Chapter 1

Absolute Quantification of TGF-β Signaling Proteins Using Quantitative Western Blot

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Abstract

Cell signaling governs the basic functions of cells by molecular interactions that involve of many proteins. The abundance of signaling proteins can directly influence cellular responses to external signal, contributing to cellular heterogeneity. Absolute quantification of proteins is important for modeling and understanding the complex signaling network. Here, we introduce how to measure the amount of TGF-β signaling proteins using quantitative immunoblotting. In addition, we discuss how to convert the measurements of protein abundance to the quantities of absolute molecules per cell. This method is generally applicable to the absolute quantification of other proteins.

Key words Quantitative immunoblotting, Protein abundance, Transforming Growth Factor-β, Smad3

1 Introduction

Cells interact with their immediate environment through complicated cell signaling network that involves protein–protein interactions and enzymatic reactions. The concentrations of signaling proteins within a cell affect the speed of the signaling processes and influence cellular responses. Protein concentration is determined by the balance between protein production and protein degradation. It is relatively stable when cells are in steady state. As many processes regulate protein synthesis and stability, the proteome within a cell is highly dynamic and varies under different environmental conditions [1]. It has shown that variation of protein abundance contributes to the heterogeneity in individual cells and populations [2, 3]. In addition, mathematical modeling of biochemical networks needs the knowledge of absolute protein quantities [4, 5]. Therefore, getting protein abundance information is important to understand and predict the dynamics of cell systems.

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Many methods have been developed to quantify protein abundance in a high-throughput way, which include mass spectrometry [6, 7] and fluorescence microscopic analysis [8, 9]. While these approaches are powerful in measuring protein abundances in a large scale, they have some special requirements for the equipment, sample preparation, and the detection range of protein abundance, which preclude their daily applications in normal labs. On the other hand, protein amounts have been routinely quantified by western blot for decades [10]. Immunoblotting of proteins can provide measurements of absolute protein levels from a given number of cells as the absolute quantities for the immunoblotting signal of cell extracts could be inferred based on the standard curve coupled to purified protein standards [11]. When the recombinant protein standards and correct antibodies are available, quantitative western blot experiments are easily implemented for protein abundance quantification.

Transforming growth factor β (TGF-β) is a multifunctional cytokine that regulates many cellular functions in developmental processes and tissue homeostasis [12, 13]. In the canonical TGF-β signaling, TGF-β induces the formation of ligand-receptor complex with type I (TβRI) and type II TGF-β receptors (TβRII) [14]. The oligomerization of the receptors promotes activation of the TβRI, which phosphorylates the receptor-regulated Smad proteins (R-Smads, i.e., Smad2 and Smad3 for TGF-β-like signaling pathway). The R-Smads then bind to the common mediator Smad4 (co-Smad) and translocate into the nucleus [15], where they regulate the transcription of various target genes [16]. In this chapter, we described the quantitative western blot method to measure the absolute abundance of protein using a TGF-β signaling protein (Smad3) as an example. As western blot is commonly implemented in labs, we focus on the critical considerations [17–19] and data analysis in quantitative immunoblotting.

2 Materials

2.1 Cell Culture

1. Cell culture medium: Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 2 mM L-Glutamine (*see* **Note 1**), 100 units/mL penicillin, and 100 μg/mL streptomycin.
2. Cell culture plasticware: 60 mm cell culture dishes with TC surface treatment.
3. 0.05% trypsin-EDTA.
4. Dulbecco's phosphate-buffered saline (DPBS), without calcium and magnesium.
5. 0.025% EDTA/DPBS solution.

6. HaCaT cell line, a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin.
7. Cell counter.

2.2 Protein Sample Preparation

1. Cell lysis buffer (*see* **Note 2**): Radio-immunoprecipitation assay (RIPA) buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin, supplemented with 1 mM PMSF, 1 mM NaF, protease inhibitor cocktail and phosphatase inhibitor cocktails.
2. Recombinant Human Smad3 protein (*see* **Note 3**).
3. 4× Laemmli Sample Buffer: 62.5 mM Tris–HCl, pH 6.8 10% glycerol, 1% SDS, 0.005% Bromophenol Blue (*see* **Note 4**).
4. β-mercaptoethanol (or dithiothreitol).
5. BCA protein assay kit.
6. Cell scrapers for adherent cells.
7. 1.5 mL microfuge tubes.
8. 96-well microplate.

