Methods to support protein extraction efficiency from plant tissues

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Introduction

A major determinant of accurate protein quantification is the effective and comprehensive extraction of the protein of interest (POI) from plant tissues. There are many factors that impact protein extraction, including molecular weight, isoelectric point, amino acid composition, and extinction coefficient of the POI. These factors influence how a protein extraction needs to be optimized as part of assay development for each POI and plant tissue combination. Extraction of POI can vary by plant species and plant tissue type along with the extraction protocol. Variations in processing, tissue disruption, extraction buffer selection, and tissue-to-buffer ratio can all impact the effectiveness of the extraction protocol. Due to the complexity of the physiological and biochemical environments of tissue types, the same POI may extract differently. Thus, there is always a chance that some of the POI remains in the pellet even after extraction optimization. After optimizing the extraction method, it is important to determine the percentage of the POI successfully extracted from a tissue sample before the downstream applications.

Extraction efficiency (EE) is defined as the ratio of the amount of POI extracted from a plant tissue sample in a single extraction compared to the total amount of POI in a given plant tissue. In each plant tissue, the POI can be present in both the soluble and the insoluble fractions. The ability to extract the POI is dependent on the extraction buffer and the conditions applied to the sample during extraction. In this white paper the soluble POI fraction will be defined as the protein that is extracted using buffers that are compatible with the downstream protein analysis; for example, ELISA and mass spectrometry (MS). The insoluble POI fraction will be defined as any unextracted POI remaining in the pellet that may be extracted using harsher conditions which may not be compatible with downstream protein analysis. To determine EE, the soluble fraction(s) of POI can be assessed by typical detection methods (e.g., ELISA, MS, western blot). The insoluble portion is more technically challenging to evaluate since harsh denaturing buffers and conditions must be deployed, which may not be compatible with some detection methods. Western blot can be used to estimate the POI in the insoluble fractions.

There are many different approaches to estimate extraction efficiency due to variations in protein expression levels, biophysical and biochemical properties. Here we will present several robust methodologies for determining extraction efficiency.

Methods

There are many different extraction methods that are dependent on the POI and specific tissue matrix. n this section, common methods for the extraction of soluble and insoluble POI will be described. Additional details or modifications to these methods will be described in the results section. To avoid false positive results, the same extraction procedures described below should be applied to the appropriate negative controls which could be conventional, other crops or buffer controls.

Soluble POI Extraction

An optimized extraction buffer (e.g., Phosphate-Buffered Saline with 0.05% Tween™20) compatible with downstream applications is added to finely ground plant tissue (typically using triplicate replicates). A predetermined tissue-to-buffer ratio is used. The mixture is then homogenized using a mechanical force (e.g., Geno/Grinder or homogenizer). The mixture undergoes phase separation via filtration or centrifugation and the liquid phase (i.e., extract) is retained. The POI potentially remaining in the pellet may be further extracted by iteratively applying the extraction procedure and saving the extracts after each iteration. The number of iterative extractions may vary based on the method utilized. In all cases, the removed extracts are kept chilled during all steps to minimize temperature effects on the protein over time.

Insoluble POI Extraction

The remaining non-extracted POI is extracted using harsh conditions, which may include the use of detergents, reducing agents, chaotropic chemicals and high temperatures. An example of this would include using Laemmli with DTT buffer followed by heating at ≥ 75°C for 5-10 minutes. The sample is clarified, and the liquid phase is retained.

Determination of Extraction Efficiency

Once the soluble and insoluble POI have been extracted, the extraction efficiency could be determined. Two approaches are described below to calculate the extraction efficiency. The first approach measures the amount of POI in both the soluble and insoluble fractions; while the second approach measures the ratio of intensities via a western blot from the soluble and insoluble fractions.

