Measuring Protein Concentration in Cell Lysates by Direct UV280 Absorbance in the NanoPhotometer® P330

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Introduction

The most rapid and reproducible method to determine protein concentration over a wide range is by measuring absorbance at 280 nm by spectrophotometry[1]. Accomplishing this task in the past has often been met with the challenge of working with limited volumes of valuable samples. The advent of technology and spectrophotometers allowing the use of sample volumes as low as 0.5 µl to quantify the concentration of protein has markedly facilitated this task. However, protein samples are commonly prepared in buffers containing components which may interfere with direct absorbance. This is often the case when total cell lysates are prepared using certain detergent-based buffers and protein concentration needs to be determined to ensure equal amounts of protein are used for any subsequent experiments (e.g. immunoblotting, enzymatic assays, binding assays, etc.). The most common example is the use of RIPA lysis extraction buffers formulated with SDS and/or NP-40 for preparing cell lysates[2]. These detergents exhibit strong absorbance in the 280 nm region (A280), making RIPA unsuitable for direct UV measurements in lysates. There are, however, alternative detergents available that do not absorb significantly in the 280 nm region and can be used for preparing cell lysis buffers including Mammalian Protein Extraction Reagent (M-PER and Tissue Protein Extraction Buffer (T-PER)[3,4].

Another important issue pertains to the purity of protein samples and information about their spectral properties, including their extinction coefficients. It is common to have samples containing complex mixtures of unknown proteins in which concentration needs to be measured, such as cell lysates. In this case, lack of knowledge about the sample composition or the specific extinction coefficients of their protein components creates a challenge to use direct A280 measurements, as this information is necessary to calculate the protein concentration based on Beer-Lambert law using the A280 method[5].

Under these circumstances, colorimetric methods are commonly used since they do not depend on intrinsic protein absorbance. However, in order to make them quantitative, they must rely on the use of a protein standard (e.g. BSA) to create a calibration curve[1]. This practice assumes that the extinction coefficient of the standard protein is the same or equivalent to the proteins in the sample in terms of their chromogenic reaction in a colorimetric assay, such as a Bradford or bicinchoninic acid (BCA) assay[3,4].

In this application note, the NanoPhotometer® P-Class Model 330 was used to quantify protein concentrations in total cell extracts by direct A280 readings. These extracts were obtained by lysis of cultured cells using three different common extraction buffers RIPA, M-PER and T-PER which are all commercially available (Thermo Scientific). In contrast to the stringent denaturing RIPA buffer, M-PER and T-PER are formulations of buffers containing a proprietary non-denaturing detergent in a bicine-based buffer and used to prepare total extracts from cultured cells or tissues[3,4]. Non-denaturing buffers have the benefit of maintaining native protein conformation and may be used for extraction when the protein of interest is detergent-soluble[6].

Results obtained from A280 measurements were compared with those using a colorimetric method (BCA) in the same samples. A conversion factor was calculated, which allows the application of the much simpler and expeditious A280 method to comparable samples, thereby saving time and reagents while providing accurate results.

Material & Methods

HEK293T fibroblasts (ATCC) were plated on 12-well plates at a density of 250,000 cells/well and grown to confluence in DMEM, 10% FBS in a humidified CO_2 incubator. For immunoblot experiments, stable transfectant derivatives of HEK293T cells expressing the human iron transporter ferroportin (Fpn) fused to green fluorescent protein (GFP) were used and processed in the same manner[7]. Confluent monolayers were quickly rinsed twice with ice-cold PBS and then lysed by the addition of 150 µl of the various extraction buffers RIPA, T-PER or M-PER (Thermo Scientific), in the presence or absence of a protease inhibitor cocktail (cOmplete, Mini, EDTA-free, Roche) and adjusted to 5 mM EDTA. Plates were incubated on a rocking platform for 30 min at 4°C and lysates harvested, transferred to 1.5 ml tubes and centrifuged at 20,000 x g for 15 min. Supernatants were collected and saved for protein assays and immunoblot analysis.

