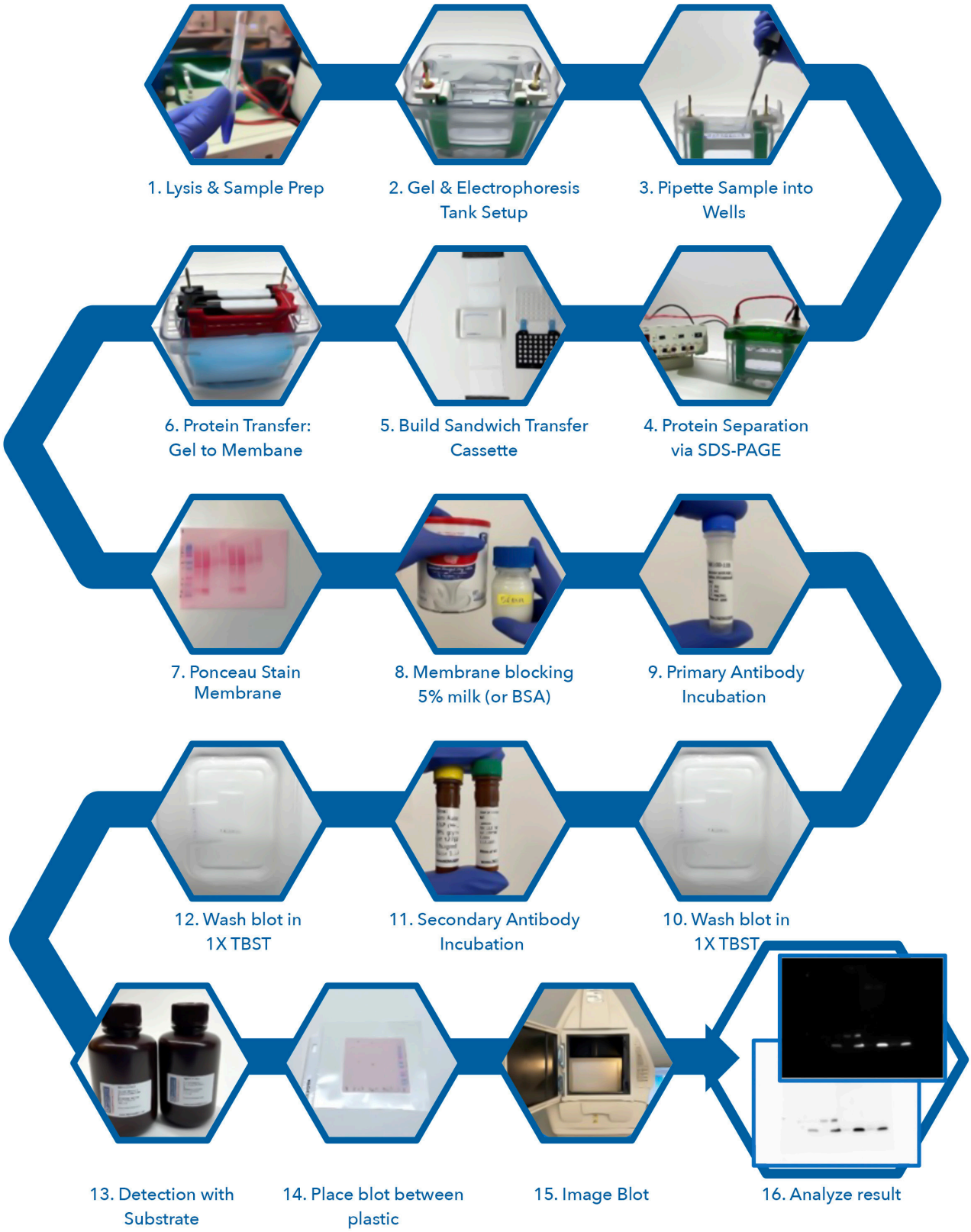
ALTERNATIVES TO LOADING CONTROLS

Total protein quantification, or normalization, assays can be performed as an alternative to loading controls. A quick and reversible membrane stain such as Ponceau S Staining Solution can be applied to visually confirm equal lane loading and successful transfer of proteins to the membrane. Total protein assay is also utilized in Simple Western systems. With instruments like Jess™, total protein normalization assays can be performed at the same time as the immunoassay.



Alternatives to loading controls. (Left) KO Validated Western blot showing lysates from U20S parental cell line and Gelsolin KO cell line at 50 µg each. Membrane was probed with Mouse Anti-Gelsolin Monoclonal Anitbody (893205) (R&D Systems Catalog # MAB8170) at a 1:5000 dilution, followed by HRP-conjugated Secondary Antibody and detected with ECL reagent. A specific band was detected for Gelsolin (as indicated) in the parental U20S cell line but is not in the KO U20S cell line. The corresponding Ponceau stained transfer of the blot is shown to confirm equal protein loading. Data image, protocol, and testing courtesy of YCharOS Inc. **(Right)** Simple Western lane view of protein normalization visualized as the traditional total protein membrane stain overlayed with 14-3-3 target protein.

WORKFLOW: WESTERN BLOTTING



***Note:** Please see page 27 for specific Buffer & Reagent Recipes mentioned throughout Western Blot workflow and protocol

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 into 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 many antibodies recognize linear epitopes of reduced and denatured proteins, some antibodies are specific for conformational epitopes and 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 be used as a starting point to determine the optimal lysis buffer for obtaining high protein purity and yield. Proteins that are found predominantly in a sub-cellular location, such as the nucleus or mitochondria, can be enriched by fractionating these specific compartments. This technique is also beneficial for investigating whether an experimental treatment influences localization of a protein of interest. Since every protein is different, lysis buffer and detergent conditions may require optimization for individual western blotting experiments. Refer to the table below for choosing a lysis buffer and see Buffer & Reagent Recipes section for standard lysis buffer recipes.

RECOMMENDED LYSIS BUFFERS BASED ON CELLULAR LOCATIONS OF PROTEIN OF INTEREST

SUBCELLULAR LOCALIZATION	RECOMMENDED BUFFER
Whole Cell Lysate	NP-40
Nucleus	RIPA
Mitochondria	RIPA
Cytoplasm	Tris-HCL
Membrane-bound Protein	RIPA (SDS is generally considered harsh and thus is often well-suited for difficult to solubilize proteins)

PROTEIN ENRICHMENT

Fractionation is particularly useful for probing weakly expressed proteins. Additionally, immunoprecipitation (IP) is commonly used to enrich for less abundant proteins and determine protein-protein interactions. Immunoprecipitated proteins can then be detected via western blot.

PROTEASE & PHOSPHATASE INHIBITORS

Immediately following cell lysis, proteolysis, dephosphorylation, and denaturation begin to occur. To keep these activities to a minimum, samples should be prepared on ice and freshly made lysis buffers should include protease and phosphatase inhibitors.

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

COMMON & PHOSPHATASE INHIBITORS

INHIBITOR	TARGET	FINAL CONCENTRATION
Aprotinin	Trypsin, chymotrypsin, plasmin	2 µg/mL
Leupeptin	Lysosomal, trypsin, 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
ß-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. By contrast, lysis of adherent cells is typically as simple as scraping cells in lysis buffer. Researchers should choose their lysis protocol based on standard protocols in the field for the given tissue.

DETERMINING LYSATE 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.

