Normalization in western blotting to obtain relative quantitation

Introduction

Western blotting combined with advanced digital imaging has become a powerful tool for measuring protein abundance and protein modifications. With the latest advances in imaging software and instrument sensitivity, quantitative western blot analysis is now easier to achieve.

Normalization is a critical step in obtaining reliable and reproducible quantitative western blotting. Under ideal conditions, normalization would not be necessary, but factors such as sample loading and transfer efficiency make normalizing the western blot essential. This technical note provides the basic principles of normalization using internal loading controls and describes how to accurately normalize western blots to obtain meaningful, reproducible data. All data were imaged using the Invitrogen™ iBright™ FL1000 Imaging System.

Normalization using housekeeping proteins (internal loading controls)

Choosing a housekeeping protein is an important aspect of western blot normalization. For various reasons, not all housekeeping proteins can be equally utilized for normalization studies in all biological test systems. The housekeeping protein of choice depends largely on the target protein of interest. For example, if a chemiluminescence or one-color fluorescence system is being used for target detection, the housekeeping protein should not interfere with detection of the target (e.g., should not be of similar molecular weight). To begin using internal loading controls, the quantitative accuracy and linear range of any housekeeping protein must be assessed before performing western blot normalization. The signal obtained for the housekeeping protein should be linear over a wide concentration range, such that it can be used as a reliable reference for normalization.

Prerequisite 1: Determine the linear range of the desired housekeeping protein's signal with a dilution series

The key to using an internal loading control is to ensure use within the range where there is a linear relationship between the amount of protein on the membrane and signal intensity observed. The linear range of any housekeeping protein can be assessed using a dilution series of the cell or tissue extracts of interest.

As an example, the usability and linear range of five common housekeeping proteins (β-tubulin, cyclophilin B, GAPDH, β-actin, and heat shock protein 90α (HSP90α)) were tested in HeLa cell extracts using a 2-fold dilution series, starting with 50 µg of lysate. Replicate immunoblots were developed and analyzed, where the primary antibody concentrations and exposure times were both varied (Figures 1 and 2).

With the experimental conditions utilized, the β-tubulin signal was found to be linear up to 50 µg of HeLa cell lysate using a 1 min exposure time and a primary antibody dilution of 1:1,000 (Figure 1). However, at the same primary antibody dilution, longer exposure times of 2 and 5 min caused signal saturation at the higher amounts of total protein. Saturated band signals cannot be used for normalization because the amount of detected signal is not proportional to the amount of protein in the corresponding band. Saturation of the β-tubulin signal was eliminated at longer exposure times when a higher dilution (lower concentration) of primary antibody was utilized (1:2,500).

