Video Article

Western Blotting: Sample Preparation to Detection

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Abstract

Western blotting is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.

Video Link

The video component of this article can be found at https://www.jove.com/video/2359/

Protocol

1. Sample Preparation

The first step in Western blotting is sample preparation. To prepare samples for running a gel cells and tissues need to be lysed to release the proteins of interest.

- A convenient, ready-to-use reagent that is versatile and easy to use for soluble protein extraction is CytoBuster or PhosphoSafe. These extraction buffers contain detergents optimized for efficient extraction of soluble proteins from mammalian and insect cells. The gentle, non-ionic composition of CytoBuster Protein Extraction Reagent enables isolation of functionally active endogenous or expressed proteins without a need for secondary treatment such as sonication or freeze/thaw. PhoshoSafe has the added benefit of containing four phosphatase inhibitors.
    1. Pellet the cells by low speed centrifugation (e.g. 5 min at 2500 x g), drain the cell pellet well.
    2. Resuspend the cells in CytoBuster Protein Extraction Reagent using 150 µL per 10^6 cells (optimal amount of CytoBuster Protein Extraction Reagent may vary based on cell size).
    3. Incubate at room temperature for 5 min.
    4. Transfer to a suitable tube and spin for 5 min at 16,000 x g at 4°C.
    5. Transfer cleared supernatant (cell extract) to a fresh tube and proceed with analysis.
- For specialized protein extraction, we recommend using our high-quality ProteoExtract Kits. EMD has over a dozen kits for mitochondrial isolation, subcellular proteome extraction, transmembrane protein extraction, and many others. Please visit our Western blot landing page for further details.
- EMD also sells a wide variety of protease and phosphatase inhibitor cocktail sets. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer. Please visit our Western blot landing page for further details.

2. SDS-PAGE

The second step in Western blotting is protein separation. Proteins are separated based on molecular weight.

- You can make your own PAGE gel, however we will be using a commercially pre-cast gel.
    1. After preparing your sample, you are ready to determine the protein concentration. EMD has two kits for this to measure concentration, one being the Non-Interfering Protein Assay Kit and the other being the BCA Protein Assay Kit.
    2. Once the protein concentration is assessed we are ready to prepare our sample for loading the gel. Antibodies typically recognize a small portion of the protein of interest (referred to as the epitope) and this domain may reside within the 3D conformation of the protein. To enable access of the antibody to this portion it is necessary to unfold the protein.
    3. To denature, use a loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS), and boil the mixture at 95-100°C for 5 minutes. An easy and convenient loading buffer to use is 4X Sample Buffer.

- Load the first lane of the well with Trail Mix Protein Marker. This marker has three reference bands which allow you to monitor electrophoresis. When the gel is stained, 10 bands are visible ranging from 10-225 kDa.
- Fill the electrophoresis unit with running buffer. For PAGE gels, this is usually 1X Tris-glycine. For detailed buffer recipes, visit the Western blotting page. EMD offers high-quality buffers reagents with our OmniPur chemical line. Run the gel at 220V for 1 hour.
- Once the proteins have separated, stain the gel with Coomassie blue to ensure the proteins have migrated evenly and uniformly. RAPIDstain is an ultra-sensitive stain which is ready-to-use. No destaining is required.

3. Protein Transfer

Just as proteins with an electrical charge can be induced to travel through a gel in an electrical field, so can the proteins be transferred in an electrical field from the gel onto a membrane, being either PVDF or nitrocellulose.

- Today we will do a wet transfer. In wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring no air bubbles have formed between the gel and membrane. The sandwich is submerged in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on.
- Two types of membranes are available: nitrocellulose and PVDF. Both work well. Today we will be using PVDF. PVDF membranes require soaking in methanol for a few minutes, followed by ice cold transfer buffer for 5 minutes. The gel also needs to equilibrate for a few minutes in ice cold transfer buffer. Not doing so may shrink the gel during transfer.
- The buffer for wet transfer is 1X Tris-glycine with 20% methanol. Detailed buffer preparations are available on our website.
- Once the transfer is complete, it's a good idea to visualize proteins to ensure even transfer with no air pockets. This can easily be done with RedAlert Western Blot Stain. This stain is ready-to-use and destaining can be done easily with water.

4. Antibodies and Accessory Reagents

- The first step in doing probing and antibody to antigen is to block the membrane. Blocking the membrane prevents non-specific background binding.
- Either non-fat milk or BSA can be used as a blocking solution. However, when using phospho-specific antibodies, it is not recommended to use milk as it will cause high background.
- EMD offers several high-quality blocking reagents, including SeaBlock, which is a non-mammalian blocking agent and BLOT QuickBlocker, which is prepared, ready-to-use blocking agent. We also have high-quality BSA (Fraction V) available.
    1. Incubate the membrane 1 hour at room temperature under gentle agitation.
    2. Wash three times in TBST or PBST after incubation. An easy and convenient way to make TBST or PBST is to use our tablets (PBS TWEEN Tablets/TBS TWEEN Tablets)

- Once the membrane has been blocked, you are ready to incubate with the primary antibody. EMD has been offering a comprehensive portfolio of exceptional antibodies with the best antibody guarantee in the industry for over 30 years. Remember that with all EMD antibodies, we offer a full 100% no-risk guarantee. Purchase an antibody and use it for any species specificity or application you desire. If the antibody does not work to your satisfaction, we will provide a full credit to be used on any other product. For more details, please visit our Western blotting web page.
    1. Today we will be incubating our primary antibody using solution 1 of SignalBoost. SignalBoost is a great product allowing your signal to be enhanced significantly. Simply incubate your primary antibody in Solution 1 and incubate for 1 hour or as you normally would. There is no need to add further blocking agents. Alternatively, you can use BSA or nonfat dry milk as a blocking agent.
    2. Wash with TBST. We make TBST using the ready-to-dissolve TBST tablet.
    3. Incubate your secondary antibody using solution 2 of SignalBoost. Incubate the secondary antibody in blocking buffer for 1 hour. Wash the membrane again several times with TBST.

5. Detection

For HRP conjugating secondary antibodies, ECL is traditionally used. An excellent ECL reagent we offer is RapidStep. The advantages of RapidStep are that no mixing of luminal or enhancer is required. Simply spray the membrane a few times and develop using x-ray film. RapidStep offers low pictogram sensitivity as well as emitting light for up to 2 hours after the addition of substrate.

Discussion

In this video presentation, we have highlighted the major steps in Western blotting which are sample preparation, SDS-PAGE, membrane blocking/probing with antibodies, and detection. For each step of the process, proper protocols and appropriate reagents should be used to attain high-quality results.

Disclosures

The authors Anna Eslami and Jesse Lujan are employed by EMD Chemicals Inc that produces reagents and instruments used in this Article.

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