SAMPLE PREPARATION FOR GEL LOADING

The linear epitope for antibody binding can be hidden within the 3D conformation of the protein. Thus, it is generally recommended to unfold, or denature, the protein to expose all available epitopes. Denaturing is performed by briefly boiling the sample in a loading buffer containing SDS.

UNDERSTANDING COMPONENTS OF LOADING BUFFERS

The most common loading buffer is Laemmli Buffer, comprised of SDS, Dithiothreitol (DTT) or ß-Mercaptoethanol (ßME), glycerol, and Bromophenol-Blue. Traditionally, it is made as 2X concentrate, but can be made at other concentrations such as 4X or 6X, which may be helpful if loading larger volumes of lysates with low protein concentration.

When **SDS** is added to proteins, they become negatively charged by their attachment to SDS anions. The SDS wrapping around the polypeptide backbone causes some protein denaturation. The negative charged conferred by SDS to polypeptide chains is proportional to their length. Because of this, proteins are separated by SDS-PAGE according to their molecular weight. Both the loading buffer and the gel running buffer contain SDS to maintain the SDS polypeptide complex.

DTT and **ßME** function as reducing agents. While SDS serves to unfold proteins, DTT and ßME further remove tertiary and quaternary structure by reducing disulfide bonds. DTT or ßME is only excluded from the loading 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 to increase sample density so, when samples are loaded into the gel, they sink to the bottom of the well. This minimizes sample overflow and promotes even protein loading between wells.

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

LOADING AND RUNNING BUFFER CONDITIONS

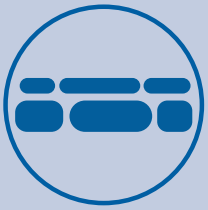
PROTEIN STATE	SAMPLE LOADING BUFFER	GEL RUNNING BUFFER
Reduced and Denatured (most common)	SDS + ßME or DTT	2 µg/mL
Reduced and Native	(No SDS) + ßME or DTT	1-10 µg/mL
Oxidized and Denatured	SDS (No ßME or DTT)	1 µg/mL
Oxidized and Native	(No SDS and No ßME or DTT)	1 mM

Note: Reducing/denaturing conditions are recommended unless the antibody datasheet indicates otherwise. Some proteins are more effectively reduced with DTT than ßME, so a change in reducing agent might be required for certain targets. Some antibodies recognize conformational epitopes which are only present when a protein is in its native, non-denatured state. In this case, denaturing the protein would prevent antibody recognition. Non-denaturing conditions can be achieved by leaving SDS out of the loading and running buffers and skipping the sample boiling step. Furthermore, certain epitopes are only present when a protein is in a non-reduced, or oxidized form. In this case, the reducing-agents, such as ßME or DTT, should not be included in the buffers. Refer to the table above for loading buffer and gel running buffer guidelines.

Note: 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.

TROUBLESHOOTING TIPS

VISUAL RESULT:



Wrong Band Size
or Multiple Bands

POSSIBLE REASON & SOLUTIONS:

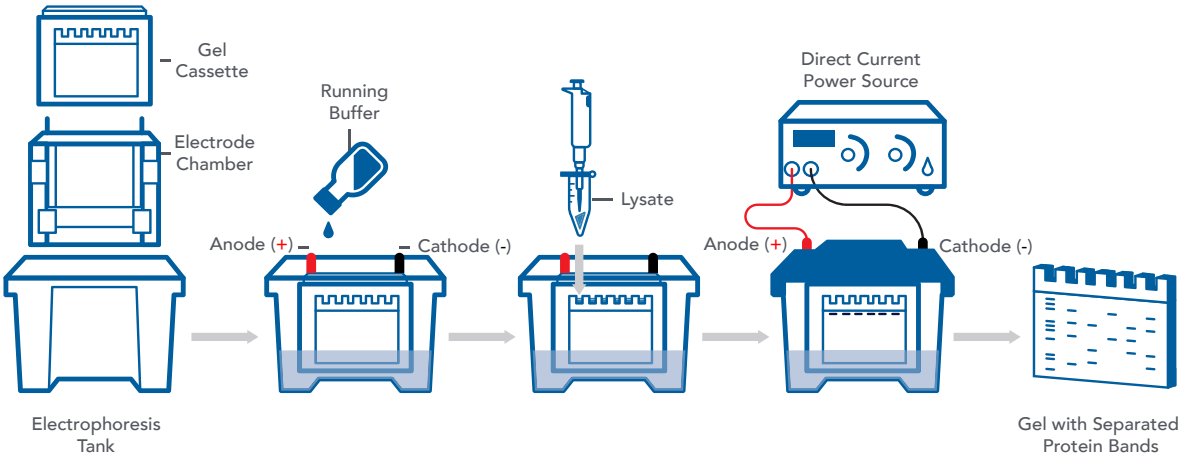
SAMPLE DEGRADATION:

- Use fresh lysates.
- Keep sample on ice until just before sample buffers addition and boiling.
- Always include protease inhibitors and phosphatase inhibitor in loading buffer.

LOADING & RUNNING 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, in turn, proteins can be separated by size. Running buffer with conducting properties is added to the electrophoresis apparatus and when the voltage from the power source is applied, the running buffer heats up the gel, driving protein separation and migration. Electrophoresis time and voltage can vary, but a suggested recommendation is to run the gel at 100V for approximately 1-2 hours, or until the dye has run to the bottom of the gel. 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.

Note: If the protein of interest has multiple isoforms ranging from low to high molecular weight sizes or if probing 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.



Schematic of gel electrophoresis steps. The electrophoresis apparatus is set up by placing the gel cassette into the electrode chamber and then into the electrophoresis tank. Running buffer is added to the apparatus and sample lysates are added to the wells. The lid is securely attached to the tank and connected to the power source at a set voltage. After gel has run for the appropriate amount of time, the cassette is disassembled, and the gel is removed. The next step after gel electrophoresis is protein transfer from the gel to a membrane.

TROUBLESHOOTING TIPS

VISUAL RESULT:

Smeared Bands/Lanes

POSSIBLE REASON & SOLUTIONS:

SAMPLE OVERLOADING

- Use less total protein loaded into each lane.
  - o Perform an immunoprecipitation first to enrich the target in the lysate. Always include protease inhibitors and phosphatase inhibitor in loading buffer.

Smiling Bands

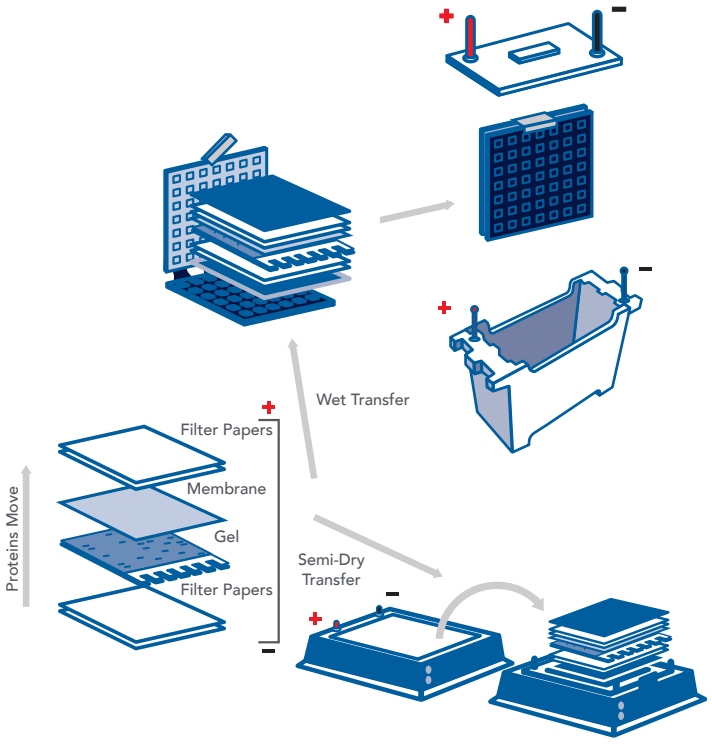
SDS-PAGE GEL ELECTROPHORESIS MIGRATION

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

PROTEIN TRANSFER: 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 polyvinylidene difluoride (PVDF) or nitrocellulose. PVDF and nitrocellulose membranes have distinct properties making each one suitable under specific experimental conditions. Once the desired membrane is selected, the transfer sandwich is assembled, and proteins are transferred to the membrane via electrical current through either wet or semi-dry transfer.