In the first method, the concentration of POI in the soluble fractions will be determined using ELISA, MS, or another quantitative method. Western blot will be utilized to determine the concentration of POI in the insoluble fractions. A reference POI (i.e., protein standard) will be prepared at multiple concentrations in negative control extract or buffer. These can be used to estimate the concentration of the POI in the insoluble fraction. The extracted samples, the prepared reference POI samples, and controls, if used, will then be loaded on an SDS-PAGE gel. Following protein migration, the proteins on the gel will be transferred to a blot followed by western blot analysis using relevant antibodies. The western blot will be imaged and analyzed using software (e.g., Image Lab™). If visual analysis of the western blot image demonstrates the absence of the POI band in the insoluble fractions, then the amount of insoluble POI will be

considered negligible, and no further analysis is necessary. If visual analysis of the western blot image demonstrates the presence of the POI band in the insoluble fractions, the blot is further analyzed using densitometry. Acceptance criteria for densitometry may be set as follows:

- Reference POI should include at least three levels
- Avoidance of band saturation

Signal intensity (normalized densitometry volume of the extracted POI bands) relative to the signal of matrix or buffer POI bands	Interpretation
Below range of the spiked reference POI bands	Concentration cannot be estimated but can be assumed to be lower than the lowest reference POI sample
Within range of spiked reference POI bands	Concentration can be estimated using the reference POI samples
Above range of spiked reference POI bands	The signal is saturated and the sample should be diluted and retested

In the second method, the soluble and insoluble fractions may be analyzed side-by-side on the same assay platform, for example using western blot. The dilution-adjusted signal intensity produced by the fractions can be used for extraction efficiency assessment. Similar controls as defined above may be used for densitometric analysis with some modification. The dose-response of the signal can be determined with plant-matrix or buffer-spiked reference POI at multiple dilutions to ensure that overexposure of either signal is not observed. A comparison of the two signals may be performed if the signal intensity from densitometry for these samples is between the highest and lowest spiked samples that meet the criteria established above. If the signal of the soluble fraction is above the highest spiked reference POI, then the sample should be further diluted and western blot analysis performed again. If the signal of the insoluble fraction is below the lowest spiked reference POI, then the amount of insoluble protein will be considered negligible. Some imaging software packages have the ability to determine if the signal intensity from the samples is saturated, allowing for the omission of spiked reference POI.

Calculation of Percent Extraction Efficiency

The extraction efficiency is determined using the following equation, where %EE is percent extraction efficiency, soluble POI is the concentration or densitometry values for the POI in each of the soluble fractions, and insoluble POI is the concentration or densitometry values of the POI in the insoluble fractions.

$$\%\mathrm{EE} = \frac{\text{Soluble POI first extract}}{[\text{Total Soluble POI} + \text{Total Insoluble POI}]} \times 100\%$$

Results

Examples of data analysis for determining extraction efficiency are provided below.

Example #1 ELISA coupled with comparative western blot

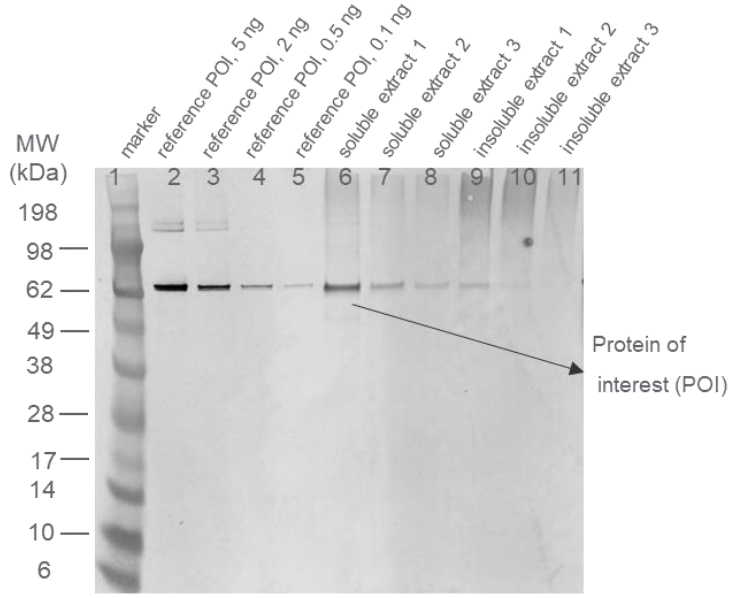
In this example, the sample was iteratively extracted three times to obtain soluble POI. The concentration of POI in the soluble extracts was determined using a quantitative method (e.g., ELISA, MS), these values were used to determine the Soluble Extract Efficiency (Soluble EE). The three iterative soluble extracts were also prepared for western blot (Figure 1A, lanes 6-8)

Three iterative insoluble extractions from the remaining pellet were prepared using harsh conditions (Figure 1A, lanes 9-11).

To establish the limit of detection for the assay, four concentrations of reference POI were also prepared (Figure 1A, lanes 2 to 5).

