A Method for Greater Reliability in Western Blot Loading Controls: Stain-Free Total Protein Quantitation

Tech Note

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Reliable assessment of changes in target protein levels by western blot requires measurement of both the target and loading control proteins in the linear dynamic range. Stain-free technology is a novel method introduced by Bio-Rad to visualize and quantify proteins in gels and blots. In this study, we compared the linearity of a series of dilutions measured by stain-free total protein measurement as well as immunodetection of three housekeeping proteins (ß-actin, ß-tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We found that immunodetection-based measurements of ß-actin, ß-tubulin, and GAPDH protein levels neither showed good linearity nor accurately indicated 10–50 µg of HeLa cell lysate loading levels. By contrast, stain-free total protein measurements exhibited great linearity in the same loading range and its linearity correlated with the immunodetection of a selection of low abundant protein targets: MEK, Akt, and Erk. This study demonstrated that stain-free total protein measurement serves as a more reliable loading control than housekeeping proteins, particularly in the loading range commonly used for cell lysates, 10–50 µg.

Introduction:

A western blot experiment generates at least two important pieces of data: a target protein blot image usually showing changes in expression levels among different samples and a loading control blot image showing whether the samples are fairly compared. The loading control is usually done by checking the level of a reference protein in each sample to see if they are equally presented. Researchers often use a housekeeping protein as loading control such as (ß-actin, ß-tubulin, or GAPDH. Reliable assessment of the changes in target protein expression levels requires the measurement of both the target protein and the loading control protein in their linear dynamic ranges (Heidebrecht et al. 2009, Suzuki et al. 2011).

Unfortunately, it is not uncommon to see oversaturated ß-actin, ß-tubulin, or GAPDH protein band signals used as loading controls in western blotting, indicating that the detection of such housekeeping proteins was more than likely not in the linear dynamic range. The reason is simple: housekeeping proteins are the most abundant proteins in a cell or tissue while target proteins are often in low abundance. Consequently, one has to load large amounts of cell lysate (for example, 10–50 µg) in order to sufficiently detect the target protein of interest, which results in housekeeping proteins being in such high abundance so as to be out of the linear dynamic range for immunodetection.

One approach to address this dilemma is to use total protein measurement as the loading control. Researchers have explored this option by staining the membrane with protein stains such as Coomassie, SYPRO Ruby, Flamingo™, amido black, and Ponceau S (Lanoix et al. 2012, Welinder et al. 2011, Hagiwara et al. 2010, Romero-Calvo et al. 2010, Aldridge et al. 2008, Collella et al. 2012, Gürtler et al. 2013). Since total protein stains are less sensitive than antibody-based immunodetection, they are far less likely to result in an oversaturated signal. As a result, they exhibit great linearity in the common loading range of 10–50 µg of cell lysate. This way, both target protein levels—of low abundance measured using sensitive immunodetection techniques—and loading control protein levels—of high abundance measured using less sensitive total protein staining techniques—are likely to be measured in the linear dynamic range.

Stain-free technology is a novel method introduced by Bio-Rad to visualize and quantify proteins in gels and blots. This technology is a critical component in Bio-Rad's V3 Western Workflow™, which provides total protein loading control in western blots (Collella et al. 2012, Gürtler et al. 2013). In this study, we compared the stain-free technology to the immunodetection of housekeeping proteins ß-actin, ß-tubulin, and GAPDH to see which method better reflects the protein loading difference among samples at the common loading range of 10–50 µg of cell lysate.

Methods

HeLa cells were grown in 100 mm dishes and lysed in RIPA buffer supplemented with phosphatase and protease inhibitors. Protein concentration was determined using the *RC DC*™ protein assay kit. The protein lysate was mixed with 2x Laemmli buffer at 1:1 and heated for 5 min at 95°C before

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being loaded for gel electrophoresis. A serial dilution of the same HeLa cell lysate (10, 20, 30, 40, and 50 µg, or 0.5, 1, 2, 3, 4 µg) was loaded onto an 18-well Criterion™ TGX Stain-Free™ gel three times. At least two such gels were used for immunodetection of each housekeeping protein or kinase (ß-actin, ß-tubulin, GAPDH, Akt, Erk, or MEK).