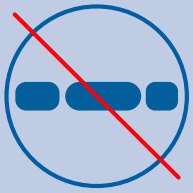
	PVDF	NITROCELLULOSE
Physical Properties:	Durable	Brittle and fragile
Protein Binding Capacity:	150 - 160 µg/cm2	80 - 100 µg/cm2
Membrane Activation:	Methanol (followed by rinse in distilled water and soak in transfer buffer)	Methanol-FREE transfer buffer
Binding Interactions:	Hydrophobic and Dipole	Hydrophobic
Pore Sizes Available:	0.1, 0.2, and 0.45 µm	0.1, 0.2, and 0.45 µm
Additional Information:	Better for low molecular weight proteins- for proteins less than 30 kDa, 0.2 µm PVDF is recommended over 0.45 µm.	-



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.

TROUBLESHOOTING TIPS

VISUAL RESULT:



No Bands/Signal

POSSIBLE REASON & SOLUTIONS:

PROTEIN TRANSFER FROM GEL TO MEMBRANE WAS UNSUCCESSFUL

- Confirm that proteins were successfully transferred by Ponceau S staining of the membrane.
- Confirm the quality transfer of proteins by staining gel with Coomassie Blue.
- Confirm equal transfer of proteins to the membrane by analyzing loading control expression.
- Assure that membrane was correctly activated.
  - PVDF should be wet in methanol prior to soaking briefly in distilled water followed by 1X transfer buffer.
  - Nitrocellulose membrane should be wet in methanol-free transfer buffer.

IMMUNOBLOTTING

MEMBRANE BLOCKING

The first step in immunoblotting is to rinse and block the membrane with non-specific proteins, 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 the antibody from binding non-specifically to the membrane and contributing to 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 number of phosphorylated proteins and can result in non-specific binding of the primary antibody to milk proteins, resulting in high background or no signal at all.

ANTIBODY BINDING

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

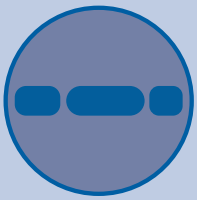
Note: While the primary antibody dilution can be reused (no more that 3-4 times), it is best to use fresh antibody dilutions every time as the signal can become reduced and the re-used solution is more prone to contamination.

Secondary antibody: After washing to remove unbound primary antibody from the membrane, the secondary antibody is added. The 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). In addition to enzymatic conjugation, fluorescently labeled secondary antibodies have become an increasingly popular method for western blot detection.

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 wash steps. Bio-Techne offers a large selection of primary antibodies conjugated to HRP.

TROUBLESHOOTING TIPS

VISUAL RESULT:



High Uniform Background

POSSIBLE REASON & SOLUTIONS:

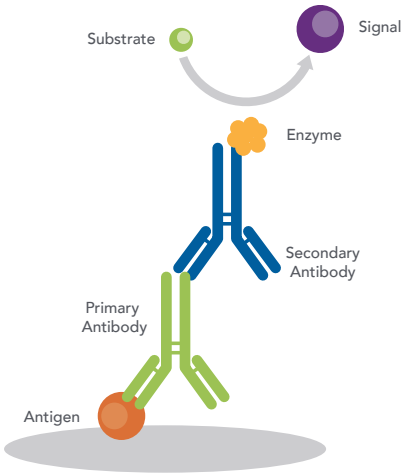
NON-SPECIFIC BINDING DUE TO HIGH ANTIBODY CONCENTRATION

- Lower concentration of primary or secondary antibody (titrations may be helpful).
- Use a stronger blocking agent in antibody diluents.
  - Milk is typically a stronger blocking agent than BSA.
- Confirm that the background is not due to issues with the secondary antibody by omitting the primary antibody.

DETECTION & IMAGING

In western blotting, the method of detection is dependent on the label of the secondary antibody or, in using direct detection, the label of the primary antibody. Chemiluminescence relies on an enzyme-substrate reaction and is historically the most common detection method in western blotting, compared to fluorescence or colorimetric detection.

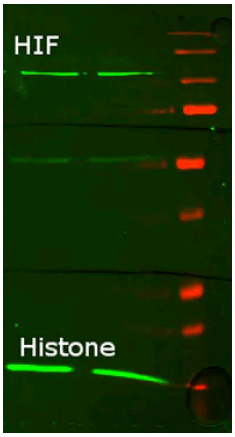
The method of detection will determine the suitable imaging method. When choosing the optimal detection and imaging method, it is important to consider the experimental dynamic range. The **dynamic range** refers to the span of signal, or band, intensities that can be measured by the detection system in a single scan of the blot. A wide dynamic range means that the system is highly sensitive and able to capture both weak and strong protein expression without signal saturation. High antibody sensitivity along with a broad dynamic range improves quantitative western blot analysis.



Schematic depicting an enzyme-substrate reaction. The addition of substrate results in a reaction with the enzyme conjugated to the secondary antibody, resulting in a detectable signal, such as light emission or colored precipitation.

CHEMILUMINESCENCE	FLUORESCENCE	CHROMOGENIC/COLORIMETRIC
Relies on an enzyme-conjugated antibody and the addition of a substrate which results in emission of a light signal.	Utilizes a fluorophore directly conjugated to an antibody that emits a detectable signal.	Relies on an enzyme-conjugated antibody reaction and addition of a substrate that results in colored precipitation on the blot.
Advantages: • High Sensitivity **(high femtogram range)** • Quick detection • Compatible with X-Ray film or digital imaging	Advantages: • Multiplexing more than one antibody in a single assay • Greater linear dynamic range than chemiluminescence • Signal stability **(signal length: weeks to months)** • Best for digital imaging	Advantages: • Cost-effective and fast • Colored precipitate stable for long periods of time **(signal length: months)** • No special instruments required
Disadvantages: • Multiplexing requires stripping and reprobing the blot • Signal stability and timing– the blot must be imaged immediately following substrate addition **(signal length: 6 – 24 hours)**	Disadvantages: • Less sensitive than chemiluminescence **(picogram range)** • Need special imaging equipment	Disadvantages: • Lack of sensitivity **(nanogram range)** • Hard to quantify

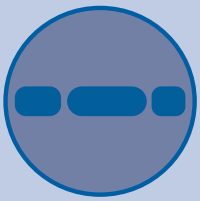
Following chemiluminescence or fluorescence detection, the blot is then imaged to visually identify the proteins of interest. X-Ray film is the traditional imaging method for capturing and documenting chemiluminescent western blot signal. While film is useful for detecting the presence or absence of a protein, illustrating a large signal difference, it is semi-quantitative and limited by a narrow dynamic range. Strong signals on film can more easily become saturated, resulting in underestimating protein expression. Advances in digital imaging systems with charge-coupled device (CCD) cameras offer a broader dynamic range and quantitative imaging analysis. Digital imaging systems tend to capture both stronger and weaker signals compared to x-ray film. Additionally, digital imaging is compatible with multiplex detection with fluorescent westerns.