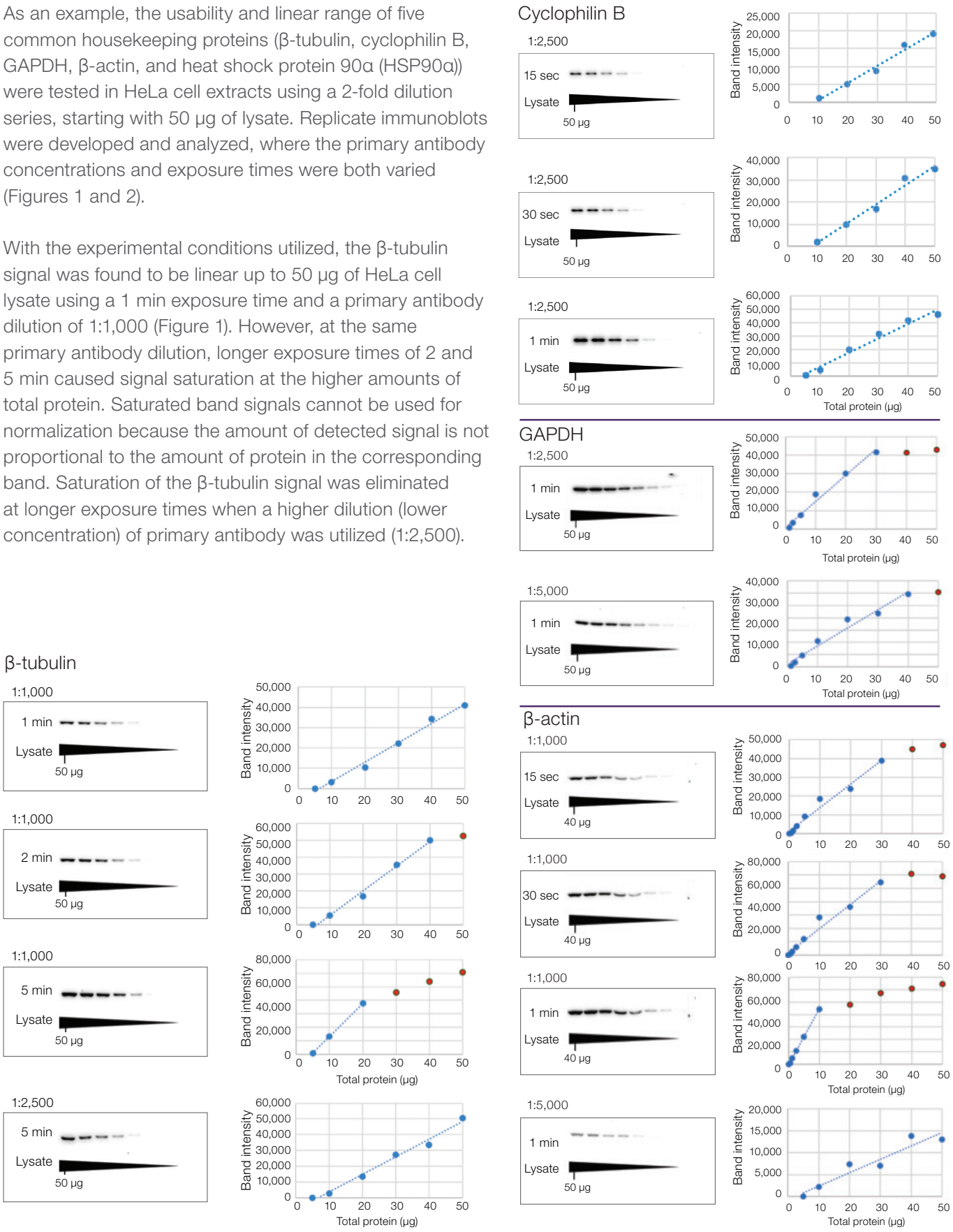

Figure 1. Linearity and signal saturation of housekeeping proteins β-tubulin, cyclophilin B, GAPDH, and β-actin in HeLa cell extracts. The points on each graph that fall within the linear range are shown in blue; the points outside the linear range are shown in red.

Because the linear range for each protein varies depending on abundance and experimental conditions, the concentration of primary antibody used in a particular experimental system should be dictated by the target that is being quantified—and the target protein must be detected within the same linear range as the housekeeping protein. For example, if the target protein requires longer exposure times due to low abundance, a lower concentration of primary antibody for the housekeeping protein may need to be used. It should also be noted that linear ranges of proteins can change if a different cell or tissue type is being used.

When testing other commonly used housekeeping proteins in HeLa cell extracts, such as cyclophilin B, GAPDH, and β-actin, various linear ranges were observed (Figure 1). For cyclophilin B, the signals were linear up to 50 µg of total protein, regardless of exposure time. The GAPDH signals displayed a linear range up to 30 µg when the primary antibody was used at a 1:2,500 dilution, which could be increased to 40 µg when the antibody concentration was reduced to a 1:5,000 dilution. Similarly, the linear range of β-actin signals was observed to be relatively narrow at a 1:1,000 primary antibody dilution, but decreasing the primary antibody concentration to a 1:5,000 dilution increased the linear range to 50 µg of total protein.

An example of a housekeeping protein that was a poor choice for normalization for the HeLa cell extract system utilized in this study is HSP90α (Figure 2). In this example, the signal of HSP90α was too high for the system used, and saturation was reached at a relatively low amount of protein. The linear range was relatively narrow, and the relationship between signal and protein amount became hyperbolic at higher total protein concentrations, making use of this protein prone to inaccurate quantitation, even when using a higher dilution of primary antibody.

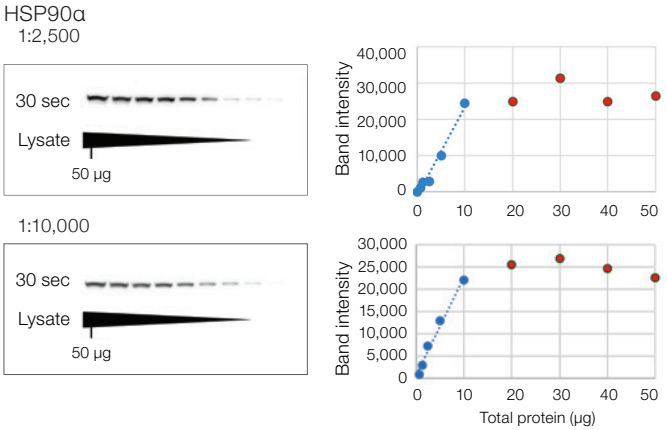

Figure 2. Linearity and saturation of housekeeping protein HSP90α in HeLa cell extracts. The points on each graph that fall within the linear range are shown in blue, whereas the points outside the linear range are shown in red.