The proteins were separated in Criterion TGX Stain-Free precast gels at 300 V for 20–25 min. The gel was then activated by exposure to UV light for 1 min to visualize the proteins using the ChemiDoc™ MP system. Proteins were transferred to a nitrocellulose membrane blot in 7 min using the Trans-Blot® Turbo™ transfer system. A stain-free blot image was taken using the ChemiDoc MP system for total protein measurement in each sample lane.

The blot was blocked in a blocking buffer (Rockland Antibodies & Assays, Gilbertsville, PA) for 1 hr at room temperature and probed with primary antibody overnight at 4°C at the following dilutions: GAPDH, 1:10,000; ß-actin, 1:4000; ß-tubulin, 1:4000; Akt1, 1:20,000; MEK1/2, 1:5000; Erk1/2, 1:10,000. GAPDH, ß-actin, and ß-tubulin antibodies were from Rockland. Akt, Erk, and MEK antibodies were from Cell Signaling Technologies, Danvers, MA. HRP conjugated goat anti-mouse and goat anti-rabbit antibodies from Jackson ImmunoResearch Laboratories, West Grove, PA, were applied to the blot at a dilution of 1:50,000 for 1 hr at room temperature. All antibodies were diluted in Rockland blocking buffer. Chemiluminescent signals were developed using Bio-Rad's Clarity™ western ECL substrate and captured by the ChemiDoc MP system. Image data were analyzed using Image Lab™ 4.1 software. Statistical analysis was done using Microsoft Excel software.

Results and Discussion:

1. If 10–50 µg of cell lysate were loaded in a western blot experiment, which method can truly tell the difference in loading levels among the samples?

A loading control, ideally, should truly reflect the protein load in a western blot. For example, if 10, 20, 30, 40, and 50 µg of a HeLa cell lysate were loaded on a gel, one would expect the loading control measurement from the 50 µg sample lane to be 5x the 10 µg sample lane. This measurement is labeled as "quantitative response" in our data analysis (dashed line in Figure 1).

In the first experiment, ß-actin, ß-tubulin, and GAPDH levels in 10–50 µg of HeLa cell lysate were measured by immunodetection. Although the actin and tubulin signals appeared linear in this loading range, the ratio between the housekeeping protein signals and sample load was far from 1:1. The ß-actin signal from the 50 µg lysate loading was only 2.2x the signal from the 10 µg lane. The ratios for ß-tubulin and GAPDH were 2.2 and 1.14, respectively (Figure 1). These results clearly show that housekeeping protein level measurements were not an accurate reflection of the actual total protein loaded in each lane, indicating that all the housekeeping protein signals tested were approaching the saturation level due to overload.

The stain-free blot image was then used to measure the total protein signal in each lane. The total protein signal of the 50 µg sample lane was found to be 4.8x that in the 10 µg sample lane, indicating that, by contrast, stain-free total protein measurements are an accurate reflection of the actual total protein loaded in each lane, and can therefore truly tell the load difference among the samples in the loading range of 10–50 µg.