Example of multiplexing in a fluorescent western. The blot shows simultaneous probing for two different antibodies, Rabbit Anti-HIF-1 alpha Polyclonal Antibody (Novus Biologicals Catalog # NB100-449) and the loading control Histone, followed by fluorescent dye-conjugated secondary antibody. The protein standard is in the far right lane. Scanned using LI-COR Odyssey system. Image provided by verified customer review from Dr. Lidan Zhao.

TROUBLESHOOTING TIPS

VISUAL RESULT:



High Uniform Background

POSSIBLE REASON & SOLUTIONS:

FILM EXPOSURE

- Lower the exposure time of the film or wait ~15 minutes before exposing.

ECL INCUBATION

- Lower the incubation time to 1-3 minutes.

ANALYSIS

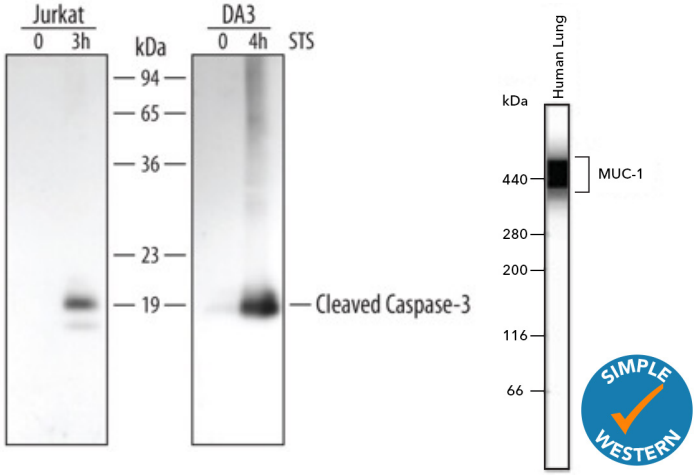
Accurate analysis of western blot data and validating results requires 1) the use of molecular weight ladders to confirm protein size, 2) determining the linear range of detection for the target proteins and loading control, and 3) data normalization.

PROTEIN MOLECULAR WEIGHT

Molecular weight markers, or ladders, consist of a mixture of proteins of known molecular weights that should be run along with samples in every gel. During analysis, the target band is compared to the molecular weight marker standard to estimate the size of the protein of interest. Molecular weight markers are available in a variety of formats, including pre-stained and unstained, and can vary in the number of bands (5 to 10) and molecular weight range (~1 kDa to 250 kDa). In addition to assisting with protein size estimation, pre-stained ladders allow for visualization of protein migration and separation during electrophoresis, as well as protein transfer from gel to membrane.

At times, the predicted molecular weight of a protein may not match the observed size on a western blot. The appearance of a molecular weight band larger than the calculated value can be indicative of a post-translational modification (PTMs) such as glycosylation, phosphorylation, or ubiquitination. A single phosphoryl group only has a molecular weight of ~ 80 Da so a noticeable shift in size in a western blot may mean the protein is phosphorylated at multiple sites. While an increase in molecular weight is suggestive of a PTM, specific antibodies to detect PTMs, like phospho-specific antibodies, are required to confirm the type of modification.

The appearance of smaller than expected molecular weight bands can be a result of protein cleavage or alternative splicing. In the case of a cleaved protein such as caspase 3, the primary antibody may specifically recognize the cleaved product or both the unprocessed and cleaved forms, dependent on the site of the epitope. With alternative splicing, multiple protein isoforms result from a single gene, either with similar or diverse functions.

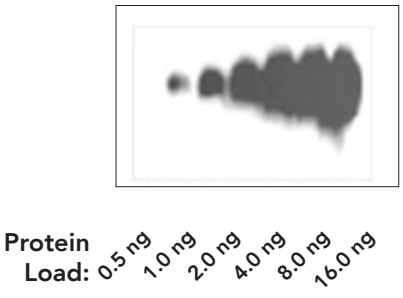


Examples of protein cleavage and post-translational modification in western blot. (Left). Western blot shows lysates of Jurkat human acute T cell leukemia cell line and DA3 mouse myeloma cell line untreated (-) or treated (+) with 1 µM staurosporine (STS). PVDF membrane was probed with Rabbit Anti-Cleaved Caspase 3 Monoclonal Antibody (Asp175) (R&D System Catalog # MAB835), followed by HRP-conjugated Anti-Rabbit IgG Secondary Antibody (R&D Systems Catalog # HAF008). A specific band was detected for Cleaved Caspase 3 (Asp175) at approximately 18 kDa (as indicated). **(Right)** Simple Western lane view shows lysates of human lung tissue. Specific bands were detected for MUC-1 at approximately 300-500 kDa (as indicated) using Mouse Anti-Human MUC-1 Monoclonal Antibody (604804) (R&D Systems Catalog # MAB6298). These high molecular weight bands are suggestive of glycosylation as MUC-1's core protein theoretical molecular weight is only 122 kDa, but increases following glycosylation. This experiment was conducted under reducing conditions using the 66-440 kDa separation system.

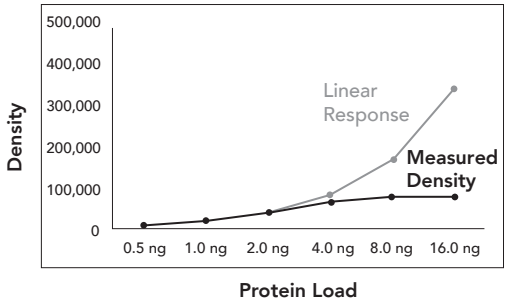
DETERMINING LINEAR RANGE

Because many target proteins are less abundant relative to loading control proteins, a large amount of sample is often loaded on the gel to detect the target protein. However, loading high amounts of total protein often places the loading control outside of the linear range of detection. The optimal amount of total protein to load on the gel should be determined to ensure the accurate quantification of both the target and loading control proteins.

To determine the linear range of the samples, a standard curve is created using serial dilutions. This can be accomplished by running duplicate samples (e.g. ABCD, ABCD) and cutting the blot vertically to separate the samples. One blot is then used to determine the linear range of the loading control, while the other is used for the target protein. The standard curve can then be used to identify the saturation point of the assay and the total amount of starting protein required.



Amt of Sample Loaded	Measured Density	Linear Response
0.5 ng	10811	10811
1.0 ng	26158	21622
2.0 ng	48585	43244
4.0 ng	71737	86488
8.0 ng	79795	172976
16.0 ng	75830	345952



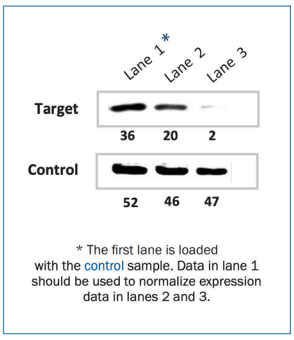
Comparison of the densitometry readings of each sample to the linear response indicates the signal becomes saturated above 4.0 ng. Above 4.0 ng, the band intensity is no longer proportional to total protein abundance: the signal falls outside the linear range of detection. These data determine the optimal total protein load to be 4.0 ng or less for accurate quantification of protein expression.