Prerequisite 2: Confirm abundance of housekeeping protein is constant across experimental conditions

It is important that the protein chosen for the internal loading control has an abundance that is as close to constant as possible across the different experimental conditions that will be evaluated. This can be determined by performing a western blot on equal amounts of the different experimental lysates or samples that will be used to detect the target. As an example, HeLa cells were treated with staurosporine, a broad-spectrum inhibitor of protein kinases. Two housekeeping proteins were tested for consistency and abundance across the two experimental conditions. As seen in Figure 3, similar levels of β-tubulin and GAPDH were detected in untreated and treated cells, and detection of both housekeeping proteins remained linear in the range of exposure times used for signal detection.

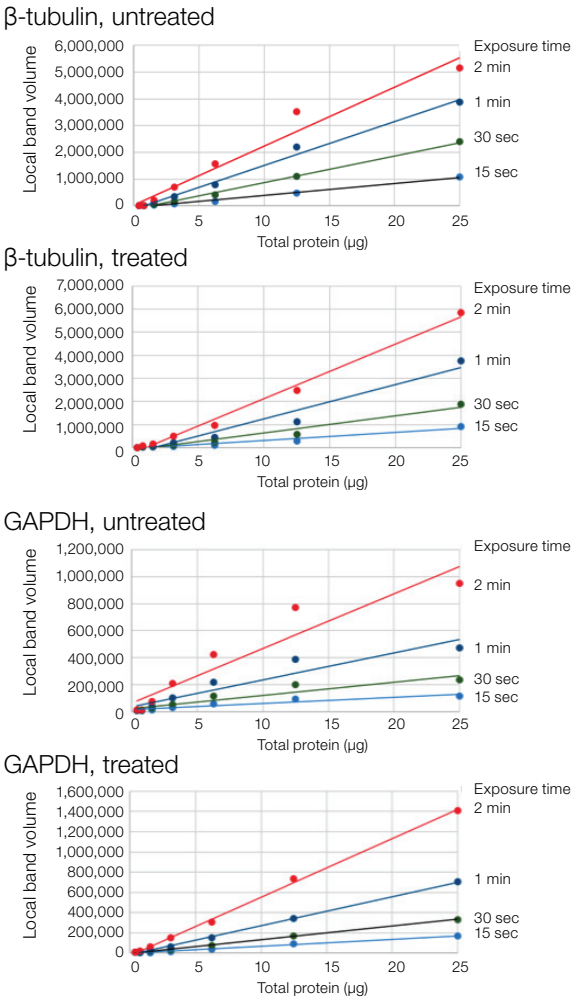

Figure 3. Detection of β-tubulin and GAPDH in extracts of untreated and staurosporine-treated HeLa cells.

Data analysis—normalizing your western blot data

Once a housekeeping protein has been validated, it can be used to normalize the data. Normalization factors must be calculated for each blot; do not use the same normalization factors among different blots.

Step 1: Quantify experimental target and loading control signal in each lane

The first step in western blot normalization is to quantify the protein signals (e.g., intensity or density) of the experimental target and housekeeping protein in each lane. This can be performed using Invitrogen™ iBright™ Analysis Software or other analysis software. Each software program will provide a different numerical value for signal, depending on the algorithm used, but the relative signal relationships between bands will be the same. Once signal values are determined for each band, the background signal should be subtracted from the signal of each individual band. This may be done automatically, depending on the software used.

As an example, HeLa cells were subjected to a range of concentrations of staurosporine for 3 hours to assess the level of cleaved poly (ADP-ribose) polymerase (PARP), a family of proteins involved in a number of cellular processes such as DNA repair, genomic stability, and programmed cell death. The relative level of cleaved PARP was normalized to GAPDH (Figure 4). Signal intensities were determined using iBright Analysis Software.

Step 2: Calculate the lane normalization factor

To determine the normalization factor for each lane that will be used to normalize the experimental intensity values, the highest signal detected for the housekeeping protein must be located. The value of this band will be used to normalize the rest of the housekeeping bands on the blot. To determine the lane normalization factor, the value of the observed signal for the housekeeping protein in each lane should be divided by the highest observed housekeeping protein signal on the blot. In our example, because lane 4 had the highest GAPDH signal, the signal values for GAPDH in all other lanes were divided by the signal value of GAPDH in lane 4 (Figure 5).