2.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Precast polyacrylamide gel (*see* **Note 5**).
2. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1% SDS.
3. Prestained NIR Protein Ladder.
4. Power Supply.
5. Electrophoresis chamber.

2.4 Immunoblotting

1. Nitrocellulose membranes.
2. Western blot transfer buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20% methanol. We used premixed transfer buffer in Bio-Rad Trans-Blot Turbo RTA transfer kit.
3. Protein transfer apparatus. We used Bio-Rad Trans-Blot Turbo Transfer System, which provides rapid transfers with high efficiency.
4. Tris-buffered saline Tween (TBST): 137 mM Sodium Chloride, 20 mM Tris, 0.1% Tween-20. Supplied at pH 7.6. Store at room temperature.
5. Blocking buffer: 1× TBST with 5% w/v BSA.
6. Primary antibodies: Smad3 (Cell signaling, Smad3 Rabbit mAb #9523), diluted to 1:1000 in blocking buffer.
7. Secondary antibody: Anti-mouse IgG (H + L) (DyLight 680 Conjugate) diluted in blocking buffer.

8. Filter paper.
9. Plastic container.
10. Tweezer.
11. Western blot incubation box.
12. Rocking platform.
13. Western blog imaging system (*see* **Note 6**). We used the Odyssey CLx Near-Infrared Fluorescence Imaging System.

2.5 Data Analysis

1. Computer.
2. Image quantification software (e.g., LI-COR Empiria/Image Studio or ImageJ).
3. Microsoft Excel.

3 Methods

All the experimental procedures should be performed at room temperature unless different temperature is specified.

3.1 Cell Culture

1. HaCaT cells are cultured with cell culture medium in a humidified CO2 incubator (37 °C and 5% CO_2).
2. Seed 1.5×10^6 HaCaT cells per 60 mm cell culture dish, let cells settle and grow overnight (*see* **Note 7**).
3. In day 2, trypsinize the cells and count cell numbers. Remove the culture medium and rinse HaCaT cells with DPBS. Add 0.025% EDTA/DPBS solution and incubate for 10 min at 37 °C. The cell sheet must be covered completely. Remove EDTA, add fresh trypsin/EDTA solution and incubate at 37 °C until the cells detach (approximately 3–5 min). Stop the trypsin activity using cell culture medium and collect them in 15 mL conical tubes. Dilute the cells with cell culture medium to a proper volume and count cell numbers with the hemocytometer or automated cell counter. Cell numbers from at least 3 dishes should be counted, record cell numbers for each dish. The average of cell numbers from multiple dishes will be used for calculating absolute protein abundance later.

3.2 Cell Lysis and Protein Extraction for Western Blotting

1. Remove the cell culture medium from the dishes used for cell lysate preparation. Wash cells twice with ice-cold DPBS and aspirate DPBS. If cell lysate is not prepared immediately, freeze the cells by carefully pouring liquid nitrogen into each dish. Store dishes at −80 °C until ready to lyse.
2. Lyse cells by adding 250 μL cell lysis buffer to each 60 mm dish. Immediately scrape the cells off the dishes and transfer the extract to 1.5 mL microcentrifuge tubes.

3. Rotate the thawed cell extracts for 1 h at 4 °C cold room. Then spin down the insoluble material in a microfuge for 10 min at 16,000 × *g* at 4 °C.
4. Gently remove the tubes from the centrifuge and place on ice, transfer the supernatant extracts to labeled 1.5 mL microcentrifuge tubes and kept on ice. Aliquot 10 μL lysate and measure protein concentrations in a 96-well microplate with BCA protein assay kit following the manufacturer's instructions.
5. Add 4× Laemmli Sample Buffer with reducing reagent (e.g., β-mercaptoethanol) to the cell lysates (loading buffer to cell lysate volume, 1:3). Cap the microcentrifuge tubes tightly and boil them in a heat block at 95 °C for 5 min, let them cool down, followed by spinning down with a centrifuge.
6. Either freeze the samples at −20 °C (or −80 °C) for later use or proceed directly to the SDS-PAGE steps.