HOW TO NORMALIZE WESTERN BLOT DATA

Data normalization is required in western blot analysis to accurately compare target protein expression across multiple samples. First, the band intensity of each sample is determined by densitometry. Next, the intensity of the target protein is divided by the intensity of the loading control protein. This calculation adjusts the expression of the protein of interest to a common scale and reduces the impact of sample-to-sample variation. Relative target protein expression can then be compared for all lanes to assess changes in target protein expression across samples.

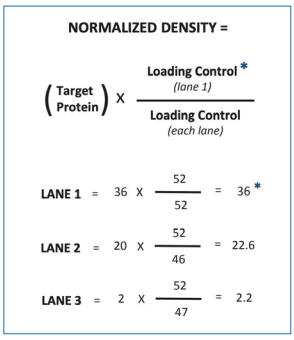
Step 1

Determine the intensity of each band using densitometry software



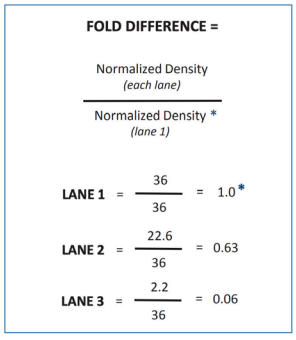
Step 2

Normalize the target protein levels to the loading control protein



Step 3

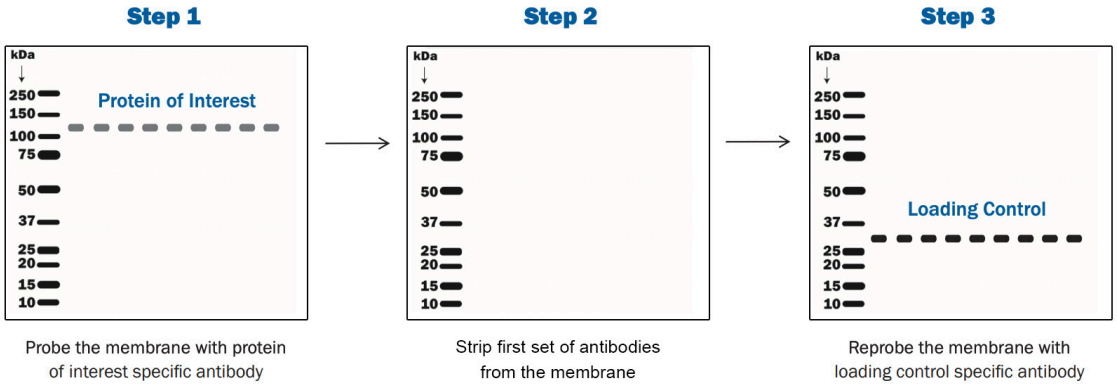
Compare relative target protein levels across samples



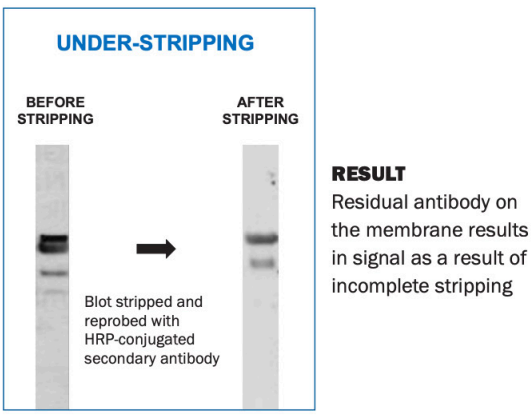
After band intensity is determined by densitometry software, the loading control protein is used to normalize target protein expression. To normalize expression, multiply the density of the target protein in each lane by the ratio of loading control density from the control sample (lane 1) to the loading control density of other lanes. The fold change can then be calculated by dividing the normalized expression from each lane by the normalized expression of the control sample in lane 1.

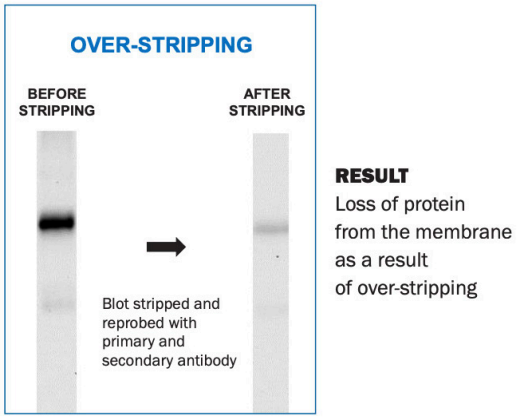
STRIPPING & REPROBING

A single blot can be analyzed sequentially with multiple antibodies by stripping one antibody from the blot and subsequently incubating with an antibody for a different target protein. This practice may be useful to obtain more information about protein abundance from a single sample or when sample is limited. Best practice is to probe for low-abundance proteins first to reduce the likelihood of losing antigen during the stripping process. This includes probing for the protein of interest first, prior to the often more abundant loading control. When using this method, it is important that the first antibody is fully stripped from the membrane before reprobing. Residual antibody on the blot may result in artifacts, impacting interpretation of expression data for the second target. If reprobing for the loading control, a significant loss of sample protein from the membrane could impact expression data and normalization calculations. For this reason, quantitative comparisons of protein expression before and after membrane stripping are not recommended.



After stripping, before reprobing, the membrane should be checked to verify the first set of antibodies was completely removed. To confirm complete antibody removal, the membrane should be washed, blocked, and then stained with a secondary antibody. If stripping was complete, then the secondary antibody will remain unbound and produce no detectable signal. If the signal is still present, then the stripping conditions must be optimized. Often small changes in incubation time or temperature are sufficient to remove the remaining antibody. However, a stronger stripping buffer may be necessary in certain cases for antibodies which are difficult to remove.





Under-Stripping: A primary antibody was used to probe expression of a target protein. Following target protein detection, the blot was stripped to remove antibodies, and then reprobed with HRP-conjugated secondary antibody to confirm complete stripping. The signal generated post-stripping demonstrates the stripping process was incomplete. The HRP-conjugated secondary antibody bound to residual primary antibody left on the membrane, resulting in a chemiluminescent signal.

Over-Stripping: A primary antibody was used to probe expression of a target protein. Following target protein detection, the blot was stripped to remove antibodies, and then reprobed with the same target protein-specific antibody. The lower signal generated post-stripping demonstrates protein sample was stripped from the membrane. Unequal stripping of protein sample across lanes can result in inaccurate normalization. Special care should be taken when stripping blots containing multiple sample types.

Two methods for stripping and reprobing the membrane are outlined below. The first method uses low pH to inactivate the antigen binding site of the antibody and the second uses heat and detergent to release antibodies from the membrane. As a general rule, it is recommended to begin with a milder stripping solution (Stripping with acidic pH, stripping solution #1). If stripping is incomplete and antibody is detected, then a harsher stripping protocol (Stripping with heat and detergent, stripping solution #2) can be used. For fluorescent western blots, harsher stripping conditions may be required to adequately remove the initial signal. While many labs choose to make their own stripping buffers, pre-made stripping solutions are available to specifically work with the membrane type and detection method used. Given the higher binding capacity and sturdiness, PVDF membranes are recommended over nitrocellulose for stripping and reprobing purposes.

