Avoiding Housekeeping Protein Detection Saturation

Bulletin 6359

Protocol

Reliable western blot data require detection of the target and loading control proteins in the linear dynamic range. Many published western blot images show saturated protein bands, especially for the housekeeping proteins (HKP) including ß-actin, ß-tubulin, and GAPDH, indicating that they were not measured in the linear dynamic range. Quantitative analysis based on this type of data is not reliable. This protocol describes common ways of avoiding signal saturation for the detection of HKP loading controls.

Method 1: Decrease sample load for HKP detection

Utilizes two gels to ensure quantitative detection of both target and HKP loading control proteins. One gel is used for target protein detection using a high sample load. A second gel is used for HKP detection using a very small amount of cell lysate to ensure the HKP detection is in the linear dynamic range.

Protocol:

1. Prepare two identical gels.
2. Load 20 µg cell lysate for each sample in gel 1 for target protein detection.

   Note: Many protocols use 10–50 µg of cell lysate for the detection of target proteins. The appropriate amount of protein load should be determined before an actual experiment is performed. Please refer to *Determining the Appropriate Sample Load for Western Blots*, bulletin 6362, for details.
3. Load 1 µg of cell lysate for each sample in gel 2 for HKP detection (such as ß-actin and ß-tubulin).

   Note: The samples should be loaded in the same order as in gel 1. The appropriate amount of protein load should be determined before an actual experiment is performed. Please refer to *Determining the Appropriate Sample Load for Western Blots*, bulletin 6362, for details. A reasonable starting point is 1 µg.
4. To ensure similar transfer conditions for both gels, use the same tank for a wet transfer, or the same cassette if using a fast transfer system.
5. Following the antibody manufacturer's instructions, apply the primary and secondary antibodies for detection of the target and loading control proteins on blots 1 and 2.
6. Develop the blots with a substrate, preferably one with a long signal duration such as Clarity™ western ECL substrate.
7. Use a CCD camera-based imager to capture the chemiluminescent signals on both blots. It is not recommended to use film.
8. The chemiluminescent image of blot 1 reveals the change in target protein expression level in different samples. The image of blot 2 reveals the load difference between the samples and can be used to normalize the target protein signals ensuring that the change observed in blot 1 is a true response to a biological event.

Note:

- Procedure details are omitted for steps such as protein sample prep, gel load, gel electrophoresis, transfer, or antibody incubation. For details, please refer to the *General Protocol for Western Blotting*, bulletin 6376
- Some journal reviewers may critique the fact that the loading control is from a different blot in a western blot experiment (Neill, U 2009)
- Once the experimental setup and conditions are established for the assay, do not change the sample load, transfer method, transfer time, antibody dilution, antibody incubation time, or temperature in subsequent experiments, as these factors may significantly change the detection signals

Method 2: Titration of antibody concentration

Utilizes only one gel for both the target and loading control protein detection. If the target is a low abundance protein and difficult to detect, the sample load is frequently increased in order to detect the target protein. It is common to titrate down the HKP antibody concentration to avoid saturation and achieve quantitative detection.

Protocol:

1. Select a typical sample in which an average amount of target and loading control proteins are present.
2. Load 20 µg of the sample in each lane of a 15-well gel. Separate every two sample lanes with a prestained protein standard. Utilize two of the wells to load 1 µg of the sample as reference.

   Note: Typically, 10–50 µg of cell lysate are used to detection target proteins. The appropriate amount of protein load should be determined before a real experiment is performed. Please refer to *Determining the Appropriate Sample Load for Western Blots*, bulletin 6362, for details.
3. Select a wet transfer or fast transfer method to transfer the gel. Following the transfer, cut the membrane along the prestained standard lanes to generate five blots.

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4. Prepare the primary antibody against the housekeeping protein (e.g. ß-actin) at different dilutions:
   a. Manufacture recommended dilution (e.g. 1:1,000)
   b. 5x more dilute than recommended (e.g. 1:5,000)
   c. 10x more dilute than recommended (e.g. 1:10,000)
   d. 20x more dilute than recommended (e.g. 1:20,000)
5. Apply the four different dilutions of HKP primary antibody to the four blots loaded with 20 µg of cell lysate. Treat the reference blot loaded with 1 µg of cell lysate with the primary antibody using the manufacturer's recommended concentration.
6. Develop the blots using a substrate with a very long signal duration, for example, Clarity western ECL substrate.
7. Use a CCD camera-based imager to capture the chemiluminescent signals. It is not recommended to use film.
8. Based on the results from the blots, select the antibody concentration that most closely matches the data for the 1 µg blot.

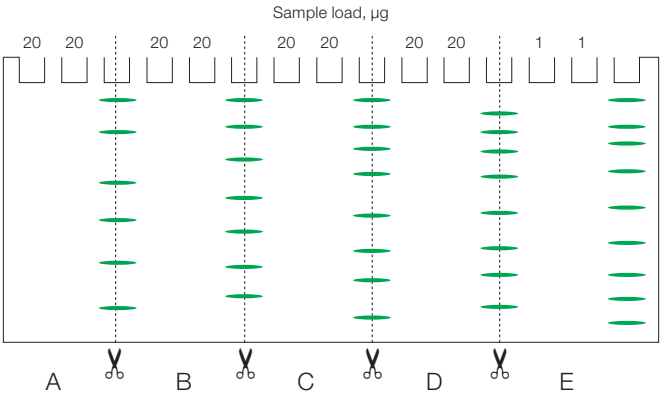
Note:

- Procedure details are omitted for steps such as protein sample prep, gel load, gel electrophoresis, transfer, or antibody incubation. For details, please refer to the *General Protocol for Western Blotting*, bulletin 6376
- Once the experimental setup and conditions are established for the assay, do not change the sample load, transfer method, transfer time, antibody dilution, antibody incubation time, or temperature in subsequent experiments, as these factors may significantly change the HKP signals

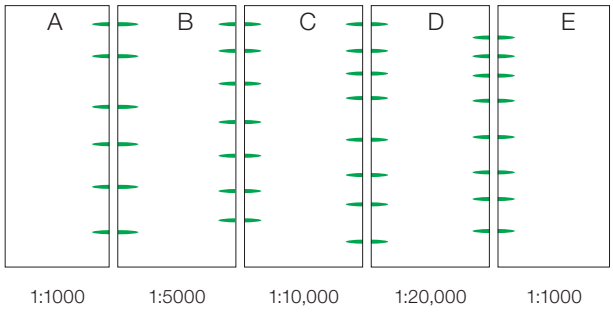
Reference

Neill U (2009). All data are not created equal. J Clin Invest 119, 424.

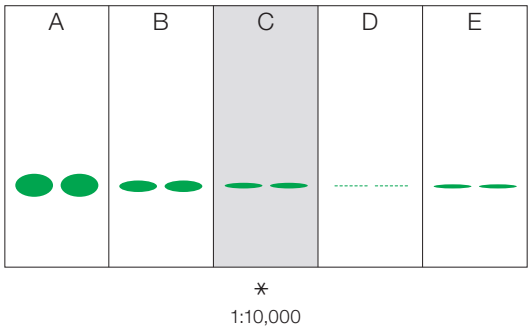
Gel Electrophoresis and Transfer



Antibody Incubation



Imaging and Analysis



Method 2: Titration of antibody concentration

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