Detecting low-abundance proteins in western blotting

Detection of low-abundance proteins or detection of proteins from small samples can be a major challenge when performing western blotting. The abundance of the target may be low because the protein itself is expressed at low levels in the source or is difficult to extract (and thus recovery is low), or because the sample is limited (leaving a small overall volume available to load onto the gel), or because of a combination of conditions. The outcome in such situations is often faint or undetectable band(s) during the imaging step, resulting in inconclusive data analysis. This often leads to repeat experimentation or re-optimization of conditions, both of which use up time and resources. Here we will discuss specific methods, from sample preparation through immunodetection, that can help overcome challenges and improve the signal to noise of low-abundance proteins for more successful western blot detection.

When detecting a low-abundance target on a western blot, strong signal intensity is critical for reaching scientific conclusions. Several key factors can help achieve high-quality detection of low-abundance targets:

- Efficient protein extraction
- Effective protein separation
- Complete protein transfer
- Specificity of primary antibodies
- Increased low-end detection sensitivity
- High-resolution image capture

Protein extraction

- For efficient extraction, use buffers optimized for your sample source and target protein location
- To prevent protein loss, use **broad-spectrum protease inhibitors**

Not all proteins are easily extracted, and inefficient extraction can lead to low yields, which makes it difficult to detect the target in downstream western blotting. Protein extraction techniques should vary depending on the source of the starting material and the location of the protein of interest within the cell. Obtain high protein yields from tissues, cells, or subcellular fractions using reagents that are optimized for mammalian, bacterial, yeast, insect (baculovirus), and plant samples. To prevent protein loss, use **broad-spectrum protease inhibitors** to protect your proteins during extraction and lysate preparation.

Recommended protease and phosphatase inhibitors

Thermo Scientific™ Inhibitor Cocktail	Format	Components
Halt™ Protease Inhibitor Cocktail	Concentrated liquid	AEBSF-HCl, aprotinin, bestatin, E-64, leupeptin, EDTA*
Pierce™ Protease Inhibitor Tablets	Tablet	
Halt™ Protease and Phosphatase Inhibitor Cocktail	Concentrated liquid	Aprotinin, bestatin, E-64, leupeptin, sodium fluoride, sodium orthovanadate, β-glycerophosphate, sodium pyrophosphate, EDTA*
Pierce™ Protease and Phosphatase Inhibitor Tablets	Tablet	

* Formulations are available without EDTA.

Overview of sample types and recommended protein extraction reagents and kits

Sample type	Goal	Recommended Thermo Scientific™ reagents or kits
Mammalian cells or tissues	Total protein extraction	• RIPA Lysis and Extraction Buffer • M-PER Mammalian Protein Extraction Reagent • T-PER Tissue Protein Extraction Reagent • N-PER Neuronal Protein Extraction Reagent
Cultured mammalian cells or tissues	Subcellular fractionation or organelle isolation	• NE-PER Nuclear and Cytoplasmic Extraction Reagents • Subcellular fractionation kits • Mitochondria isolation kits • Pierce Cell Surface Protein Isolation Kit • Syn-PER Synaptic Protein Extraction Reagent • Lysosome Enrichment Kit for Tissues and Cultured Cells
Bacterial cells	Total protein extraction	• B-PER Complete Bacterial Protein Extraction Reagent
Yeast cells		• Y-PER Yeast Protein Extraction Reagent
Insect cells (baculovirus)		• I-PER Insect Cell Protein Extraction Reagent
Plant tissue (leaf, stem, root, flower)		• Pierce Plant Total Protein Extraction Kit

Protein separation

For optimal resolution: choose the **right gel chemistry** for your protein target

Bis-Tris: 6–250 kDa
Tris-acetate: 40–500 kDa
Tricine: 2.5–40 kDa

Optimal separation of low-abundance targets is essential to ensuring detection of your target protein by allowing it to be fully accessible to antibody binding during immunoblotting steps. The key to getting optimal separation is to choose the right gel for your target protein. The appropriate gel chemistry depends on the size of the protein you're separating. As a general rule, the proteins being targeted should migrate through about 70% of the length of the gel for optimal resolution (Figure 1). For separation of a broad range of proteins, two chemistries—Bis-Tris and Tris-glycine—are well suited. However, the alkaline pH of a Tris-glycine gel can cause protein modifications. Bis-Tris gels can provide greater sensitivity for protein detection than Tris-glycine gels. The neutral-pH formulation of Bis-Tris gels preserves protein integrity by minimizing protein modification or degradation, resulting in better band resolution.