Lane number	Condition (µM staurosporine)	GAPDH signal	Cleaved PARP signal
1	0	1,691,755	0
2	0.125	1,527,534	416,165
3	0.25	1,780,149	1,034,564
4	0.50	1,869,046	1,342,778
5	1.00	1,370,796	1,783,420
6	2.00	1,072,007	1,247,725

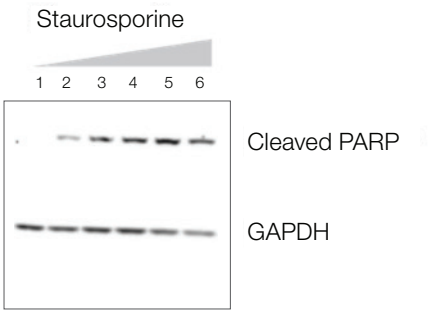

Figure 4. Signal intensities of cleaved PARP and GAPDH from HeLa cells subjected to a range of concentrations of staurosporine.

$$\text{Lane normalization factor} = \frac{\text{Observed signal of housekeeping protein for each lane}}{\text{Highest observed signal of housekeeping protein on the blot}}$$

Lane number	GAPDH signal	Normalization factor
1	1,691,755	0.91
2	1,527,534	0.82
3	1,780,149	0.95
4	1,869,046	1.00
5	1,370,796	0.73
6	1,072,007	0.57

Figure 5. Determination of lane normalization factor. Normalization factors were determined using the lane normalization factor equation above.

Step 3: Calculate the normalized signal of target to be used for relative quantitative comparison

To calculate the normalized signal of each experimental target band, the observed signal intensities of each experimental target band should be divided by the lane normalization factor (Figure 6).

The combination of steps 1–3 results in normalized signals for the experimental targets. With these values, the experimental data can now be compared across biological replicates. In our example, the experiment was repeated with three biological replicates using GAPDH and β-tubulin as loading controls to observe trends after treatment with staurosporine (Figure 7). Whether GAPDH or β-tubulin was used for normalization of cleaved PARP, the trends observed with staurosporine treatment were similar.

$$\text{Normalized experimental signal} = \frac{\text{Observed experimental signal}}{\text{Lane normalization factor}}$$

Lane number	Cleaved PARP signal	Lane normalization factor	Normalized signal
1	0	0.91	0
2	416,165	0.82	507,518
3	1,034,564	0.95	1,089,015
4	1,342,778	1.00	1,342,778
5	1,783,420	0.73	2,443,041
6	1,247,725	0.57	2,188,991

Figure 6. Determination of normalized signals of target bands.

