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

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Abstract

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 quantitate 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 loading levels in 10–50 µg of HeLa cell lysate. 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 abundance 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 such as ß-actin, ß-tubulin, or GAPDH as a loading control. 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 band signals for ß-actin, ß-tubulin, or GAPDH protein when they're 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 low in abundance. Consequently, large amounts of cell lysate (for example, 10–50 µg) must be loaded in order to provide sufficient target protein of interest for detection. This results in such high abundance of housekeeping proteins that they no longer fall in the linear dynamic range for immunodetection.

One approach to 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 and Ekblad 2011, Hagiwara et al. 2010, Romero-Calvo et al. 2010, Aldridge et al. 2008, Colella 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, levels of both a low abundance target protein, measured using sensitive immunodetection techniques, and high abundance loading control proteins, 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 quantitate proteins in gels and blots.

This technology is a critical component in Stain-Free western blotting from Bio-Rad, which provides total protein loading control in western blots (Colella 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 in the common loading range of 10–50 µg of cell lysate.

Methods

HeLa cells were grown in 100 mm dishes and lysed in radioimmunoprecipitation assay buffer supplemented with phosphatase and protease inhibitors. Protein concentration was determined using an *RC DC* Protein Assay Kit (Bio-Rad Laboratories, Inc.). The protein lysate was mixed with 2x Laemmli buffer at 1:1 and heated for 5 min at 95°C before 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, and 4 µg) was loaded onto an 18-well Criterion TGX Stain-Free Gel (Bio-Rad) three times. At least two such gels were used for immunodetection of each housekeeping protein or kinase (ß-actin, ß-tubulin, GAPDH, MEK, Akt, or Erk).

The proteins were separated in Criterion TGX Stain-Free Precast Gels at 300 V for 20–25 min. In order to visualize the proteins, the gels were then placed in a ChemiDoc MP Imaging System (Bio-Rad) for activation by exposure to UV light for 1 min. Proteins were transferred to a nitrocellulose membrane blot in 7 min using the Trans-Blot Turbo Transfer System (Bio-Rad). 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 Immunochemicals, Inc.) for 1 hr at room temperature and probed with primary antibody overnight at 4°C at the following dilutions: ß-actin, 1:4,000; ß-tubulin, 1:4,000; GAPDH, 1:10,000; MEK1/2, 1:5,000; Akt1, 1:20,000; Erk1/2, 1:10,000. ß-actin, ß-tubulin, and GAPDH antibodies were from Rockland. MEK, Akt, and Erk antibodies were from Cell Signaling Technology, Inc. HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies from Jackson ImmunoResearch Laboratories, Inc. 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 Clarity Western ECL Substrate from Bio-Rad and captured by the ChemiDoc MP System. Image data were analyzed using Image Lab 4.1 Software (Bio-Rad). Statistical analysis was done using Microsoft Excel Software.

Results and Discussion

1. **If 10–50 µg of cell lysate are 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 are loaded on a gel, one would expect the loading control measurement from the 50 µg sample lane to be five times that of 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 signals from the housekeeping protein and the sample load was far from 1:1. The ß-actin signal from the 50 µg lysate loading was only 2.2x the signal from that of the 10 µg lane. The ratios for ß-tubulin and GAPDH were 2.2x and 1.14x, respectively (Figure 1). These results clearly show

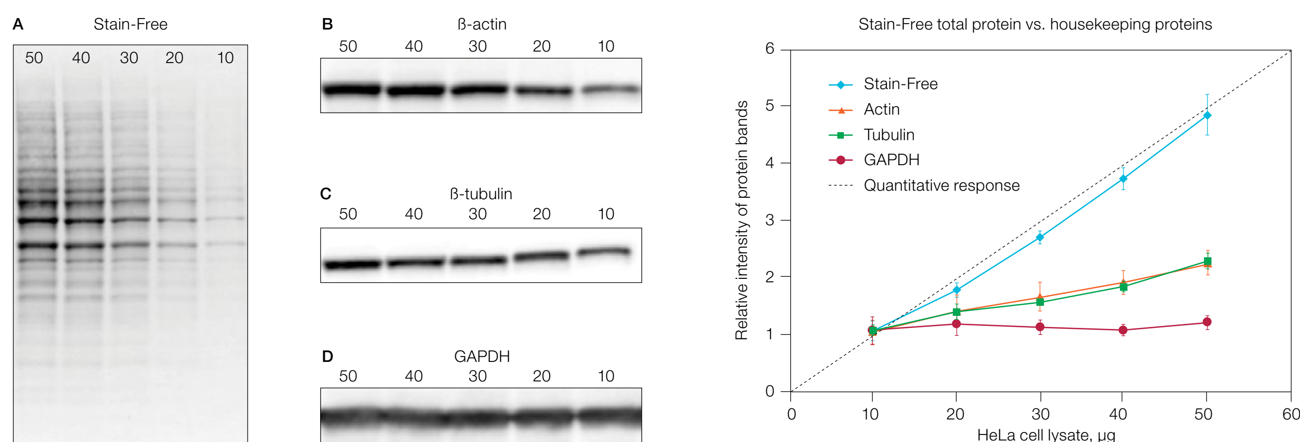

Fig. 1. Linearity comparison of Stain-Free total protein measurement and immunodetection of three housekeeping proteins in 10–50 µg of HeLa cell lysate. On the left are representative images of **A**, Stain-Free blot; **B**, ß-actin chemi blot; **C**, ß-tubulin chemi blot; and **D**, GAPDH chemi blot.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