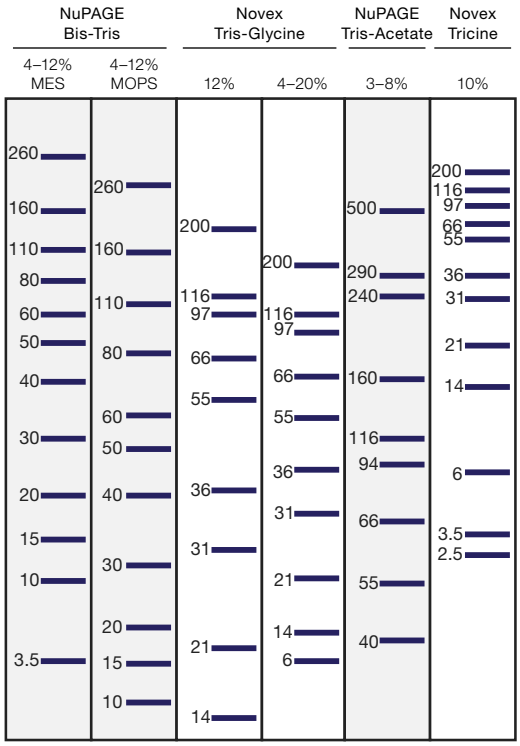

Figure 1. Find the right gel for your research needs based on the molecular weight of your target protein. The target protein should migrate through ~70% of the length of the gel for optimal resolution.

When studying high or low molecular weight proteins, use Tris-acetate or tricine gels, respectively. High molecular weight proteins will be compressed into a narrow region at the top of Tris-glycine and Bis-Tris gels but can migrate further through a Tris-acetate gel. This increased resolution leads to increased transfer efficiencies and higher sensitivity. Low molecular weight proteins run on Bis-Tris or Tris-glycine gels can migrate too close to the bottom of the gel, limiting their resolution (Figure 2). Using a tricine gel can help ensure proteins migrate within the optimal range of the gel and are fully accessible during immunoblotting steps.

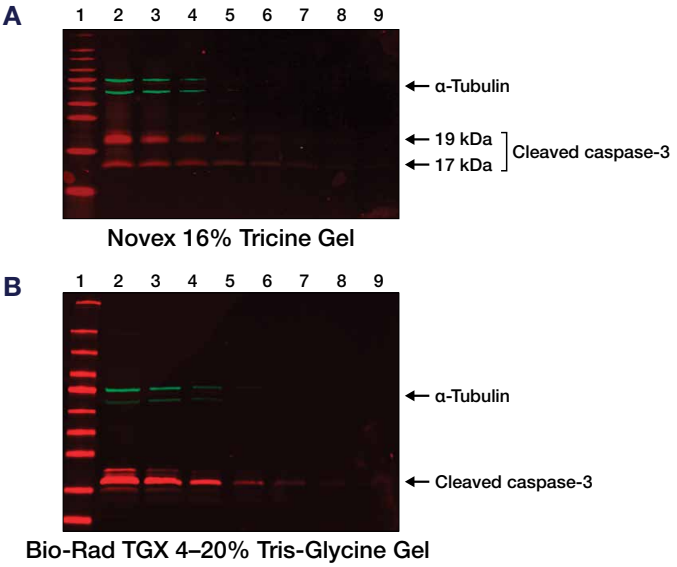

Figure 2. Tricine gels provide better resolution of low molecular weight proteins. (A) A Novex 16% Tricine Gel resolved the 17 and 19 kDa bands of cleaved caspase-3, whereas **(B)** a Bio-Rad™ TGX™ Tris-Glycine Gel did not provide such resolution.

Protein transfer

- For optimal transfer efficiency, use neutral-pH gels such as **Bis-Tris** (6–250 kDa) or **Tris-acetate** (40–500 kDa) gels.
- To limit handling inconsistencies, use **ready-to-use membrane filter stacks**.

