Published in final edited form as:

Anal Biochem. 2007 February 15; 361(2): 305–307.

ON THE USE OF RATIO STANDARD CURVES TO ACCURATELY QUANTITATE RELATIVE CHANGES IN PROTEIN LEVELS BY WESTERN BLOT

Aaron Pitre, (BSc), Yihang Pan, (MSc), Steve Pruet, (PhD), and Omar Skalli, (PhD)

Dept. of Cellular Biology and Anatomy and Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA

Relative changes in the expression of a specific protein are commonly measured on western blots by forming the ratio of the densitometric values of bands containing the protein between control and experimental samples. It is generally assumed that this analysis provides an accurate determination of relative changes in a specific protein expression if there is a linear relation between increasing amounts of that protein, as represented by bands on a western blot or a gel, and the densitometric measurements of these bands. In this study, we provide direct evidence that this assumption is invalid because even in the presence of a linear relation, densitometric ratios differ substantially from known actual ratios of protein amounts. We present also the mathematical basis for this bias, and demonstrate that it can be circumvented by using a “ratio standard curve”. Alternatively, the use of purified protein standards to plot a standard densitometry curve can solve this problem, but a survey of the literature shows that most investigators who use western blots to quantify relative changes in protein expression samples do not have standards for the specific proteins that they are investigating.

To examine whether densitometric ratios accurately reflect ratios between protein amounts, we performed experiments to silence lamin C (a nuclear envelope protein) in U-373 MG glioblastoma cells. These cells were grown as described earlier [1], and were transfected with a lamin C specific siRNA (5'-CUGGACUCCAGAAGAdTdT-3' and 5'-UGUUCUUCUGGAAGUCCAGdTdT-3' for the sense and anti-sense sequences, respectively) using siLentFect lipid reagent (Bio-Rad) according to the manufacturer's instructions. Control cells were treated with transfection reagent and vehicle. One and two days after transfection, the cells were processed for SDS-PAGE as described earlier [1]. Protein concentrations were measured with the bicinchoninic acid assay [2].

Gels were loaded with incremental amounts (4, 8, 20, 40, and 80 g) of total cellular proteins from control samples, as well as with 80 g of total cellular protein for experimental samples. Each loading was performed in duplicate, and all the loadings were applied to the same gel to insure identical treatment during the western blotting procedure, which was performed following standard methods [3]. In addition, to avoid band distortion, sample loadings were brought to the same final volume with sample buffer. After blocking in PBS plus 5% dry milk, the blots were incubated with a rabbit anti-lamin antibody (Chemicon, Temecula, CA) diluted at 1:100 in blocking solution, and then with goat anti-rabbit IgGs conjugated to peroxidase (KPL, Gaithersburg, MD) diluted at 1:1000 in blocking solution. After incubation of the blots

Address correspondence to: Dr. Omar Skalli, Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA. Tel.: (318) 675-8324. Fax: (318) 675-5889. E-Mail: oskalli@lsuhsc.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

with the peroxidase substrate, the lamin C bands were evaluated by densitometry with a flat bed, 2400 dpi resolution scanner (Epson Perfection 2450, Epson) and the Un-Scan-It software (version 6.1, Silk Scientific Co., Orem, UT). Densitometric values the bands were corrected for background. Each data point represented the mean of duplicate loadings. Linear regression and curve-fitting analysis of the data were performed with Table Curve 2D (v5.0; SPSS Science, Chicago, IL).

The densitometric values of the lamin bands in the incremental loadings of control samples were plotted against the corresponding total cellular protein amounts. Curve-fit analysis revealed that the curve obtained was described well ($r^2 > 0.9$) by an indirect linear relation, that is a relation such as $y = ax + b$ with $b \neq 0$ (Figure 1A). The densitometric ratios, however, differed from the ratios formed between the corresponding total protein amounts loaded on the gels (Figure 1B). Obviously, the ratios between total protein amounts are equal to the ratios between lamin C protein amounts because if, for instance, there is y ng of lamin C in 10 g of total cellular proteins, there will be $10y$ ng lamin C in 100 g of total cellular proteins. The discrepancy between densitometric ratios and corresponding protein amount ratios was observed with different densitometric apparatus and quantitation softwares (data not shown).