All samples were resolved on an SDS-PAGE and transferred to a western blot for analysis. Following the western blot, densitometry analysis was performed to determine the intensities of POI in each lane (Figure 1B). The percentage of insoluble protein after each extraction is calculated as the densitometry volume of the POI band in the insoluble extract divided by the sum of POI densitometry volumes in all extracts (Figure 1B).

A



B

Extaction Iteration	Densitometry Volume	% Recovered POI
SE1	463.56	ND
SE2	149.65	ND
SE3	65.38	ND
IE1	67.42	9%
IE2	24.65	3%
IE3	0	0%

Figure 1. (A) Western blot analysis of soluble and insoluble extracts. (B) Densitometic analysis SE = Soluble Extract, IE = Insoluble Extract

The percent (%) Recovered POI in the first and second insoluble extraction was found to be 9% and 3%, with no detectable protein in the third iterative harsh extraction (Figure 1B). As a result, the total percent of Insoluble protein was determined to be 12% based on the sum of the three iterations of insoluble extractions. Note, individual concentrations (ng/mL) of the POI in the soluble and insoluble extracts were not calculated. The % insoluble was determined as a ratio of densitometry volumes (or band intensities).

The overall extraction efficiency (Overall EE) was calculated by adjusting the Soluble EE with the % insoluble using following formula:

$$\text{Overall EE} = \frac{\text{Soluble EE} \times (100\% - \%\ \text{insoluble})}{100}$$

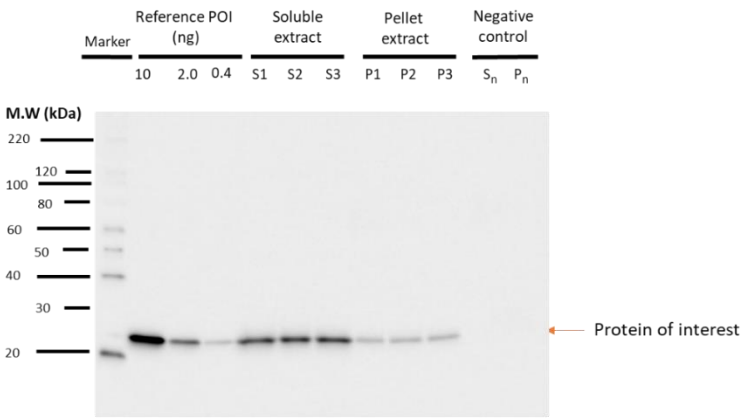
In the example above, the Soluble EE was found to be 87% and the overall EE was calculated as 77% with a %CV of 14%.

Example #2 Use of western blot for comparative analysis of extraction efficiency

Another approach to determine the overall extraction efficiency is to perform a single iteration of soluble extraction of a sample using a buffer compatible with the downstream method and then extract the remaining unextracted POI from the resultant pellet using harsh conditions. After preparing the extracts for SDS-PAGE, they can be analyzed side-by-side on the same western blot. Negative or conventional variety samples can be prepared and used as controls following the same process as the positive samples. In addition to the extracts and controls, the western blot also includes reference POI at known concentrations, which can be used to establish the limit of detection of the method.

In this example, three plant sample replicates were each extracted a single iteration for soluble extraction and the resulting pellets were each then extracted under harsh conditions for the unextracted POI. Both soluble extracts and pellet extracts were prepared, loaded and proteins resolved on an SDS-PAGE, along with three concentrations reference POI. Additionally, a negative control of plant tissue not expressing the POI was also extracted in the same manner and loaded on the gel. The result of the western blot is shown in Figure 2.

A



B

Sample #	Densitometry signal (control-adjusted)		%EE	Mean EE%	%CV
	Soluble fraction	Pellet fraction			
1	994,144	219,932	81.9%	83.0%	1.5%
2	972,607	201,839	82.8%		
3	1,010,478	187,239	84.4%		

Figure 2. (A) Comparative western blot analysis of triplicate soluble (S1, S2, S3) and pellet (P1, P2, P3) extracts with negative control soluble (Sn) and pellet (Pn) extracts of conventional variety samples. (B) Densitometric analysis and determination of %EE