The efficiency of protein transfer can be affected by both the gel chemistry and transfer method. Choosing the right gel is a key factor in the successful transfer of your target protein. A popular general-use gel is a 4–20% Tris-glycine gel, which can effectively separate a range of protein sizes. However, high molecular weight proteins will be compressed in a narrow region at the top of the gel, which can decrease transfer efficiency (Figure 3). Choosing a gel that provides optimal separation of the protein of interest will aid in its transfer. In addition, neutral-pH gels such as Bis-Tris and Tris-acetate can provide better transfer efficiencies than alkaline Tris-glycine gels. The neutral-pH environment minimizes protein degradation and allows for cleaner release of the proteins from the gel. Preservation of protein integrity is particularly important when separating and transferring low-abundance proteins.

Several transfer methods can be used to successfully transfer and ultimately detect low-abundance proteins. Traditional wet transfer (wet tank) offers high-efficiency transfer but requires significant time and hands-on effort. Handling inconsistencies are more likely to occur with this more traditional do-it-yourself transfer stack setup, affecting efficiency. Semi-dry blotting provides more speed and convenience but can have slightly lower efficiency when transferring larger molecular weight proteins (>300 kDa). Dry electroblotting offers high-quality transfer combined with speed and convenience. The ready-to-use stacks used in dry blotting eliminate the need for premade buffers or to soak filter paper and membranes, minimizing handling that can lead to inconsistent performance. The use of a copper anode in the dry blotting system does not generate oxygen gas through electrolysis of water, resulting in increased transfer consistency and performance equal to or better than wet transfer.

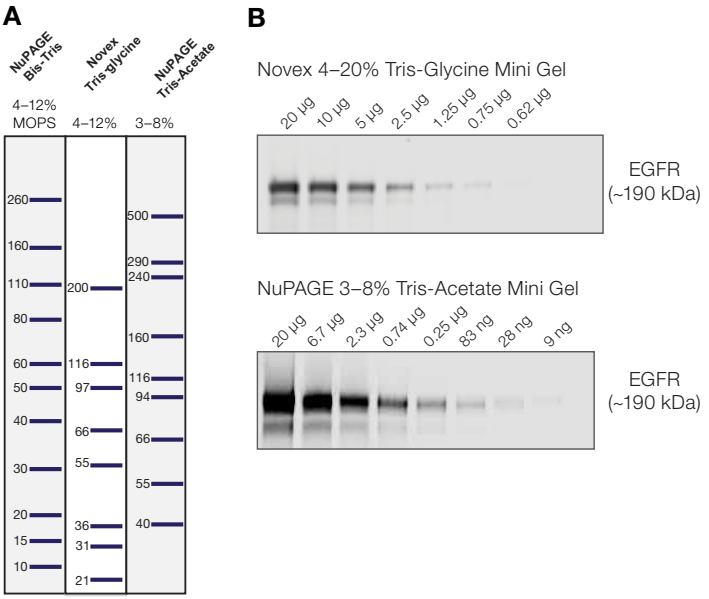

Figure 3. Improved transfer of high molecular weight proteins enhances western detection sensitivity. (A) Migration patterns for a 4–12% Bis-Tris gel, 4–12% Tris-glycine gel, and 3–8% Tris-acetate gel. **(B)** Western blot analysis of EGFR from A431 lysates. Lysates were separated on both Invitrogen™ Novex™ 4–20% Tris-glycine and NuPAGE™ 3–8% Tris-acetate gels. The use of a Tris-acetate gel improved detection sensitivity for EGFR.

Specificity of antibodies

For optimal detection, use antibodies that are verified and validated for specificity and are designed to perform in western blots.

To obtain strong signals from your target protein, choose antibodies for which the supplier provides target-specific verification and validation. In addition, use antibodies designated specifically for western blotting or that have western blotting listed as an application. This will help ensure high performance and specificity for your target protein.