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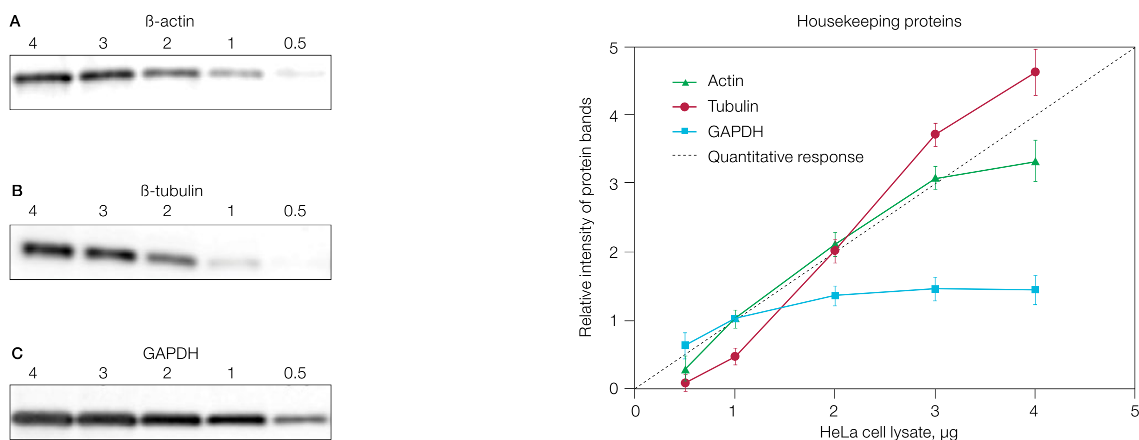

Fig. 2. Linearity of immunodetection of three housekeeping proteins in 0.5–4 µg of HeLa cell lysate. On the left are representative chemi blot images of **A**, ß-actin; **B**, ß-tubulin; and **C**, GAPDH. Numbers at the top of the blot images are the amount of HeLa cell lysate loaded to each lane (in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

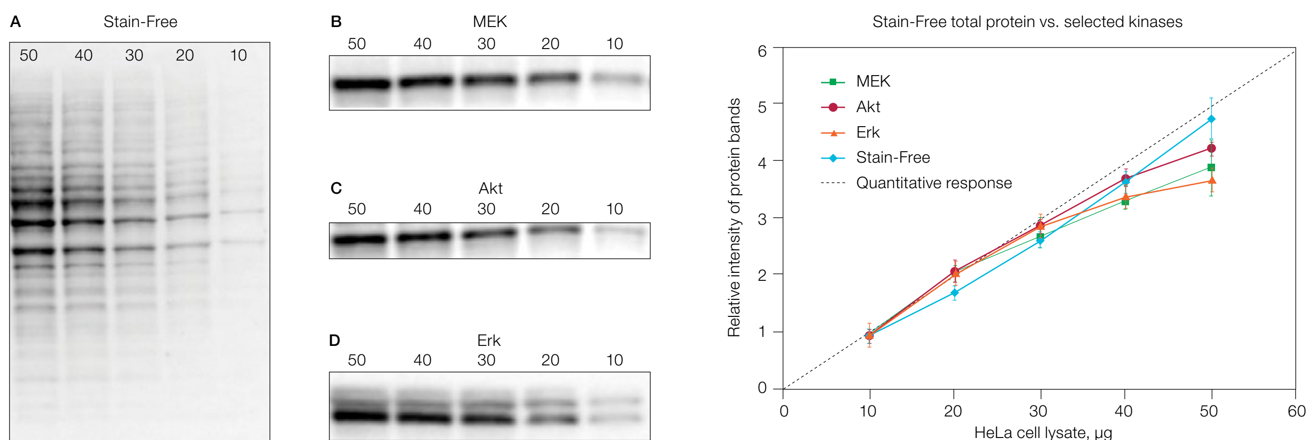

Fig. 3. Linearity comparison of Stain-Free total protein measurement and immunodetection of three kinases in 10–50 µg of HeLa cell lysate. On the left are representative images of **A**, Stain-Free blot; **B**, MEK chemi blot; **C**, Akt chemi blot; and **D**, Erk chemi blot. Numbers at the top of the 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.

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. By contrast with the housekeeping proteins, 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 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.

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 in the 1 µg sample lane (Figure 2). However, the signal in the 4 µg sample lane was only 3.3x that in 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 in the 2 µg sample lane (Figure 2). This 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 in the 1 µg

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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: MEK1/2, Akt1, 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 present in saturating quantities. Studies have shown that total protein stains provide a more accurate and reliable alternative to housekeeping proteins in this loading range (Lanoix et al. 2012, Welinder and Ekblad 2011, Hagiwara et al. 2010, Romero-Calvo et al. 2010, Aldridge et al. 2008, Colella et al. 2012, Gürtler et al. 2013). Unfortunately, using stains such as SYPRO Ruby and Coomassie requires adding tedious procedures, including staining and destaining, to the workflow; this is a major reason total protein stains have not been more widely adopted.

Stain-Free western blotting from Bio-Rad 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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