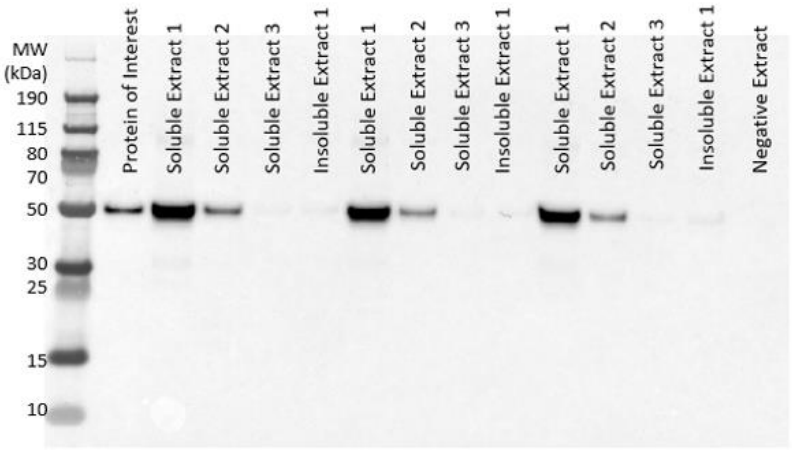
In this approach, the extraction efficiency can be readily determined in one step from densitometry volumes of the POI bands in soluble and pellet extracts using the equation in the Methods section. The densitometry volume of the positive sample, both soluble and pellet, can be corrected by subtracting the background signal from corresponding negative sample extracts or buffer background. The mean extraction efficiency is calculated as the mean of the individual estimates. The mean extraction efficiency of POI in the above sample was determined to be 83.0%. The method demonstrated reproducibility with %CV among replicates of 1.5%.

Example #3: Use of western blot for comparative analysis of extraction efficiency with multiple soluble iterative extractions

In this example, three sample replicates are iteratively extracted three times with the extraction buffer used in the downstream application to extract the soluble protein. These samples are then prepared for western blot analysis. The remaining protein, insoluble fraction, in the resulting pellet is then extracted under harsh conditions. Additionally, a negative control sample is extracted under harsh conditions and is analyzed on the western blot to account for potential non-specific binding of the antibody to other plant proteins in the tissue extracts.

The three prepared soluble extracts, insoluble extracts, and negative control are then analyzed by western blot, where %EE is determined based off densitometry values of the samples. Two examples are provided below. In the first example (Figure 3), a protein signal is detected in the insoluble extract and the contribution of that protein is used in the calculation for %EE. In the second example (Figure 4), no protein signal is observed in the insoluble fraction, thus the total %EE was determined by the intensities of the three iteratively soluble extractions.

A

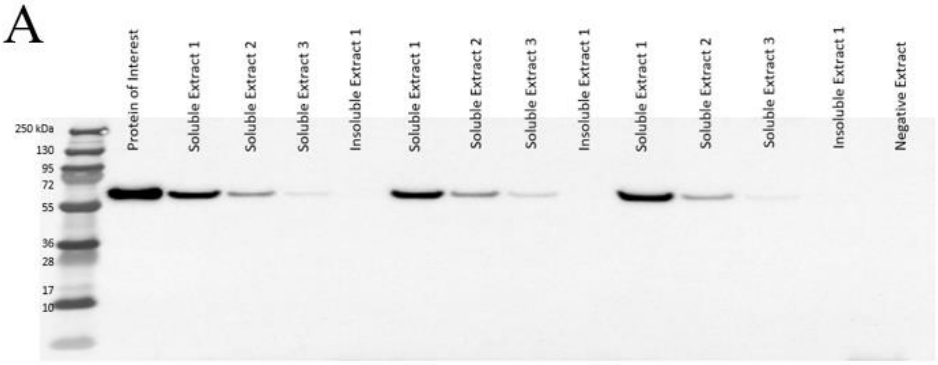


B

Replicate	Densitometry Signal				%EE	Mean %EE	%CV
	Soluble Extract 1	Soluble Extract 2	Soluble Extract 3	Insoluble Extract 1			
1	199,000	53,100	0	4470	78	79	1.3
2	180,000	41,900	0	4120	80		
3	163,000	41,000	0	4330	78		

Figure 3. (A) Western blot analysis of soluble and insoluble extracts. (B) Densitometry values are used to determine extraction efficiency %. Three replicates averaged to determine Mean %EE and %CV.