3.3 Run SDS-PAGE

1. Remove the gels from the storage pouch and pull out the comb carefully according to the manufacturer's instructions. Use a wash bottle, or disposable transfer pipet to rinse the wells with SDS-PAGE running buffer.
2. Mount the precast gel to the gel cassettes, assemble the gel apparatus, and fill the reservoirs with SDS-PAGE running buffer.
3. Calculate and load equal amount of protein samples with gel-loading pipette tips (10–30 μg). The protein ladder is loaded in the first lane (*see* **Notes 8** and **9**).
4. Connect the gel apparatus to the power supply and perform electrophoresis on constant current or voltage mode (*see* **Note 10**).
5. After electrophoresis, turn the power off and disconnect the electronic leads. Open the gel cassettes and remove the gel by floating it off the plate into a container with transfer buffer for immunoblotting.

3.4 Immunoblotting

1. After electrophoresis, assemble the blotting sandwich, load and run the transfer based on the instructions of western blotting transfer system (*see* **Note 11**). For the quantification of Smad3 protein abundance, we used a piece of nitrocellulose membrane.
2. (Optional) Upon the completion of the transfer, wash the nitrocellulose membrane once with TBST for 3 min.
3. Incubate the membrane with blocking buffer for 1 h on a rocking platform (*see* **Note 12**).
4. Wash the membrane three times with TBST for 5 min each.

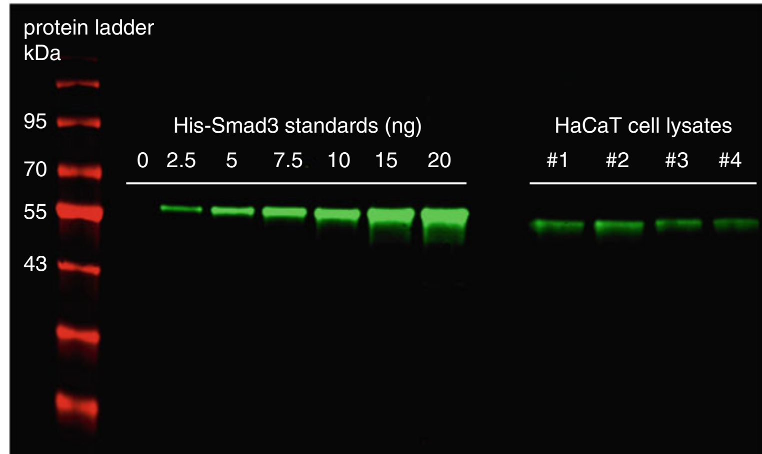


Fig. 1 Quantification of the absolute abundance of endogenous Smad3 protein in HaCaT cells using a fluorescent western blot. Serial dilutions of the His-Smad3 protein were loaded in a SDS-PAGE gel together with 4 cell lysate samples from 2.1×10^5 untreated HaCaT cells. The molecular weight of His-Smad3 is predicted as 51 kDa

5. Incubate membrane and primary antibody solution (at the appropriate dilution as recommended in the product datasheet) with gentle agitation on a rocking platform overnight at 4 °C (*see* **Note 13**).
6. Wash the membrane three times with TBST for 5 min each.
7. Incubate membrane with secondary antibody solution (at appropriate dilution as recommended in the product datasheet) for 1 h at room temperature (*see* **Note 14**).
8. Wash the membrane three times with TBST for 5 min each.
9. Drain excess TBST solution from the membrane and place it in the Western blot imaging system. Scan the membrane and detect protein signal according to the instructions of the imaging system. Save the acquired imaging data (Fig. 1).

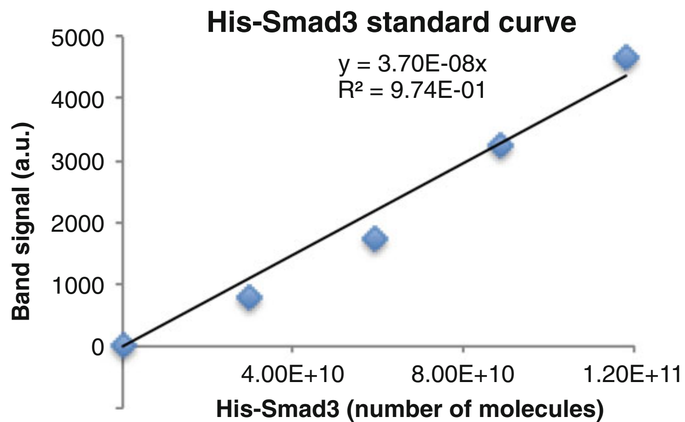
3.5 Data Analysis of Western Blot Images

The signal of the targeted protein shown in the bands of the membrane can be quantified with different imaging software tools. The general quantification pipeline described here is based on LI-COR Studio quantification software. Alternatively, ImageJ can be used to quantify the band intensity [19].

