Western Blot Normalization

Normalization is critical for obtaining accurate, quantitative data from Western blots. Normalization accounts for unequal loading of samples across the lanes on a gel, and for differences in transfer efficiency across a blot.

Traditionally, protein normalization for Western blots involves comparing the relative abundance of a protein of interest to that of an unrelated protein, the normalization or loading control. More recently, total protein normalization (TPN) methods have been developed, in which the relative abundance of the protein of interest is compared to the total protein content of the sample.

Normalization using a single protein

To perform Western blot normalization using a single protein as a control, the blot is probed with a primary antibody specific for the protein of interest, and one directed against a normalization control. Ideally, the normalization control is a protein that is present at constant levels in every sample. The normalization control can be a pure protein spiked into each sample, or a protein expected to be expressed at a constant level across all sample types used in the experiment, such a housekeeping protein. If a housekeeping protein is used, its expression must not be affected by experimental conditions. Additionally, the abundance of the normalization control in the sample should be similar to that of the protein of interest so that both proteins are within the linear range of detection in the samples loaded on the gel.

For each sample, the relative abundance of the protein of interest is determined by calculating the ratio of the intensity of the signal for the protein of interest to that of the normalization control. For qualitative comparison, images of the two bands may be shown next to each other. For accurate quantitative results, the protein of interest and the control must be detected on the same blot. Probing duplicate blots does not control for differences in transfer efficiency, or for loading errors that may occur when preparing one or both gels.

Housekeeping proteins are often used as normalization controls. These proteins are required for basic cellular functions and may be constitutively expressed at a consistent level across many cell types. Several proteins are widely used as normalization controls, including glyceraldehyde 3-phosphate dehydrogenase (GADPH), actins (such as beta-actin), and tubulins. Recent work has demonstrated that it is not always safe to assume that these housekeeping proteins are expressed at constant levels in different cell lines or tissue types, or that their expression is unaffected by experimental conditions.[1-5] Therefore, it is important to validate any protein selected as a normalization control for use with your sample types and experimental conditions.

Some of the more popular housekeeping proteins used as normalization controls are abundantly expressed, which may preclude their use as normalization controls when the protein of interest is much less abundant. Under such conditions, loading enough protein to detect the protein of interest could result in an over-saturated signal for a highly expressed loading control.

To use a single protein as a normalization control:

- The protein should be present across all cell types or tissue types used in the experiment
- Expression of the normalization control should not change due to experimental conditions
- Both the target protein and the normalization control should be within the dynamic range of detection.

Advantages to using single proteins for normalization

- Validated, cost-effective antibodies are available from many companies
- No new skills or equipment are required

Disadvantages to using single proteins for normalization

- Unless fluorescent detection is used, the normalization control must have a substantially different molecular weight than the protein of interest

- A normalization control should be validated for each different experiment to ensure its expression is the same across all sample types and is not affected by experimental conditions
- Many commonly used housekeeping proteins are very highly expressed so are most appropriate for use with highly expressed proteins of interest

Total Protein Normalization

In total protein normalization (TPN), the abundance of the protein of interest is normalized to the total amount of protein in the sample.[6] The amount of protein in the sample can be determined using protein stains or by a stain-free method. Usually, the stained gel or blot is imaged and a narrow rectangle is drawn vertically down the center of a lane and the signal intensity within this rectangle is used to represent that sample's total protein content in normalization calculations. The signal intensity of the protein of interest is normalized to this value. To use membrane protein stains, the membrane may be incubated with a protein stain before or after antibody detection of the protein of interest, depending on the type of stain. The stain binds to all proteins on the membrane and provides an immediate visual image of the loading consistencies and transfer efficiencies across the blot.

There are many stains available that can be used for total protein normalization. They differ in sensitivity, protocol, equipment needed for documentation, permanence, and whether they can be used prior to immunodetection or must be used after. Make sure the staining method selected is compatible with the detection method used and planned downstream applications. Some commonly used stains are described below.

Pre-antibody stains

Ponceau S is a reversible dye that does not affect downstream immunodetection. This negatively charged dye binds to the positively charged amino groups on proteins and also noncovalently binds nonpolar protein regions. Proteins stained with Ponceau S turn red within minutes of incubation and the stain is removed easily by washing in water. The limit of detection of Ponceau S is 100 to 1000 nanograms of protein per band. The intensity of Ponceau S staining decreases quickly over time, so documentation should be conducted immediately.

Fluorescent protein stains[7] have a broad linear range and higher sensitivity than anionic protein dyes. They are permanent, photostable stains that can be excited with either UV or visible light (depending on the stain) and require a fluorescent imager for detection. Fluorescent stains are much more sensitive than Ponceau S, having a limit of detection ranging from 0.25 to 8 nanograms. However, the staining procedure takes longer, usually 30 to 60 minutes.

Post-antibody stains

Amido black is a permanent anionic stain that is more sensitive than Ponceau S. Proteins stained with amido black turn a dark black with little to no background. Microgram quantities of protein can be visualized using amido black with a limit of detection of 50 nanograms of protein. Staining with amido black must be performed after immunodetection.

Stain-Free™ gels

Total protein can be visualized within the gel prior to transfer or on the membrane after transfer using a relatively new, stain-free technology.[8] The stain-free method relies on the incorporation of a trihalo compound into the gel. Upon exposure to UV light, tryptophan residues in proteins in the gel become modified, forming a fluorescent product. The fluorescent signal is detected by a CCD camera. The limit of detection is 1 nanogram of protein. Stain-free detection does not interfere with downstream immunodetection.

Advantages to total protein normalization

- The abundance of the protein of interest is compared to the total protein content of the sample, so normalization is not dependent on the appropriateness of a single normalization control
- Stains with a range of sensitivities are available

Disadvantages to total protein normalization

- Staining may not be even across the blot; edge artifacts have been reported in which lanes near the exterior of a blot stain more intently than those on the interior
- Some stains and stain-free technology depend on protein composition

Which method is right for you?

The answer depends on

- The availability of a validated normalization control for your experimental system
- How highly expressed your protein of interest may be. In general, TPN is preferred for low-abundance proteins and commonly used housekeeping proteins are more appropriate for highly expressed proteins

- The equipment available to you: do you have a digital imaging system to image fluorescently stained membranes?

Whichever method you choose, protein normalization will insure that you achieve the most accurate analysis of your Western blots.

References

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