Mathematical analysis showed that rather than being caused by instrumental or biological bias, the difference between densitometric and amounts ratios is rooted in the fact that linear relations $y = ax + b$ can be direct ($b = 0$) or indirect ($b \neq 0$). To demonstrate this, let q_1 and q_2 be the amounts of a specific biological molecule present in control and experimental samples, respectively. After densitometric evaluation of these samples, one obtains two measurements, m_1 for q_1 and m_2 for q_2 . Assuming a linear relation between amounts and their measurements, one has:

$$m_1 / m_2 = (aq_1 + b) / (aq_2 + b) \quad (1)$$

When $b = 0$, the relation between amounts and their measurements is directly proportional, and after simplification (1) becomes $m_1/m_2 = q_1/q_2$, that is the amount ratio is equal to the densitometric one. However, when $b \neq 0$ (indirect linear relation), then $m_1/m_2 \neq q_1/q_2$, that is the amount and densitometric ratios are different.

In this latter case, the relative error E (or $100xE$ for percentage relative error) between densitometric and amount ratios is:

$$E = [(m_1 / m_2) - (q_1 / q_2)] / (m_1 / m_2) = (m_1 q_2 - m_2 q_1) / m_1 q_2 = 1 - (m_2 q_1) / (m_1 q_2) \quad (2)$$

With $\alpha = 1/a$, q_1 and q_2 can be substituted in (2) by $\alpha(m_1 - b)$ and $\alpha(m_2 - b)$, respectively. After simplification one obtains:

$$E = 1 - [m_2(m_1 - b)] / [m_1(m_2 - b)] \quad (3)$$

Equation (3) verifies that the error between the actual and observed ratio is zero only if $m_1 = m_2$ (i.e., if there is no change in the expression of the protein analyzed) and/or $b = 0$.

The indirect linear relation existing between protein amounts and their densitometric measurements implies that a theoretical zero microgram protein sample would yield a non-zero optical density value, and this in spite of background correction. The explanation for this paradox is that the actual physical relation (the so-called Beer-Lambert law) between the incident and emergent energy of light traversing a solid substance, such as a band or a spot on a film or gel, is logarithmic (i.e. power) and not linear [4]. The different portions of this power curve can be approximated by straight segments with varying slopes that represent the

derivatives at various points. Thus, even if a good linear approximation is found for a data set, this approximation may not follow the actual power curve outside the data range.

This can be illustrated by including a “point zero” to the data set shown in Figure 1A and by analyzing this new set with the curve fitting software Table Curve 2D. This analysis shows that although a linear relation is a mediocre fit, with $r^2 = 0.84$ (Figure 1C), a power relation is an excellent representation of this data set, with $r^2 = 0.99$ (Figure 1C). This example also shows that the reason why the data set without a zero point is approximated by an indirect rather than by a direct linear relation is not related to background correction but to the changing curvature of the power curve describing the relation between protein amounts and optical density measurements. This curvature increases toward the origin, causing linear approximations of portions of the curve not including the origin to intercept the y-axis at a non-zero value (Figure 1C).

Considering that the physical law describing the relation between amounts q_i of a specific protein and their densitometric measurement m_i on western blots is actually a power law passing through the origin such that $m = aq^n$, one can still demonstrate that directly forming the ratio of densitometric measurements yields values different from ratios between actual protein amounts. To this end, we follow a reasoning similar to that detailed above for equation (1), when considering a linear relation between protein amounts and densitometric values. With q_1 and q_2 the amounts of a specific protein present in control and experimental samples and m_1 and m_2 their respective densitometric measurements on western blot, one has:

$$m_1 / m_2 = (aq_1^n) / (aq_2^n) = q_1^n / q_2^n \quad (4)$$

and thus $m_1/m_2 = q_1/q_2$ only if q_1/q_2 (i.e., if there is no change in the expression of the protein analyzed) and/or $n = 1$. In practice, $n = 1$ is not realized because this would make (4) a direct linear relation, which is not what Beer-Lambert's law stipulates.

We have examined whether the use of a ratio standard curve could circumvent the mathematical bias inherent to measuring relative changes in protein levels by directly forming the ratio of the densitometric values of bands on a western blot. To obtain this curve, the ratio of densitometric measurements are plotted against known amount ratios of the protein examined. This is similar to the procedure followed to determine unknown amounts of a molecule in a sample by using an absorbance standard curve in which optical absorbance values are plotted against known amounts of the molecule examined. This approach is not used generally to quantify specific proteins, because they are difficult and/or expensive to obtain in a purified form in sufficient amounts to plot a true densitometry standard curve (densitometry value of bands against incremental amounts of the purified protein being investigated).

A ratio standard curve for the lamin silencing experiments was obtained by using the densitometric values of the lamin bands in the incremental loadings of control samples. The densitometric value of the lamin C band in the largest amount of total cellular protein loaded was divided by that of the lamin C band in the smaller loadings. This yielded numbers representing fold-decrease in the densitometric values of the lamin C band (the reverse ratios would have been computed had there been an increase in protein levels between control and experimental sample). These measured, densitometric ratios were then plotted against those of the corresponding total protein amounts. As detailed above, these were: 80/4, 80/8, 80/20 and 80/40 (Figure 1B). As explained above, the ratios between total protein amounts are equal to the ratios between lamin C protein amounts. Therefore, unknown ratios of lamin C protein amounts (or of any other protein of interest) between control and experimental samples can be determined by using a ratio standard curve.