1. Open or import the acquired western blot image, Click the Analysis tab to open the Analysis ribbon.
2. Click Add Rectangle to add a rectangle to the image. Move the rectangle to the target protein band and adjust the size to match the band shape (*see* **Note 15**). Repeat this process until

A

His-Smad3 (ng)	His-Smad3 (number of molecules)	Band signal (a.u.)
0	0.00E+00	-0.427
2.5	2.95E+10	792
5	5.90E+10	1750
7.5	8.86E+10	3250
10	1.18E+11	4680



B

HaCaT lysates	Cell numbers in the sample	Band signal (a.u.)	Number of Smad3 molecules	Absolute abundance (molecules/cell)
#1	2.10E+05	1310	3.5E+10	**1.69E+05**
#2	2.10E+05	1410	3.8E+10	**1.81E+05**
#3	2.10E+05	936	2.5E+10	**1.20E+05**
#4	2.10E+05	805	2.2E+10	**1.04E+05**

Fig. 2 The calculation of the absolute abundance of endogenous Smad3 protein in HaCaT cells using a His-Smad3 standard curve. (**a**) The His-Smad3 dilutions generate a linear standard curve, which was fit using Excel's Trendline tool. (**b**) The number of endogenous Smad3 molecules in each HaCaT cell lysate sample is first calculated based on the linear regression equation that is fitted to the His-Smad3 standard curve. Then, the absolute abundance of Smad3 molecules per cell can be further calculated

all the target protein bands are covered with the quantification shapes (*see* **Note 16**).

3. Click the first button in background group and select "Median" from the drop-down menu (*see* **Note 17**), set the border size, segment with "All," "Top/Bottom," or "Right/

Left" to define the background area. The background options need to be adjusted depending on the noise and non-specific bands in the image.

4. Click the Shapes tab at the bottom of the table to open the Shapes Table. Select the rows for the protein bands and Click the "Copy" button.
5. Open Microsoft Excel and paste the quantified data to a new datasheet. Annotate the names for the bands (*see* **Note 18**). The "Signal" column is the quantification for the bands after background subtraction.
6. Convert the amount of loaded recombinant His-Smad3 protein standards to an absolute number of molecules with the following equation:

$$Ns = 6.022 \cdot 10^{23} \cdot \frac{\text{weight of loaded recombinant protein (g)}}{\text{Molecular weight of recombinant protein (Da)}}$$

7. Calculate the number of cells in the loaded cell lysate sample with the following equation (*see* **Note 19**):

$$Nc = \text{number of cells in a dish} \cdot \frac{\text{loaded cell lysate volume } (\mu\text{L})}{\text{lysate volume per dish } (\mu\text{L})}$$

8. Plot signal intensity vs. sample loading for the recombinant His-Smad3 protein standards as shown in Fig. 2a (*see* **Notes 20** and **21**).
9. Select the plot of the standard curve, right click and choose "Add Trendline." In the "Trendline Options," select "Linear," "Display Equation on chart," and "Display R-squared value on chart" (*see* **Note 22**).
10. Calculate the number of Smad3 protein molecules for the loaded samples with the following equation:

$$Nq = \frac{y - b}{a}$$

where y is the quantified band intensity signal for the loaded cell lysate sample, a is the slope of the linear regression line, and b is the intercept of the linear regression line.

11. Calculate the absolute abundance (number of molecules per cell) by dividing the number of molecules per band by the number of cells (Fig. 2b).
12. More information about the quality control and pitfalls for quantitative western blot analysis can be found in the refs. 18, 20.

