**Video Article**

# Western Blotting Using the Invitrogen NuPage Novex Bis Tris MiniGels

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## Abstract

Western Blotting (or immunoblotting) is a standard laboratory procedure allowing investigators to verify the expression of a protein, determine the relative amount of the protein present in different samples, and analyze the results of co-immunoprecipitation experiments. In this method, a target protein is detected with a specific primary antibody in a given sample of tissue homogenate or extract. Protein separation according to molecular weight is achieved using denaturing SDS-PAGE. After transfer to a membrane, the target protein is probed with a specific primary antibody and detected by chemiluminescence.

Since its first description, the western-blotting technique has undergone several improvements, including pre-cast gels and user-friendly equipment. In our laboratory, we have chosen to use the commercially available NuPAGE electrophoresis system from Invitrogen. It is an innovative neutral pH, discontinuous SDS-PAGE, pre-cast mini-gel system. This system presents several advantages over the traditional Laemmli technique including: i) a longer shelf life of the pre-cast gels ranging from 8 months to 1 year; ii) a broad separation range of molecular weights from 1 to 400 kDa depending of the type of gel used; and iii) greater versatility (range of acrylamide percentage, the type of gel, and the ionic composition of the running buffer).

The procedure described in this video article utilizes the Bis-Tris discontinuous buffer system with 4-12% Bis-Tris gradient gels and MES running buffer, as an illustration of how to perform a western-blot using the Invitrogen NuPAGE electrophoresis system. In our laboratory, we have obtained good and reproducible results for various biochemical applications using this western-blotting method.

## Protocol

### Gel electrophoresis

***Technical note***: Before starting the procedure have your protein samples ready.

During this first step, the proteins in the sample are separated according to their molecular weight using denaturing polyacrylamide gel electrophoresis (PAGE). The NuPAGE® LDS Sample Buffer loaded with Lithium Dodecyl Sulfate (LDS) maintains polypeptides in a denatured state once the protein sample has been heated at 70℃ for 10 minutes. A strong reducing agent is used in conjunction to remove secondary and tertiary structure (DTT, to break disulfide bonds). In addition, sample proteins become covered in the negatively charged LDS and therefore move through the acrylamide mesh of the gel toward the positively charged electrode. This allows their separation according to molecular weight (measured in kilo Daltons, kDa).

Detailed step-by-step protocols for the sample preparation and the PAGE procedure can be found on the Invitrogen website[2], or in the NuPAGE technical guide[3].

### Technical tips

1. In the Invitrogen NuPAGE Bis-Tris discontinous buffer system, the electrophoretic mobility of the proteins and the subsequent separation range of the gel is dependent on two factors: i) the acrylamid concentration of the gel (with greater acrylamide concentration resulting in better resolution of lower molecular weight proteins) and ii) the trailing ion of the running buffer, MOPS or MES. To choose the appropriate combination for the separation range that you wish to achieve, refer to the Gel Migration chart[1]. The thickness of the gel and number of wells will depend on the volume and the quantity of samples that you plan to load, respectively.
2. When you load your samples into wells of the gel, at least one lane is reserved for a molecular weight marker (or ladder). These protein standards are commercially available from several companies and typically consist of a mixture of stained proteins having defined molecular weights, so as to form visible bands that allow you to follow the migration progress. Pick a range of molecular weights that is compatible with your gel resolution.
3. To achieve a nice and even migration pattern we recommend loading ALL the wells of your gel with a similar volume of sample or 1X LDS sample buffer.

### Transfer

***Technical Note***: Before beginning the transfer step, prepare the transfer buffer (1X with 10% methanol) and pre-cool to 4°C in a cold room.

In order to make the proteins accessible to antibody detection, they are transfered by electroblotting from the gel onto a nitrocellulose membrane. Protein binding is based upon hydrophobic interactions, as well as charge interactions between the membrane and protein.

(Polyvinylidene fluoride (PVDF) membrane can also be used as an alternative. In this case, the PVDF membrane needs to be pre-wet in methanol at least 30 seconds before use.)

A detailed step-by-step protocol of the transfer procedure can be found in reference 4 if you use the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell, or in references 2-3 if your lab is equipped with the Invitrogen's XCell II™ Blot Module.

 

As a result of this process, the proteins are exposed on a thin surface layer and ready for detection. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by the reversible Ponceau S dye membrane staining.

**Ponceau S staining procedure (optional):**

1. After transferring the proteins, place the membrane in an incubation tray (proteins facing up).
2. Add enough Ponceau S Staining to cover the membrane and incubate at least 30 seconds with gentle agitation.
3. Rinse membrane with distilled water until the background is clear.
4. Destain the membrane with running distilled water for 2-3 minutes.
5. Place the membrane in blocking solution (see below).

**Technical tips:**

1. We recommend labeling your membrane with a pencil before protein transfer in order to denote the side in which the proteins were exposed and the orientation of your samples.
2. Both Ponceau S staining and the molecular weight markers may be used to cut the membrane after transfer for probing the resulting parts with different antibodies

**Immunodetection:**

***Technical Note***: This immunodetection procedure is provided as a guideline only. Optimization may be required for each antibody; specific information can be found in most data-sheets of the commercially available antibodies (e.g., working dilution, incubation time, etc.).