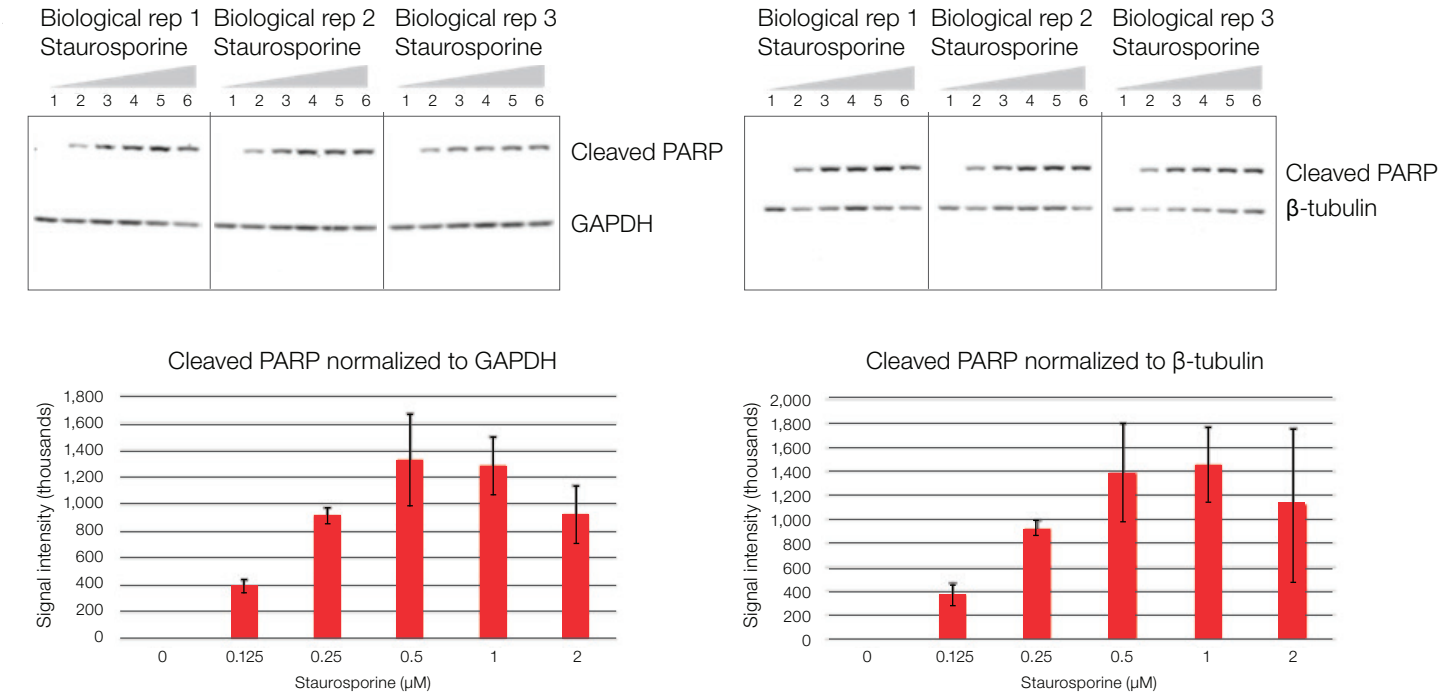

Figure 7. Effect of staurosporine on the cleavage of PARP. Signal intensities of cleaved PARP from HeLa cells subjected to a range of concentrations of staurosporine were normalized to GAPDH and β-tubulin.

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Conclusions

With appropriate calculations, sample signals can be accurately normalized to housekeeping proteins designated as internal loading controls to obtain quantitative western blot analysis. An accurate loading control should display a linear relationship between signal intensity and sample load in all experimental conditions. When implemented, normalization should correct for the sources of variability inherent in the western blotting process.

Methods

Cell treatment

HeLa cells were counted using the Invitrogen™ Countess™ Automated Cell Counter, and 2.0 x 10^6 HeLa cells were seeded per 10 cm dish and allowed to adhere overnight. The next day, cells were either left untreated or treated with various concentrations of staurosporine for 3 hours (0, 0.125, 0.25, 0.50, 1.0, 2.0 µM). Following treatment, cells were harvested by centrifugation and cell pellets were lysed using Thermo Scientific™ Pierce™ IP Lysis Buffer (Cat. No. 87788). Protein concentrations of cell lysates were determined using the Thermo Scientific™ Pierce™ Rapid Gold BCA Protein Assay Kit (Cat. No. A53225) along with the Thermo Scientific™ Pierce™ BSA Standard Pre-Diluted Set (Cat. No. 23208).

Western blots

Whole extracts of HeLa cells were mixed with Thermo Scientific™ Pierce™ Lane Marker Reducing Sample Buffer (Cat. No. 39000). Various amounts of total protein (serial dilutions containing 50 µg to 1.6 µg) were loaded onto an Invitrogen™ Novex™ 4–20% Tris-Glycine Plus 20-Well Midi Gel (Cat. No. WXP42020BOX). Following separation, proteins were transferred to a Thermo Scientific™ Nitrocellulose Membrane (Cat. No. 88018) using the Thermo Scientific™ Pierce™ Power Blotter (Cat. No. 22834) and Pierce™ 1-Step Transfer Buffer (Cat. No. 84731). Membranes were blocked in 5% nonfat dry milk in Thermo Scientific™ Pierce™ TBS Tween™ 20 Buffer (Cat. No. 28360), and then probed with one or more Invitrogen™ primary antibodies against β-tubulin (Cat. No. MA5-16308), GAPDH (Cat. No. MA5-15738), β-actin (Cat. No. MA5-15739), cyclophilin B (Cat. No. PA1-027A), HSP90α (Cat. No. PA3-013), and cleaved PARP (Cat. No. 44-698G), followed by the appropriate Invitrogen™ secondary antibody: Goat Anti–Mouse IgG, HRP (Cat. No. 31430) or Goat Anti–Rabbit IgG, HRP (Cat. No. 31460). Blots were developed using Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate (Cat. No. 34076) and imaged using the iBright FL1000 Imaging System (Cat. No. A32752). Exposure times are indicated in each figure.

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