4 Notes

1. L-glutamine is an essential amino acid nutrient for cell culture. However, it can decompose into ammonia, which is toxic to cells. To minimize this effect, researchers can use an alternative formulation of L-glutamine such as Gibco GlutaMAX and Lonza UltraGlutamine supplements, which are designed to be more stable in solution.
2. Stock RIPA buffer can be purchased from different suppliers. Store this buffer at right temperature according to the product information. PMSF is unstable in water and should be added to cell lysis buffers just prior to use.
3. Here, we assume that the stock concentration of the recombinant protein is already known. If not, the abundance or concentration of the stock recombinant protein can be measured using Coomassie staining method with bovine serum albumin (BSA) standards [19]. As protein can be degraded during transportation, it is recommended to check the purity and the concentration of the stock recombinant protein after the delivery even the concentration is labeled by the supplier.
4. Add reducing agent (2-mercaptoethanol or dithiothreitol) to the Laemmli sample buffer before mixing with cell lysate samples.
5. It is possible to use freshly prepared protein gels as protein gels contain acrylamide, which is a neurotoxin when it is unpolymerized. Please wear mask and avoid skin contact when weighing acrylamide.
6. Both chemiluminescent and fluorescent imaging systems are available for data acquisition of immunoblotting results. However, fluorescent imaging systems are recommended for quantitative western blots when the primary antibody works well. Fluorescent western blot detection has several advantages compared to enzyme-based chemiluminescent substrate detection because it provides stable signals and quantitative results.
7. Prepare multiple cell culture dishes for measuring the protein abundance with replicates. Reserve about 3 dishes for counting cell numbers.
8. Load empty wells with 1× Laemmli Sample Buffer. This can help to prevent samples from spreading out as they move through the gel, which could avoid potential band distortions.
9. Please reserve 6 lanes for loading a standard curve of the recombinant protein. In our experiments, serial dilutions with 0–20 ng of recombinant protein usually generate a good linear signal detected with LI-COR Odyssey Imaging System. The loaded volume corresponding the amount of lysate from

different numbers of cells needs to be optimized. In our experimental setup, the variations of loading volume correspond to the lysates ranging from 1×10^5 to 4×10^5 cells. The optimal loading volume depends on the cell type, the measured protein, and the primary antibody. Therefore, the linear range of the standard curve and the sample loading must be determined individually for the target protein.

10. Use the voltage or current settings recommended in the manual for the electrophoresis device you are using. Excessive voltage or current leads to decreased band resolution and band distortions. In addition, running the gel electrophoresis slowly and in cold room could improve band resolution.
11. We used the Bio-Rad trans-blot Turbo transfer system, which provides fast and efficient transfer for most of protein. For proteins with a large molecular weight, wet-tank transfer system might be needed.
12. Blocking buffers help to decrease non-specific signaling generated by non-specific binding of proteins and essentially block western blot noise. Western blot result can be improved by utilizing the appropriate blocking buffer. Please try different blocking buffers to optimize the western blot result with your primary antibody.
13. For fast western blot, it is also possible to incubate the membrane primary antibody at room temperature for about 3 h.
14. It is recommended in the Odyssey CLx Imager that the IRDye 800CW secondary antibody (800 nm channel) be used to detect the lower-abundance protein target and IRDye 680RD secondary antibody (700 nm) to detect the more abundant protein.
15. Shapes are added to all selected channels in Odyssey Imager. To add shapes to only one channel, make sure only the channel for the target protein selected in the Display panel on the right.
16. It is possible to use Copy and Paste to create new shapes for other bands. Alternatively, the bands can be automatically detected with LI-COR Studio quantification software, which might work well for good western blot results without no-specific bands nearby target protein bands.
17. In general, it is recommended to use median background signal for background subtraction because this can avoid the interference of artificial speckled background (e.g., dots with strong signal).
18. All data fields will be copied in LI-COR Studio software, but "Signal" is the field of interest for analysis.
19. Please note that "loaded cell lysate volume" in this equation is not the loaded volume in the gel because the Laemmle sample

buffer (4×) is added during sample preparation. For example, a sample loaded with 40 μL contains 30 μL cell lysate and 10 μL sample buffer (4×). Therefore, the corresponding "loaded cell lysate volume" should be 30 μL, not 40 μL.

20. Check whether the signal intensities of measured samples fall within linear range for the recombinant protein standards. If not, load different amounts of samples together with the recombinant protein standards in a preliminary experiment and choose appropriate amount of sample to load that generate a band signal fall in the middle of the linear range for the recombinant protein standards.
21. The predicted molecular weight of the recombinant protein is usually provided in the supplier's product datasheet. If this information is not available, the molecular weight for the recombinant protein sequence can be computed using the ExPASy "Compute pI/MW" tool (https://web.expasy.org/compute_pi/).
22. In the display of the regression equation, Excel might round very large or very small numbers shown in the equation, which makes the calculation not precise. To solve this problem, right click the equation, select "Format Trendline Label" and set "Scientific" for the "Number" option.

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