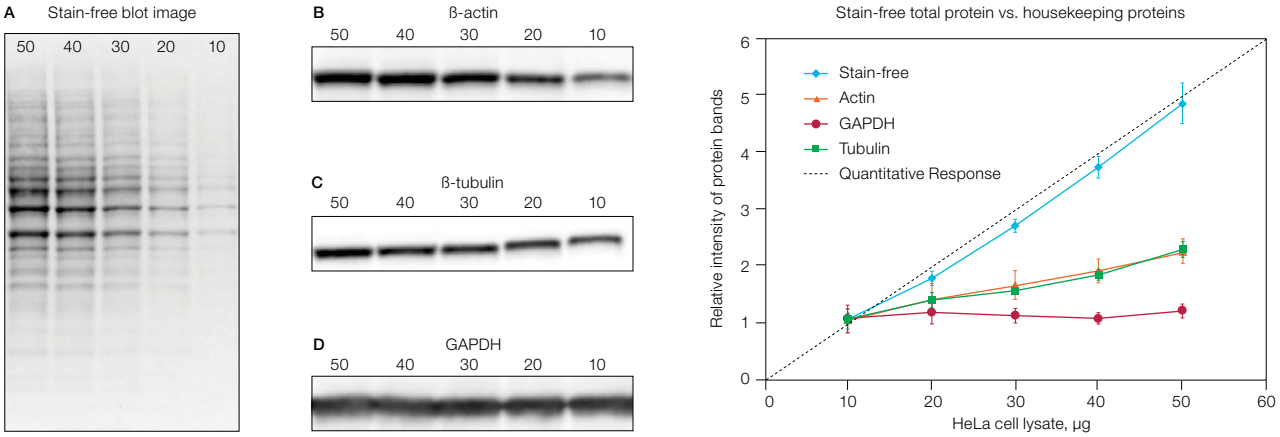

Fig. 1. Linearity comparison of stain-free total protein measurement and immunodetection of three housekeeping proteins in 10–50 µg of HeLa cell lysate. On the left are representative images of **A**, stain-free blot; **B**, ß-actin chemi blot; **C**, ß-tubulin chemi blot; and **D**, GAPDH chemi blot. Numbers on the top of the blot images are the amount of HeLa cell lysate loaded to each lane (in µg). Although the actin and tubulin signals appeared linear in this loading range, the ratio between the housekeeping protein signals and sample load was far below the predicted "quantitative response," whereas the stain-free signal correlated to the expected result.

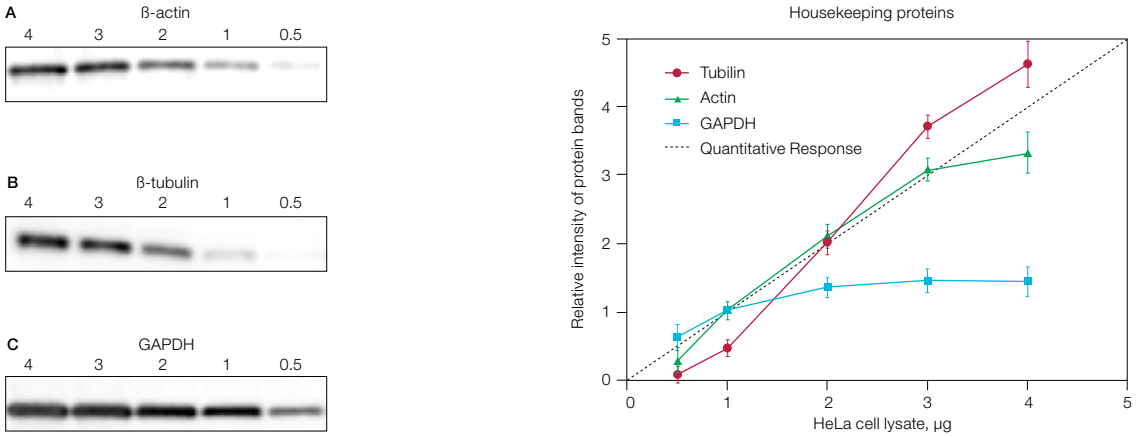

Fig. 2. Linearity of immunodetection of three housekeeping proteins in 0.5–4 µg of HeLa cell lysate. On the left are representative chemi blot images of **A**, ß-actin; **B**, ß-tubulin; and **C**, GAPDH. Numbers on the top of the blot images are the amount of HeLa cell lysate loaded to each lane (in µg). Both tubulin and actin measurement followed the predicted "quantitative response" of protein load in this loading range.