A



B

Replicate	Densitometry Signal				%EE	Mean %EE	%CV
	Soluble Extract 1	Soluble Extract 2	Soluble Extract 3	Insoluble Extract 1			
1	75,500	20,000	2,500	0	77	78	6.1
2	77,200	21,800	5,700	0	74		
3	88,100	16,400	1,600	0	83		

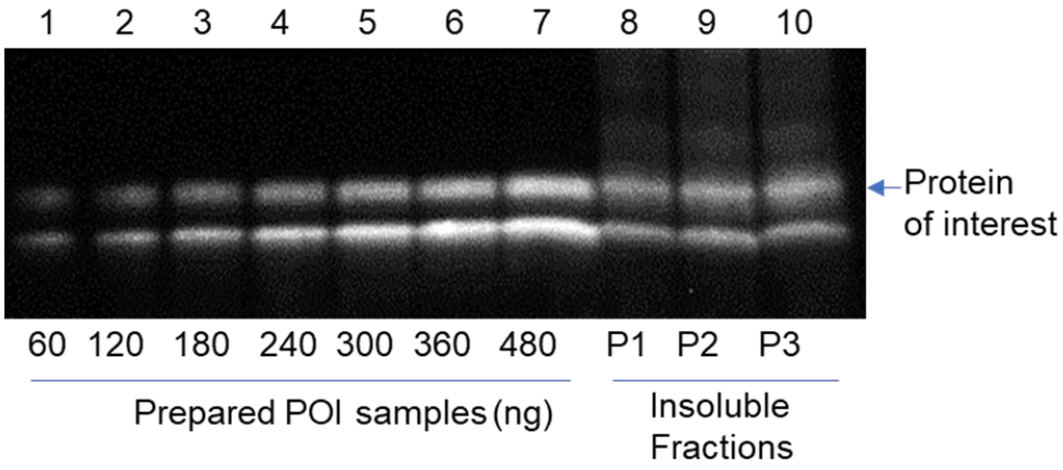
Figure 4. (A) Western blot analysis of soluble and insoluble extracts. No bands were observed in the insoluble extracts. (B) Densitometry values are used to determine extraction efficiency %. Three replicates averaged to determine Mean %EE and %CV.

Example #4: POI is an endogenous protein

In this example, the extraction efficiency of an endogenous POI was determined using concentration estimations for both the soluble and insoluble fractions. Three samples were iteratively extracted three times using a mild extraction buffer (soluble fractions) and the remaining pellets were extracted under harsh conditions (insoluble fractions). Estimation of the concentration of POI was determined by ELISA for the soluble fractions.

For the estimation of protein remaining in the insoluble fraction, a series of dilutions of the reference POI were prepared for western blot. These samples were run on SDS-PAGE along with the three insoluble fractions, a western blot was performed, and the blot was imaged (Figure 5). A linear regression analysis of the reference POI samples can be generated and used to estimate the concentration of POI in the insoluble fraction.

A



B

Sample Number	POI (µg) in Soluble Fraction (SF) - ELISA			POI (µg) in Insoluble Fraction - WB	Total POI (µg)	%EE
	1st_SF	2nd_SF	3rd_SF			
1	1299.229	59.692	9.334	28.971	1397.227	93.0%
2	1462.775	57.951	9.157	33.264	1563.148	93.6%
3	1677.101	123.649	10.800	57.542	1869.091	89.7%
Mean	**1479.702**	**80.431**	**9.764**	**39.926**	**1609.822**	**92.1%**
%CV	12.8%					2.3%

Figure 5. (A) Western blot analysis of insoluble extracts. (B) ELISA and western blot values were used to determine extraction efficiency %.

Discussion

Traditionally, extraction efficiency is evaluated through the extraction of a sample using an extraction buffer compatible with the quantitative procedure of choice (e.g., ELISA, MS). Using these methods, the amount of POI in the first extract relative to the sum of POI in all extracts was determined. These traditional methods only reported on the extraction of soluble POIs, unextractable or insoluble POIs may not be accounted for. The use of western blot analysis of the remaining pellet may be used to confirm the extraction efficiency of insoluble protein by demonstrating the absence of a significant amount of protein in the pellet or complement by accounting for unextracted protein.

Appropriate controls are foundational to extraction efficiency experiments. Often, the first choice for a negative control is a conventional sample of the same tissue matrix that does not express the POI. Transgenic samples could be used provided the expressed proteins do not interfere with the POI. For endogenous proteins, options such as lower expressing samples, specifically depleted samples, or matrix from a biosimilar plant might be useful, but not necessarily required. In such a case, using more than one type of negative control may be needed to establish specificity.