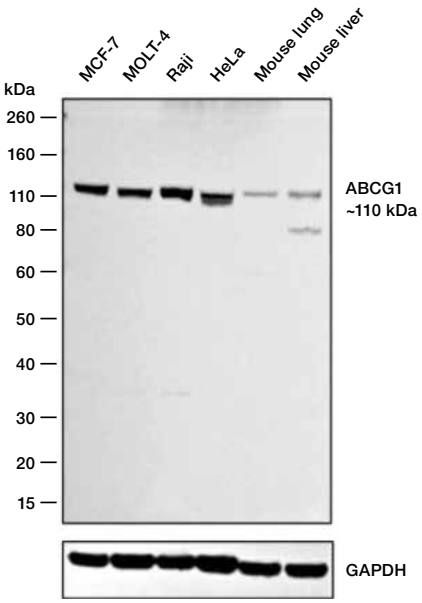

Figure 4. Western blot antibody validation.* Anti-ABCG1 Recombinant Rabbit Monoclonal Antibody (Cat. No. MA5-24857) was validated for use in western blotting by testing its ability to detect ABCG1 in several cell lines by western blot analysis.

Detection

To increase low-end detection sensitivity, use high-sensitivity chemiluminescent substrates such as the **SuperSignal West Atto Ultimate Sensitivity Substrate**.

To achieve the highest sensitivity, a chemiluminescent HRP-conjugated system should be used. Chemiluminescence yields the greatest potential sensitivity of any available detection method for western blotting. HRP is relatively small, which enables more molecules to be conjugated per IgG molecule, providing greater sensitivity than alkaline phosphatase (AP) systems. Furthermore, advances and improvements in chemiluminescent substrates for HRP have enabled even higher sensitivity over other detection systems for western blotting. When optimized, chemiluminescent systems can provide detection down to the attogram level.

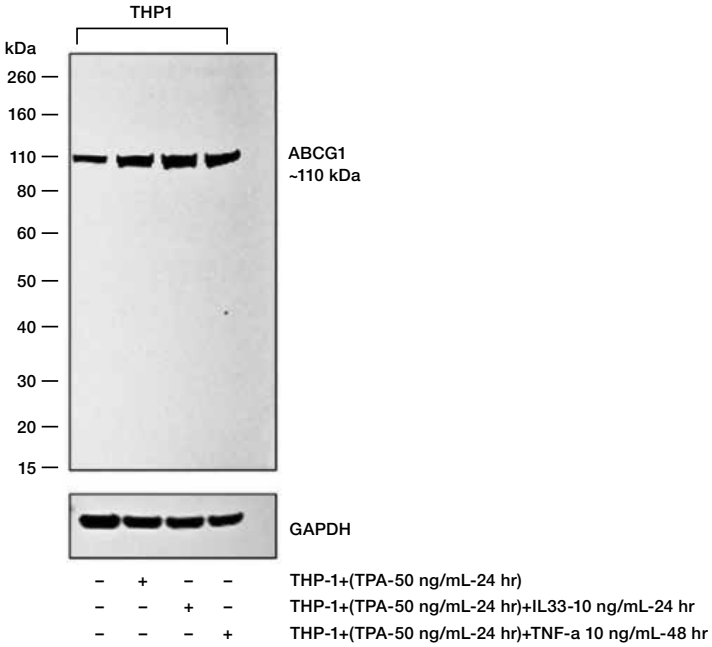

Figure 5. Antibody specificity validation.* Specificity of anti-ABCG1 antibody was validated by cell treatment to ensure that the antibody bound to the antigen stated. Altered expression of proteins upon cell treatment demonstrates antibody specificity. Western blot using **ABCG1 Recombinant Rabbit Monoclonal Antibody (2A10) (Cat. No. MA5-24857)** shows increased expression of ABCG1 in THP-1 cells upon treatment with TPA (24 hr) seen in lane 2, TPA (24 hr) plus IL-33 (24 hr) seen in lane 3, and TPA (24 hr) plus TNF-alpha (48 hr) seen in lane 4, as compared to untreated THP-1 cells seen in lane 1.