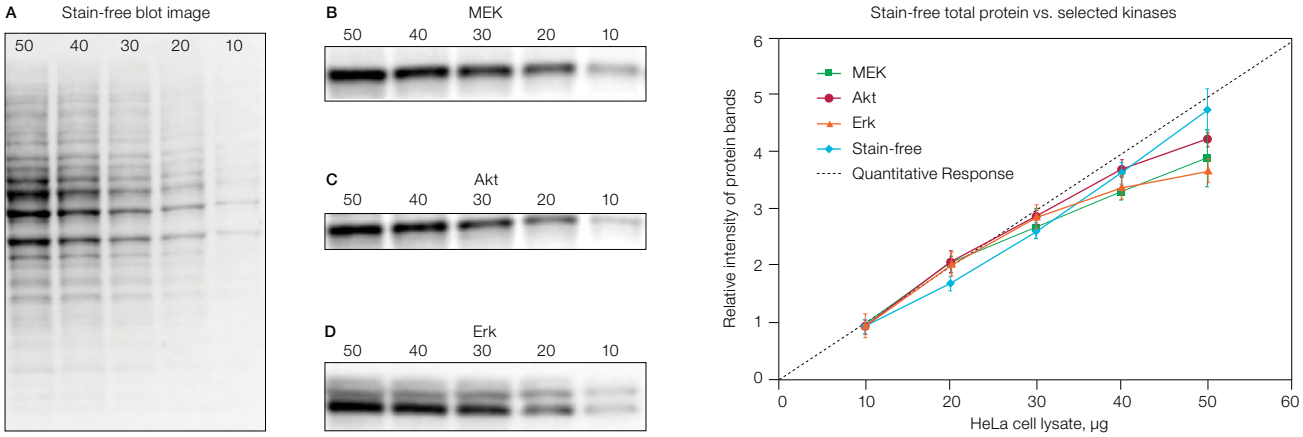

Fig. 3. Linearity comparison of stain-free total protein measurement and immunodetection of three kinases in 10–50 µg of HeLa cell lysate. On the left are representative images of **A**, stain-free blot; **B**, MEK chemi blot; **C**, Akt chemi blot; and **D**, Erk chemi blot. Numbers on the top of the blot images are the amount of HeLa cell lysate loaded to each lane (in µg). All three kinases followed the quantitative response that overlapped with the stain-free total protein measurement.

2. At what load range can housekeeping protein immunodetection truly tell the load difference?

Since the previous experiment showed that all the housekeeping proteins tested were overloaded, here we set out to find the load range that provides a quantitative response curve (dashed line in Figure 2).

In this second experiment, 0.5, 1, 2, 3, and 4 µg of HeLa cell lysate were loaded. The ß-actin signal in the 3 µg sample lane was 3.1x that of the 1 µg sample lane (Figure 2). However, the signal in the 4 µg sample lane was only 3.3x that of the 1 µg sample lane, an indication that the signal was starting to plateau. This shows that 1–3 µg was the load range where ß-actin immunodetection gave a quantitative response. ß-tubulin signal in the 4 µg sample lane was 2.3x that of the 2 µg sample lane (Figure 2). It indicates that 2–4 µg was the load range where ß-tubulin immunodetection gave a more quantitative response.

GAPDH can be detected in all five sample lanes from 0.5–4 µg of protein load. We found that the GAPDH signal in the 4 µg sample lane was only 1.4x that of the 1 µg sample lane. The result shows that much less than 1 µg of HeLa cell lysate must be loaded to find the range where GAPDH immunodetection gives a quantitative response.

3. What is the loading range where a low abundance target protein can be quantitatively measured?

The two previous experiments clearly show that stain-free total protein measurement is a more reliable and accurate loading control than housekeeping proteins in a typical western blot experiment. In many cases, researchers have to load high amounts of cell lysate in order to detect their target proteins,

which are often expressed at low levels. The next experiment sought to find out whether target proteins can be measured quantitatively in a typical load range of 10–50 µg of cell lysate.

For this third experiment, 10, 20, 30, 40, and 50 µg of HeLa cell lysate were loaded and probed for three kinases: Akt1, MEK1/2, and 44 MAPK (Erk1/2). The data show (Figure 3) that all three kinases gave a quantitative response that overlapped with the stain-free total protein measurement. However, this response started to top off at 50 µg, indicating that 10–40 µg is, indeed, an appropriate loading range to quantitatively measure these kinase levels.

Conclusions

This study demonstrated that in the typical loading range of 10–50 µg of cell lysate, the quantitation of housekeeping proteins by immunodetection is not possible because they are in saturating quantities. Studies have shown that total protein stains provide a more accurate and reliable alternative to housekeeping proteins at this loading range (Lanoix et al. 2012, Welinder et al. 2011, Hagiwara et al. 2010, Romero-Calvo et al. 2010, Aldridge et al. 2008, Collella et al. 2012, Gürtler et al. 2013). Unfortunately, in order to use these stains (for example, SYPRO Ruby and Coomassie stains), one has to use tedious procedures including staining and destaining, a major reason for their lack of widespread adoption.

Bio-Rad's V3 Western Workflow provides a practical, convenient, and reliable way to perform total protein loading control. Stain-free labeled proteins can be visualized in gels a minimum of 1 min after gel electrophoresis. Furthermore, stain-free blot visualization and image acquisition requires only a few seconds using a stain-free enabled imager. This technology makes it easy for researchers to adopt total protein loading control for western blotting.

Using stain-free total protein measurement as the loading control, researchers can ensure that both the target protein and loading control are measured in the linear dynamic range in a typical western blot experiment.

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