PROTOCOL: STRIPPING WITH ACID PH

1. Agitate the blot in stripping solution #1 for 30 minutes at room temperature.
2. Agitate the blot in 1X PBS for 10 minutes at room temperature. Repeat with fresh buffer.
3. Proceed to the blocking step of the immunoblotting protocol to reprobe the blot with a second antibody.

PROTOCOL: STRIPPING WITH HEAT AND DETERGENT

1. In a fume hood, agitate the blot in stripping solution #2 for 30 minutes at 50°C.
2. Agitate the blot in 1X PBS for 10 minutes at room temperature. Repeat with fresh buffer.
3. Proceed to the blocking step of the immunoblotting protocol to reprobe 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

1. Sandwich the dry PVDF blot between two clean sheets of Whatman 3MM paper. Agitate the blot in 1X PBS for 10 minutes at room temperature. Repeat with fresh buffer.
2. Place the sandwich between two sheets of card stock or thin cardboard.
3. Use paperclips to clip the stack together on the edges.
4. Place the stack in a plastic bag and seal the plastic bag closed.
5. 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.

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

Note: 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.

PROTOCOL: BENCHTOP WESTERN BLOTTING

MATERIALS

- Electrophoresis Apparatus (Tank, Chamber, Gel Cassette)
- Polyacrylamide Gel
- Power Source
- PVDF Membrane (or Nitrocellulose)
- Filter Paper
- Sponges
- Transfer Cassette
- Transfer Apparatus

REAGENTS

- 1X PBS
- Lysis Buffer (e.g. RIPA, NP-40, Tris-HCL)
- Loading Buffer (2X Laemmli Buffer)
- Molecular Weight Marker
- 1X Running Buffer
- Methanol
- 1X Transfer Buffer (wet)
- 1X TBST
- Blocking Solution
- Primary Antibody
- Primary Antibody Solution
- Secondary Antibody
- Enhanced Chemiluminescence (ECL) Reagent (e.g. NovaLume Pico Chemiluminescent Substrate (HRP), NBP2-61915)
- Optional: Blue Marker Antibody (NBP2-33376), Ponceau S, Coomassie Blue

METHODS

CELL LYSATE PREPARATION FROM ADHERENT CELLS

1. Wash cell culture dish on ice with ice-cold PBS. 1X Transfer Buffer (wet).
2. Aspirate PBS and add ice-cold lysis buffer (1 mL per confluent 10^7 cells/10cm dish/150 cm^2 flask). See the table below for lysis buffer recommendations based on the subcellular location of the protein of interest.
3. Using a cell scraper, scrape adherent cells off the dish and transfer the cell suspension into a microcentrifuge tube. If required, the cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer.
4. Agitate cells for 30 minutes at 4˚C.
5. Centrifuge cell lysate mixture at 4˚C.

   Note: The time and centrifugation force vary for each cell type, but a general guideline is 20 minutes at 12,000 rpm.
6. Transfer the supernatant (lysate) to a fresh tube on ice.

SUBCELLULAR LOCALIZATION	RECOMMENDED BUFFER
Whole Cell Lysate	NP-40
Nucleus	RIPA
Mitochondria	RIPA
Cytoplasm	Tris-HCL
Membrane-bound Protein	RIPA

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 the samples by boiling the lysates in Laemmli Buffer at 95-100˚C for 5 minutes.

   Note: This step should only be skipped if the antibody datasheet recommends non-reducing or non-denaturing conditions.

SDS-PAGE GEL ELECTROPHORESIS

1. Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve the 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%

2. Load samples containing equal amounts of protein (10-50 µg/lane from cell lysate or 10-100 ng/lane purified protein) 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.

   Note: 1-2 hours at 100 V is standard, but time and voltage may require optimization.

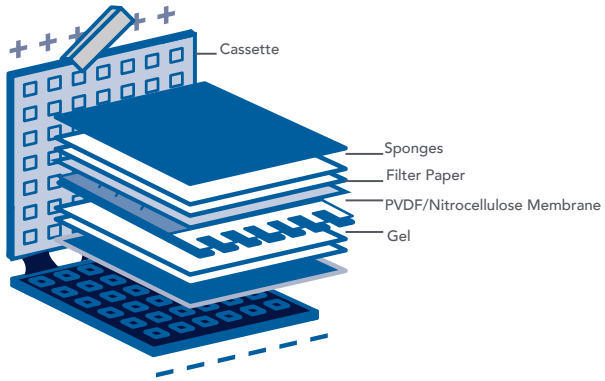
PROTEIN TRANSFER

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.

   Note: PVDF membrane must be wet in methanol but can use methanol-free transfer buffer. DO NOT wet nitrocellulose membranes with methanol or the membrane will dissolve.

   Note: PVDF membrane has a higher binding capacity, best for low expressed proteins, but nitrocellulose membrane is ideal for lower molecular weight proteins.

2. Soak filter papers and sponges in 1X 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 1X Transfer Buffer for 10 minutes.
4. Prepare the transfer 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.



5. Place the sandwich into a transfer cassette and perform semi-dry or wet transfer according to the manufacturer's instructions of the transfer apparatus.
   - **Semi-dry transfer:** generally faster, better suited for larger proteins >100 kDa. Commonly used transfer time: 1 hour at a constant current (1.25 mA/cm2).
   - **Wet transfer:** recommended for smaller proteins, especially proteins <30 kDa. Commonly used transfer time: 1 hour at 100V at 4°C.

   Note: 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

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

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

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 S (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 S with several washes in 1X TBST until membrane is clear. Additionally, Coomassie Blue staining of the gel after transfer can help determine the quality of the transfer from 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, with constant rocking.

6. To make the Primary Antibody Solution, dilute the primary antibody to working concentration in 1X TBST with 1% milk or BSA (remain consistent with Blocking Solution).

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

7. **Optional step:** To visualize the molecular weight markers, add 1 µg/mL Blue Marker Antibody to the Primary Antibody Solution. This antibody does not cross react with protein lysates and will bind specifically to the blue dye of each molecular weight marker.

8. Incubate the membrane in Primary Antibody Solution for 1 hour at room temperature or overnight at 4˚C with gentle rocking.

   Note: This time may require optimization. In most cases, overnight incubation at 4˚C increases signal strength and reduces background signal relative to 1 hour incubation at room temperature.

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

10. 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.

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

DETECTION (CHEMILUMINESCENCE)

1. Prepare the ECL Reagent just prior to use according to the manufacturer's instructions.

2. Incubate the membrane in the ECL Reagent according to manufacturer's directions. Typical incubation times are 1-5 minutes.

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

3. Carefully remove the membrane from the ECL Reagent and sandwich it between layers of plastic (i.e. a sheet protector or plastic wrap) and ensure no air bubbles form between membrane and plastic.

4. Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system, such as a ChemiDoc.

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

   Note: 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.

   Note: Membrane blot may be stripped and reprobed as needed, according to protocol.