* The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

As with other components in the western blotting systems, there are many **chemiluminescent HRP substrate** choices available. High-sensitivity substrates such as the **Thermo Scientific™ SuperSignal™ West Atto Ultimate Sensitivity Substrate** provide the most sensitive detection, delivering more than triple the sensitivity of conventional substrates (Figure 6). The SuperSignal West Atto substrate is an ultrasensitive enhanced chemiluminescent (ECL) substrate that enables protein detection down to the high-attogram level. It is the ideal choice for detection of very low-abundance targets or when using precious samples that require maximum sensitivity (Figure 7).

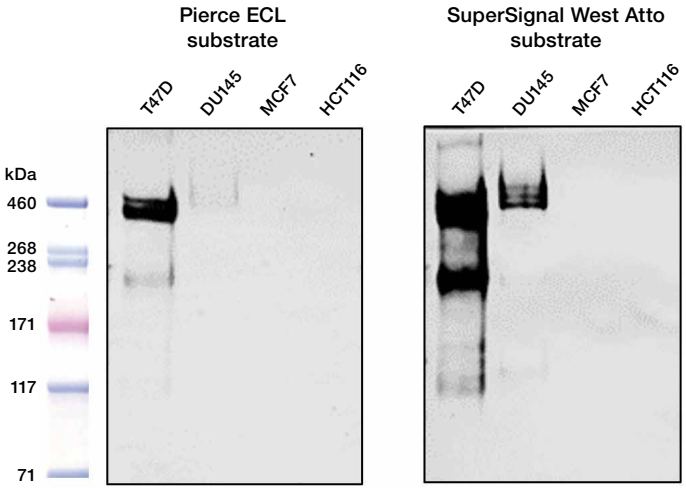

Figure 6. Increased detection of low-abundance proteins with higher-sensitivity substrates. Misfolding and hydrophobicity of membrane-bound or nuclear-expressed proteins, such as mucin 1 (MUC1), can make them difficult to extract, resulting in lower abundance for western blot detection. The molecular mass of MUC1 can vary between 250 and 500 kDa, depending on the degree of glycosylation. Both the SuperSignal West Atto Ultimate Sensitivity Substrate and Thermo Scientific™ Pierce™ ECL Western Blotting Substrate were used to detect mucin in various cell lines with various degrees of MUC1 glycosylation. The SuperSignal West Atto substrate was able to detect the less abundant glycosylation of MUC1 above 460 kDa and below 200 kDa, whereas the Pierce ECL substrate could not detect at those levels.

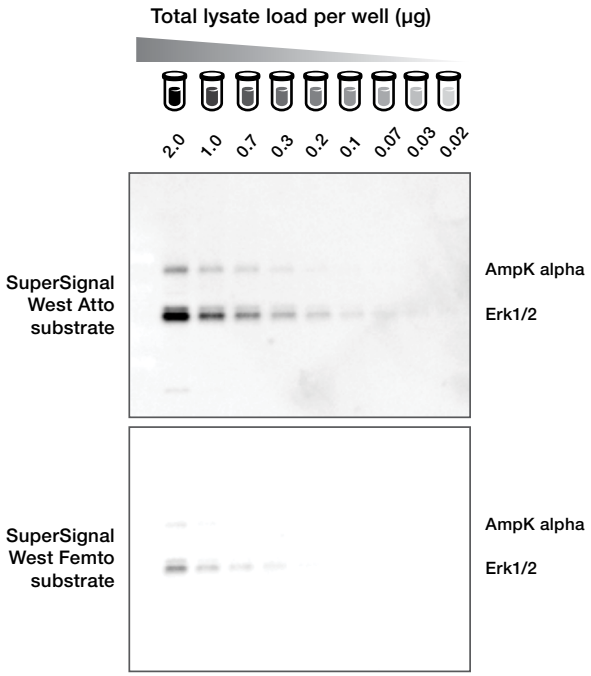

Figure 7. SuperSignal West Atto substrate allows detection of very low concentrations of target proteins. SuperSignal West Atto Ultimate Sensitivity Substrate and SuperSignal West Femto Maximum Sensitivity Substrate were used to detect AmpK alpha and Erk1/2 in HeLa cell lysates. The SuperSignal West Atto substrate was able to adequately detect both AmpK alpha and Erk1/2 with lower sample loads, whereas larger sample loads were required by the SuperSignal West Femto substrate for detection.