During this last process, the target protein will be detected using a specific antibody and will appear as a band on the film. The position of the band is dependent of the molecular weight of the target protein, whereas the band intensity depends on the amount of target protein present.

Typically, this is achieved after three substeps:

1. Blocking: To avoid non-specific interactions of the antibody with the membrane (the excess space on the membrane is covered with a dilute solution of a generic protein).
2. Probing: The protein of interest is detected by a specific primary antibody. After the unbound primary antibody is washed away, the membrane is exposed to a different antibody linked to the reporter enzyme, horseradish peroxidase (HRP). This secondary antibody is directed against the species-specific portion of the primary antibody (*e.g.* an anti-rabbit secondary antibody will bind to any rabbit-sourced primary antibody).
3. Detection: A chemiluminescent agent is used as a substrate that will luminesce when exposed to the HRP on the secondary antibody. This reaction produces luminescence in place and in proportion to the amount of probed protein. The light is then detected by photographic film.

A generic step-by-step procedure is provided below. Other detailed procedures can be found in the ECL Plus Western Blotting Detection Reagents instruction manual[5] or in most data-sheet accompanying the commercially available antibodies. Incubation time, antibody dilution, and blocking and wash solutions have to be empirically optimized for each antibody.

**Immunodetection procedure:**

1. Block the non-specific binding by incubating with enough volume of PBS 1% casein blocking solution to cover the entire membrane. Place on a rocker for at least 30 min at room temperature or overnight at 4°C.
2. Incubate the membrane with your primary antibody (specific for the protein of interest). The primary antibody should be diluted in blocking solution. For most antibodies, complete binding is achieved after 1-2 hours incubation at room temperature under gentle agitation. Alternatively, the incubation step can be performed overnight at 4°C to increase the signal to noise ratio.
3. Remove the solution (discard or keep at 4°C to -20°C for repeated use) and quickly wash the membrane once. Then 3X10 minutes with PBS, 0.05% Tween 20 (PBST) at room temperature with vigorous shaking.
4. Incubate the membrane with a HRP-coupled secondary antibody diluted in blocking solution. Choose an antibody which recognizes the IgG portion of the species where the primary antibody was raised. The incubation is performed for 1 hour at room temperature under gentle agitation.
5. Discard the solution and quickly wash the membrane once. Then 3X10 minutes with PBST at room temperature with vigorous shaking.
6. Proceed to the chemiluminescent detection according to the manufacturer instruction. We use the ECL Plus Western Blotting Detection Reagents from GE Healthcare (see reference 5 for specific instructions).
7. Place your membrane in saran wrap or a pouch and place inside an autoradiography cassette. Make sure to drain any excess liquid and remove all air bubbles.
8. Expose the photographic film to membrane in a dark room. Start with a 1 minute exposure time and adjust according to desired signal intensity.

**Technical tips:**

1. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein. We typically use 1% casein, but bovine serum albumin (~2% BSA) or non-fat dry milk (~5%) can be used as an alternative.
2. Tris-Buffered Saline (TBS) can be used throughout the procedure instead of PBS. We recommend using TBS if you plan to probe your membrane with phosphospecific antibody.
3. The use of a glow-in-the-dark sticker (e.g., Stratagene's Glogos® II Autorad Markers) tapped in the bottom of the autoradiography cassette will help you to align your film and your membrane during the analysis step.

**Analysis**

Now that you have exposed your film, you will realize that in practical terms, not all Westerns reveal protein as one nice single band. Additional bands may also appear due to the non-specific binding of both primary and secondary antibodies. This background signal can be reduced by optimizing the immunodetection procedure. In addition, an appropriate control (e.g., untransfected cells, siRNA-treated cells, etc ) will be useful to determine the specificity of your antibody and the exact location of the target protein on the membrane.

After marking on the film the position of the stained protein standard bands from the membrane, plot the log of each molecular weight of the protein standards (y-axis) against their corresponding **relative mobility** (x-axis). Relative mobility (Rf) is the term used for the ratio of the distance

 

the protein has moved from its point of origin (top of the gel) relative to the distance the tracking dye or a low molecular weight marker has moved (the gel front). Determine the regression line of the standard curve to obtain values for slope and y-intercept. The unknown molecular weight (size) of your target protein is estimated using its Rf and the following modified equation:

**log molecular weight = (slope)(mobility or Rf of the target protein) + y-intercept**

(see reference 6 for detailed instructions).

Expression level approximations are taken by comparing the band intensity of the target protein to that of a structural protein (e.g., tubulin or actin) or a housekeeping gene product such as GAPDH. This so called "loading control" should not change between samples and is revealed using a specific primary antibody. The image may be further analyzed by densitometry to evaluate the relative amount of protein staining and quantify the results in terms of optical density.

## References