BUFFER & REAGENT RECIPES

LYSIS BUFFERS

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

PBS

1X PBS

- 137 mM NaCl
- 2.7 mM KCl
- 4.3 mM Na_2HPO_4
- 1.47 mM KH_2PO_4
- Adjust pH to 7.4

LOADING BUFFER

2X LAEMELLI BUFFER

- 4% SDS
- 10% ß-Mercaptoethanol OR 100 mM DTT
- 20% Glycerol
- 0.01% Bromophenol Blue
- 0.0125 M Tris-HCl
- pH 6.8

RUNNING BUFFER

1X RUNNING BUFFFER

- 25 mM Tris base
- 192 mM glycine
- 0.1% SDS
- Adjust to pH 8.3

TRANSFER BUFFERS

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 to pH to 8.3

TBST

1X TBST

- 20 mM Tris base
- 150 mM NaCl
- 0.1% Tween 20

BLOCKING SOLUTION

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

STRIPPING BUFFERS

STRIPPING SOLUTION #1

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

STRIPPING SOLUTION #2

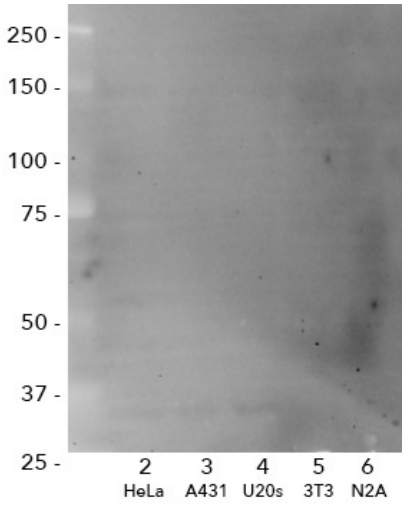
- 100 mM ß-mercaptoethanol
- 2% SDS
- 62.5 mM Tris-HCl
- Adjust pH to 6.7

TROUBLESHOOTING GUIDE

NO SIGNAL OR WEAK SIGNAL

TARGET PROTEIN CONCENTRATION IS TOO LOW

- Load more protein per well (titrations might be helpful).
- Use a positive 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) to increase the concentration of a non-abundant protein.
- Include protease inhibitors in the lysis buffer.
- Ensure the sample has not degraded.



Example of no/weak signal. Blot was probed with primary antibody at 0.5µg/mL, followed by HRP-conjugated Secondary Antibody. Target had a theoretical molecular weight of ~219 kDa. No signal or band was observed on the blot after ECL substrate addition.

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.

PROTEIN TRANSFER FROM GEL TO MEMBRANE WAS SUBOPTIMAL

- Confirm that proteins were successfully transferred by Ponceau S staining of the membrane.
- Confirm that proteins were sufficiently transferred by staining gel with Coomassie Blue.
- Confirm equal transfer of protein to the membrane by analyzing loading control expression.

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.
- Consider the binding capacity of the membrane.
  - PVDF membrane has a higher binding capacity than nitrocellulose.

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.

PRIMARY ANTIBODY CONCENTRATION IS TOO LOW

- Increase the concentration of the primary antibody (titrations may be helpful).
- 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.

ANTIBODY ONLY RECOGNIZES NATIVE PROTEINS

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

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).

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.

EXCESSIVE WASHING OF MEMBRANE

- Reduce time or number of washes.

DETECTION REAGENTS HAVE GONE BAD

- Detection reagents (e.g., ECL) 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).

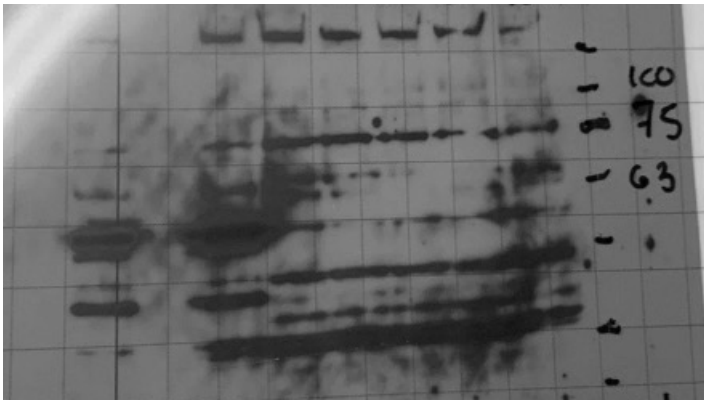
IMAGE EXPOSURE WAS TOO SHORT

- Increase exposure time (may be necessary to test a range of exposure times).

HIGH UNIFORM BACKGROUND

INSUFFICIENT BLOCKING

- Increase blocking time and/or temperature.
- Increase the concentration of blocking reagent.
    - Up to 10% blocking buffer can be used.
- Consider changing the blocking agent.
    - Milk is a stronger blocking reagent than BSA.
- Include the optional blocking agents in antibody buffers.



Example of high background and smeared bands. Likely occurred from insufficient blocking or insufficient washing.

BLOCKING NOT COMPATIBLE WITH TARGET

- Milk should not be used for phosphorylated targets.
    - Proteins in milk (caseins) are phospho-rich.
- If the 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 antibody (titrations may be helpful).
- Include blocking agents in antibody diluents.
    - Milk is typically a stronger blocking agent than BSA.
- Confirm that the background is not due to issues with the secondary antibody by omitting the primary antibody and performing a control experiment.

INSUFFICIENT WASHING OF UNBOUND ANTIBODIES

- Increase the number and/or duration of washes.

DRY MEMBRANE

- Make sure the membrane never becomes dry during the western blotting procedure.

DETECTION REAGENTS ARE TOO SENSITIVE

- Dilute the detection reagent in pure water or use a less sensitive detection reagent.

FILM EXPOSURE IS TOO LONG

- Lower the exposure time (may be necessary to test a range of exposure times).

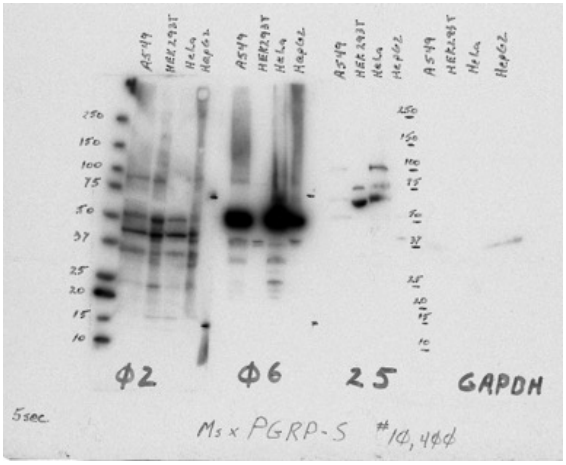
NON-SPECIFIC BANDS/WRONG SIZE OR MULTIPLE BANDS

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 Loading Buffer addition and boiling.
- Always include protease inhibitors and phosphatase inhibitors in loading buffer.



Example of non-specific bands and poor transfer. Target Protein expected at ~25 kDa is not found. Other bands shown are non-specific.

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 KD/KO lysate.

SPECKLED OR SWIRLED BACKGROUND

MEMBRANE MISHANDLING

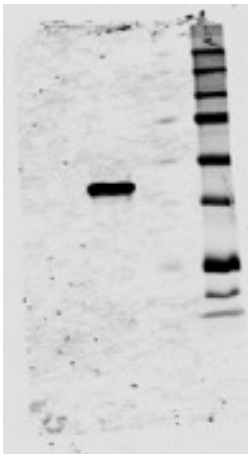
- Minimize contact with membrane. Use clean tools to handle the membrane.

BUFFER CONTAMINATION

- Use fresh buffers.