Image capture

For greater dynamic range and optimal exposure, capture western blot images using digital imaging systems.

When it comes to detecting signal, digital imaging systems will provide the greatest dynamic range and ease of determining optimal exposure to detect low-abundance proteins. With advances in camera technology and developments in imaging software, the limitations of traditional signal capture with X-ray film have become increasingly evident. Charged-coupled device (CCD) cameras are based on light-sensitive silicon chips that convert photons to digital signals. Improvements in chip design have enabled the development of sensitive, cooled CCD–based cameras with higher light-capturing performance than X-ray film. Powerful high-resolution cooled CCD cameras in instruments such as **Invitrogen™ iBright™ Imaging Systems** enable the capture and analysis of western blots with greater sensitivity, linearity, and dynamic range than X-ray film. X-ray film has only 1.5 orders of magnitude of dynamic range, compared to iBright Imaging Systems and other equivalent 16-bit CCD cameras, which can display up to 4 orders of magnitude of dynamic range (Figure 8). This larger dynamic range permits the capture of strong chemiluminescence signals without sacrificing detection of faint bands. Reference luminometer plates show evidence of a greater dynamic range of signal using an iBright Imaging System than with traditional X-ray film (Figure 8).

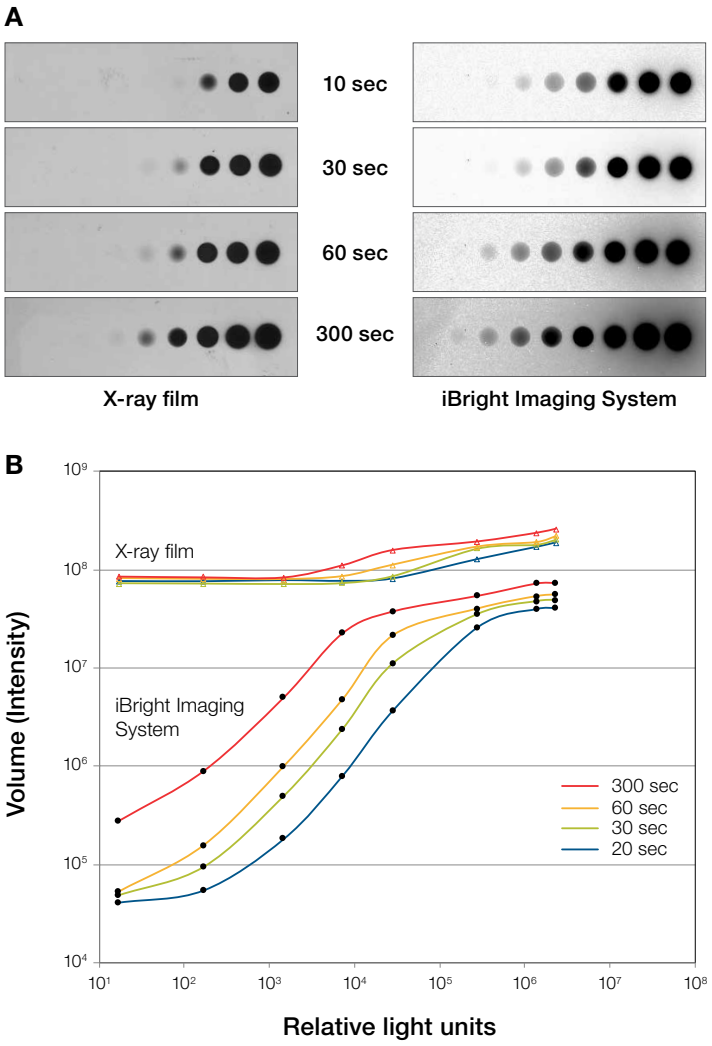

Figure 8. Comparison of image sensitivity and dynamic range of X-ray film and an iBright Imaging System. (A) At a binning of 3 x 3, images of a reference luminometer plate on an iBright Imaging System show greater sensitivity than those of X-ray film. **(B)** Analysis of the reference luminometer plate images demonstrates a greater dynamic range of signals using an iBright Imaging System compared to X-ray film.

Find additional western blotting tips and protocols at
thermofisher.com/westerneducation

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