Western blot method conditions need to be optimized before use for extraction efficiency, starting with a high-quality and specific antibody. To ensure valid results, antibodies need to be evaluated for the intended application. In some cases, an optimal antibody for western blot may not be optimal for protein expression determination by ELISA since antibodies can recognize linear or discontinuous epitopes, which may have a variable presentation in the folded or unfolded state. Either monoclonal or polyclonal primary antibodies can be used for detection. The antibody selection process should evaluate the specificity of the antibody to minimize potential cross-reactivity. Alternatively, a potentially cross-reactive antibody may be used if the detected protein is readily differentiated from any cross-reacting proteins by the experimental setup. Purification and concentration can be performed prior to use of the primary antibody. Antibody dilutions should be optimized to achieve a strong signal with minimal background. Washing, blocking, and detection steps can be adjusted until consistent results are achieved. Factors that may impact the intensity of the immunoreactive bands include the efficiency of transfer, the concentration of primary and secondary antibodies, the duration of incubation in the antibody solution, the ambient temperature, the intensity of shaking, the use of blocking agents, and finally the blot development time.

Extraction efficiency should be determined on bands within the expected molecular weight range for the protein in comparison with the negative control and co-migrate with the bands from the positive control. However, bands on the western blot might appear different from standard protein in a tissue-specific manner for various reasons. Protein degradation, post-translational modification, and background can vary. Inherent variability in the western blot technique (Koller, 2005) can also impact band appearance. Importantly, all relevant protein-specific bands should be accounted for in the final analysis. Densitometry tools and applications are numerous for estimating protein concentration by comparison of fluorescence/chemiluminescent signals. Important control checks include accounting for background signals, signal saturation, and normalization techniques (Butler, 2019).

Western blotting is often less sensitive than ELISA, so some low-expressing proteins may not be observed. Assessment of low-expressing proteins is not straightforward. Reliable detection by western blot may not be achievable even after all efforts to optimize extraction parameters have been exhausted. In such cases, extraction efficiency will not be determined using western blot. In cases where there is a low expression of the POI, it is still advisable to demonstrate that the selection of extraction buffer for the soluble fraction was appropriate, thus a harsh condition extraction of the pellet should still be performed and assayed by western blot. The number of soluble extractions may be adjusted to ensure that protein may be quantified by ELISA, during method optimization, if no protein is detected by ELISA in a third iterative extraction, then it is advisable to perform only two iterative soluble extractions and perform a harsh extraction on the remaining pellet.

By performing multiple extract efficiency experiments, the level of reproducibility of the various methods can be determined. In the examples presented above, the %CVs are below 14%. These results suggest that multiple sample replicates are not required to determine %EE.

Additional considerations on extraction efficiency

The following guide may help in determining the %EE

- For tissues where POI is detected in both soluble and insoluble fractions, the extraction efficiency may be determined by the methods described above, including the use of western blot
- For tissues where POI is not observed by western blot in either soluble or insoluble fractions but is detectable by a more sensitive method (e.g., ELISA or MS), the extraction efficiency may be determined by the sensitive method instead of western blot
- In the case that the POI is detectable in the soluble fractions by western blot but not in the insoluble fractions, the extraction efficiency can be determined using only the soluble fractions

Conclusion

We have outlined commonly used methodologies and considerations that can be used to determine extraction efficiency for most plant transgenic proteins incorporating western blot. It is not possible to cover all circumstances and technical challenges that may arise during experimental design. Alternate technologies and methodologies can be investigated on a case-by-case basis. Furthermore, new technologies may present opportunities to further enhance the quality and reliability of these experiments.

References

Koller A., and Wätzig H. (2005) Precision and variance components in quantitative gel electrophoresis. *Electrophoresis. 2005 Jun;26(12):2470-5. doi: 10.1002/elps.200500024*

Butler TAJ, Paul JW, Chan EC, Smith R, Tolosa JM. (2019) Misleading Westerns: Common Quantification Mistakes in Western Blot Densitometry and Proposed Corrective Measures. *Biomed Res Int. 2019 Jan 21;2019:5214821. doi: 10.1155/2019/5214821. eCollection 2019.*