HRP AGGREGATION

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



Example of either bacterial contamination or undissolved blocking buffer. Speckled background as a result.

INSUFFICIENT WASHING

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

AIR BUBBLES

- Roll out any bubbles between the gel and membrane before transfer.
- Ensure no bubbles between plastic and membrane before image development.

OTHER ISSUES

WHITE/HOLLOW BANDS

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

SMEARED BANDS/LANES (SAMPLE OVERLOADING)

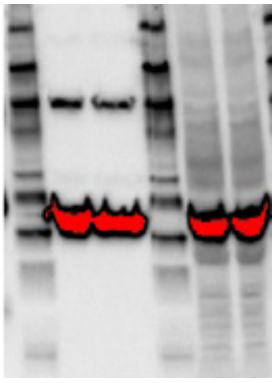
- Load less protein into each lane.

"SMILING" BANDS

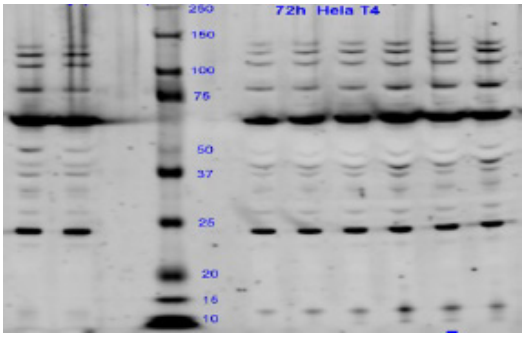
- Migration was too fast; decrease the voltage while running the gel.
- Migration was too hot; run the gel in the cold room.

MOLECULAR WEIGHT MARKER LANE IS BLACK

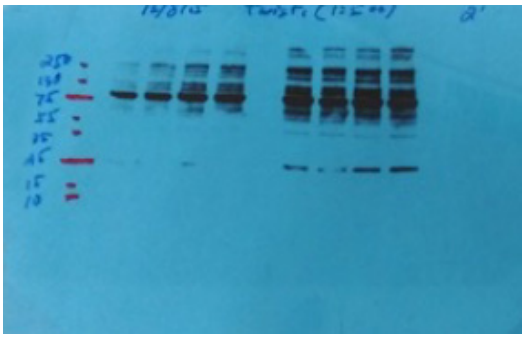
- The antibody may react with the molecular weight marker.
- Add a blank lane between the molecular weight marker and the first sample lane.



Example of overloaded protein sample. Can occasionally observe a white region inside the overloaded protein band, referred to as a ghost band.



Example of gel run too hot or too high voltage. Results in smiling or misshapen bands. This phenomenon can also occur with poor gel preparation and polymerization.



Example of wrong percentage gel or gel not run far enough. Poor band resolution and thus can't distinguish individual bands.

REFERENCES

1. Adamski J, Price A, Dive C, Makin G. (2013) Hypoxia-induced cytotoxic drug resistance in osteosarcoma is independent of HIF-1Alpha. PLoS One. 8(6):e65304. PMID: 23785417

2. Alegria-Schaffer A, Lodge A, Vattem K. (2009) Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods Enzymol. 463:573-99. PMID: 19892193

3. Bass JJ, Wilkinson DJ, Rankin D, Phillips BE, Szewczyk NJ, Smith K, Atherton PJ. (2017) An overview of technical considerations for Western blotting applications to physiological research. Scand J Med Sci Sports. 27(1), 4–25. PMID: 27263489

4. Burnette WN. (1981) "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. 112(2), 195–203. PMID: 6266278.

5. Burnette WN. (2009) Western blotting: remembrance of past things. Methods Mol Biol. 536, 5–8. PMID: 19378039

6. Ghosh R, Gilda JE, Gomes AV. (2014) The necessity of and strategies for improving confidence in the accuracy of western blots. Expert Rev Proteomics. 11(5), 549–60. PMID: 25059473

7. Lee SY (2018) Current knowledge for the western blot normalization. Medical Oncology. 3, 3-6. Retrieved from alliedacademies.org

8. MacPhee DJ (2010) Methodological considerations for improving Western blot analysis. J Pharmacol Toxicol Methods. 61(2), 171–7. PMID: 20006725

9. Mahmood T, Yang PC. (2012) Western blot: technique, theory, and trouble shooting. N Am J Med Sci. 4(9):429-34. PMID: 23050259

10. McDonough AA, Veiras LC, Minas JN, Ralph DL. (2015) Considerations when quantitating protein abundance by immunoblot. Am J Physiol Cell Physiol. 308(6), C426–33. PMID: 25540176

11. Moritz CP. (2017) Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots. Proteomics. 17(20). PMID: 28941183

12. Pillai-Kastoori L, Schutz-Geschwender AR, Harford JA. (2020) A systematic approach to quantitative Western blot analysis. Anal Biochem. 593, 113608. PMID: 32007473

13. Sun X, Gao H, Yang, Y, He M, Wu Y, Song Y, Tong Y, Rao Y. (2019) PROTACs: great opportunities for academia and industry. Signal Transduct Target Ther. 4, 64. PMID: 31885879

14. Taylor SC, Posch A. (2014) The design of a quantitative western blot experiment. Biomed Res Int. 2014:361590. PMID: 24738055

15. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T. (2016) A proposal for validation of antibodies. Nat Methods. 13(10), 823–7. PMID: 27595404

ACKNOWLEDGEMENTS

Author: Jennifer Jones

Other Contributors:

Emily Cartwright, Janine Mills, Ela Stolarczyk, Scott Baker, Jeff Cooper, and Callie Sagarsky

Torry Knodell, Amy Zimmerman, Anya Dillahunt, and Gary Mack (Novus Biologicals, Lab)

Charles Haitjema (Protein Simple)

Tyler Anderson (Handbook Design) and Charles Perdices (3D Cover Image)

NOTES:

proteinsimple
a bio-techne brand

Any lab.
Any imaging need.

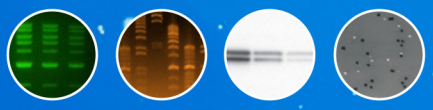
FluorChem systems have you covered.

DYE/METHOD	LIGHT SOURCE	EMISSION FILTER	R	M	E
FLUORESCENT WESTERNS					
MultiFluor Blue	475 nm LED	537/26 nm	●	●	
MultiFluor Red	632 nm LED	710/40 nm	●	●	
MultiFluor Green	534 nm LED	607/36 nm	●	●	
DyLight 550	534 nm LED	607/36 nm	●	●	
IRDye 680/680LT	632 nm LED	710/40 nm	●	●	
Dylight800	747 nm LED	835/70 nm	●		
BLOTTING					
Chemiluminescence	None	None	●	●	●
NUCLEIC ACID STAINING					
Ethidium Bromide	Trans UV	Orange (FCM - 593/40 nm) (FCE - 590/50 nm)	●	●	●
SYBR® Safe	Trans UV 475 LED (FCM only)	Green (537/26 nm)	●	●	●
PROTEIN STAINING					
Coomassie Blue	Trans UV with White Conversion Screen	Orange (FCM - 593/40 nm) (FCE - 590/50 nm)	●	●	●

Systems that let you grow

Science is always changing, and the work you're doing in the lab does too. We've got three FluorChem systems to choose from and you can add filters as you go. Pick the one that fits your budget and applications needs for the right now – you can always upgrade down the road when your needs change.

Fluoro Chem E



Fluoro Chem M

Fluoro Chem R