A ratio standard curve was plotted to accurately determine relative changes in lamin C protein expression in U-373 MG cells treated with lamin siRNA. For this determination, the densitometric value of the lamin C band in 80 g of total proteins from control cells was divided by that of the lamin C band in the experimental samples where 80 g of total cellular proteins were loaded on the gel. Triplicate experiments were performed for statistical analysis. For each of these experiments, incremental loadings of the control samples were used to obtain known incremental ratios of lamin C amounts and to plot these known increments against the corresponding ratios of the densitometric values of the lamin C bands. This yielded ratio standard curves such as that shown in Figure 1D. The average values of the densitometric ratios suggested that lamin C protein levels decreased 4.4 ± 0.6 and 3.9 ± 0.3 fold in cells silenced for 24 and 48 hours, respectively. In contrast, using ratio standard curves, these values were 9.9 ± 2.4 and 12.2 ± 2.5 , representing a two- and three-fold difference with those obtained by forming densitometric ratios.

Determining the error of measurements is an important part of physical experimentation, but is often neglected in biological sciences. Herein, we found that considering the ratio between densitometric values to evaluate relative fold change in the level of a specific protein results in errors that are larger than 10%. In the example presented here, as well as in many other experiments that we have performed, the absolute value of densitometric fold-changes are lower than fold-changes in protein amounts, suggesting that variations in gene product expression are frequently underestimated when determined by the ratio of densitometry values.

In our accumulated experience, we have not yet encountered an instance in which densitometric ratios gave the incorrect direction of a change in gene product expression (i.e. increase rather than decrease or conversely). When it comes to the interpretation of biological phenomenon, many investigators may consider that the direction of a change in gene product expression is more critical than the exact magnitude of this change. Increased numerical rigor is important, however, as a general scientific principle and for the development of valid mathematical models of biological processes.

In summary, we report here that forming the ratio between the densitometric values of bands on western blots to determine relative changes in gene expression yields inaccurate results. We also provide mathematical demonstration that this is due to the fact that there is not a direct linear relation between increasing amounts of a given protein in bands on a western blot or gel and the densitometric measurements of these bands. If the protein of interest is available in a purified form, this error can be avoided by using a standard curve to determine the actual amounts of that protein in control and experimental samples. When the protein investigated is unavailable in purified form, we show that this error can be circumvented by the use of a ratio standard curve. This simple method should prove useful in laboratories routinely determining relative changes in protein levels by densitometry of western blots.

Acknowledgements

We thank William Goodwill (Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, Shreveport, LA, USA) for excellent technical work. This work was supported by National Institutes of Health grant NS-35317 to OS.

References

1. Sultana S, Zhou R, Sadagopan MS, Skalli O. Effects of growth factors and basement membrane proteins on the phenotype of U-373 MG glioblastoma cells as determined by the expression of intermediate filament proteins. *Am J Pathol* 1998;153:1157–1168. [PubMed: 9777947]
2. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76–85. [PubMed: 3843705]

3. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354. [PubMed: 388439]
4. Tinoco, I., Jr; Sauer, K.; Wang, JC. *Physical Chemistry: Principles and Applications in Biological Sciences*. 3. Prentice Hall; Englewood Cliffs: 1995.

Figure 1.

(A) plot of the densitometric values of lamin C bands on western blots against increasing amounts of total cellular protein. Each data point is the average of duplicate loadings. Inset indicates the value of the linear correlation coefficient r^2 , and of a and b in the equation $y = ax + b$ that describes the linear relation between the densitometric measurements of the lamin bands and the total amounts of cellular proteins. (B) table showing the discrepancy between the known ratios of incremental amounts of total cellular protein and the corresponding ratios between the densitometric measurements of the lamin C bands. (C) Curve fitting of the data shown in (A) after the addition of a zero point shows that although the linear equation $y = 0.46x + 8.81$ can be adjusted to the data set with $r^2 = 0.84$ (black curve, arrow), a better fit is represented by the power equation $y = 6.2x^{0.44}$ with $r^2 = 0.99$ (grey curve, arrowhead). (D) Ratio standard curve in which densitometric ratios are plotted against known ratios between total cellular proteins. Arrows demonstrate how to use this curve to determine an unknown protein amount ratio when knowing a densitometric ratio (for details, see text).