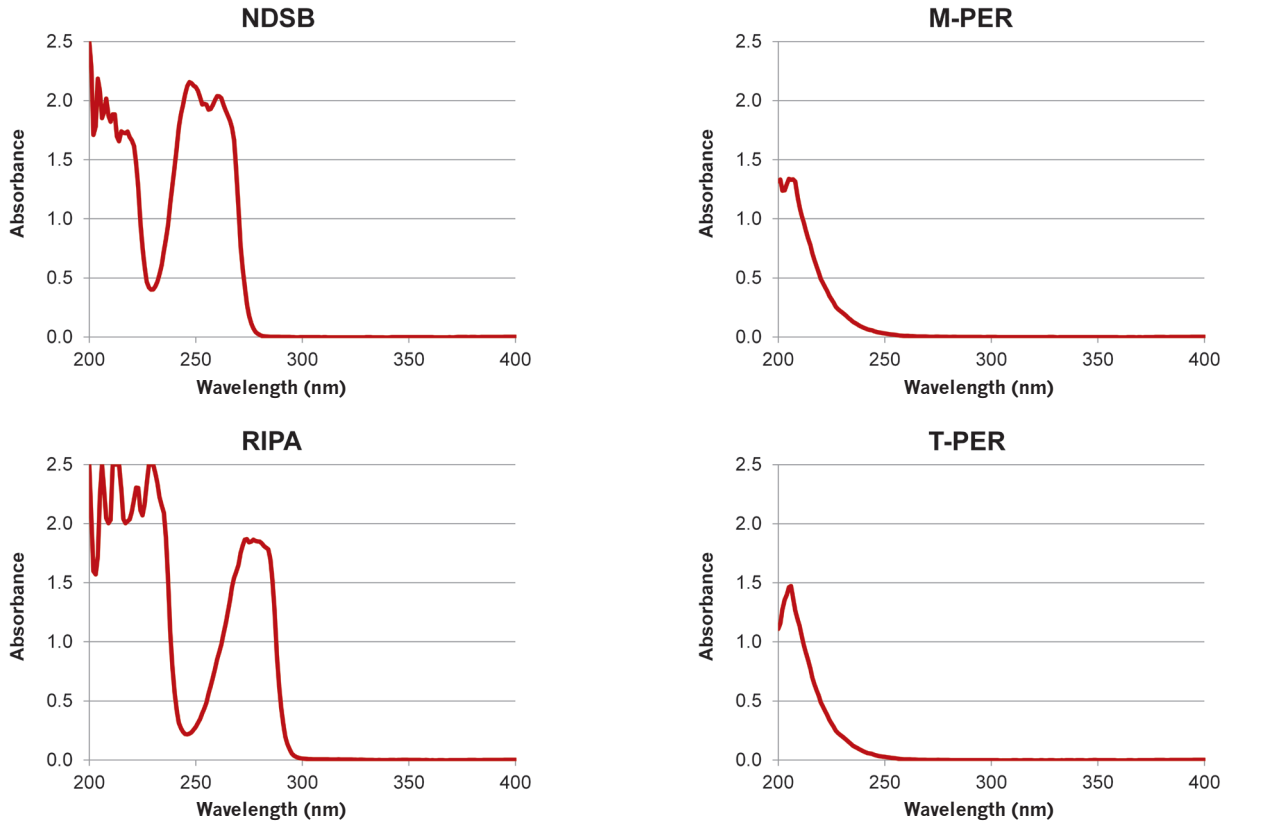
Protein assays consisted mostly of direct A280 UV quantification and in some cases, colorimetric BCA assays. For A280 measurements, the NanoPhotometer® P-Class Model 330 was used by applying 1.2 µl samples from each lysate onto the measurement pedestal and compressing the sample with a 1:10 virtual dilution lid (1 mm path length). Readings were performed in triplicate for each sample against a blank of deionized water. For colorimetric BCA assays, protein concentration was quantified with a Pierce BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions and using a SPECTRAmax PLUS 384 plate reader (Molecular Devices). For these assays, BSA was used as a reference standard to generate protein calibration curves. For immunoblot analysis, proteins in HEK293T/Fpn-GFP cell lysates were separated by SDS gel electrophoresis and transferred onto PVDF membranes (Mini Transfer Packs, BioRad Trans-Blot Turbo Transfer System). Blots were blocked with 5% nonfat dry milk and incubated with a rabbit anti-GFP polyclonal antibody, washed and then incubated with a goat anti-rabbit-HRP conjugated antibody. Blots were washed and the resulting immune complexes visualized by using a chemiluminescent HRP substrate (Thermo Scientific Super Signal West Pico).

Results

Interference by RIPA

The spectral properties of common lysis buffers reflect the UV absorption characteristics of their individual components, thereby dictating their suitability for direct A280 measurements. As mentioned above, RIPA is not the optimal choice for performing direct UV quantitation of protein due to its strong A280 interference. This is due to the intrinsic UV absorption properties of the detergent components used in the RIPA formulation, such as SDS or NP-40[8]. Likewise, certain solubilizing reagents commonly present in cell extracts, such as non-detergent sulfobetaines (NDSB), or non-ionic detergents such as Triton X-100, are also known to interfere with absorbance in or near the 280 nm region[8].

Some of these observations are confirmed by the spectral analyses shown in Figure 1, which also include spectra from other reagents and buffers typically used for preparing total cell extracts (e.g. CHAPS, NDSB, protease inhibitors, M-PER, T-PER, DTT and Hepes). As expected, it is evident that RIPA exhibits strong UV absorption in the 280 nm region, making it unsuitable for protein concentration assays by direct A280 readings. In contrast, M-PER and T-PER, two commonly used non-denaturing protein extraction reagents for preparing cell lysates[3,4], display a broad shoulder in the far UV range with a trailing absorbance of ≤0.07 at 240 nm, but virtually zero absorbance at 280 nm. Likewise, the zwitterionic detergent CHAPS, as well as commonly used reagents (protease inhibitors) or buffers (Hepes) do not significantly absorb in the 280 nm region and are therefore compatible with direct protein UV absorbance methods (Figure 1). These spectra were performed using typical concentrations of the reagents tested i.e. 1X for the lysis buffers and protease inhibitors, 1 mM DTT or 25 mM Hepes.



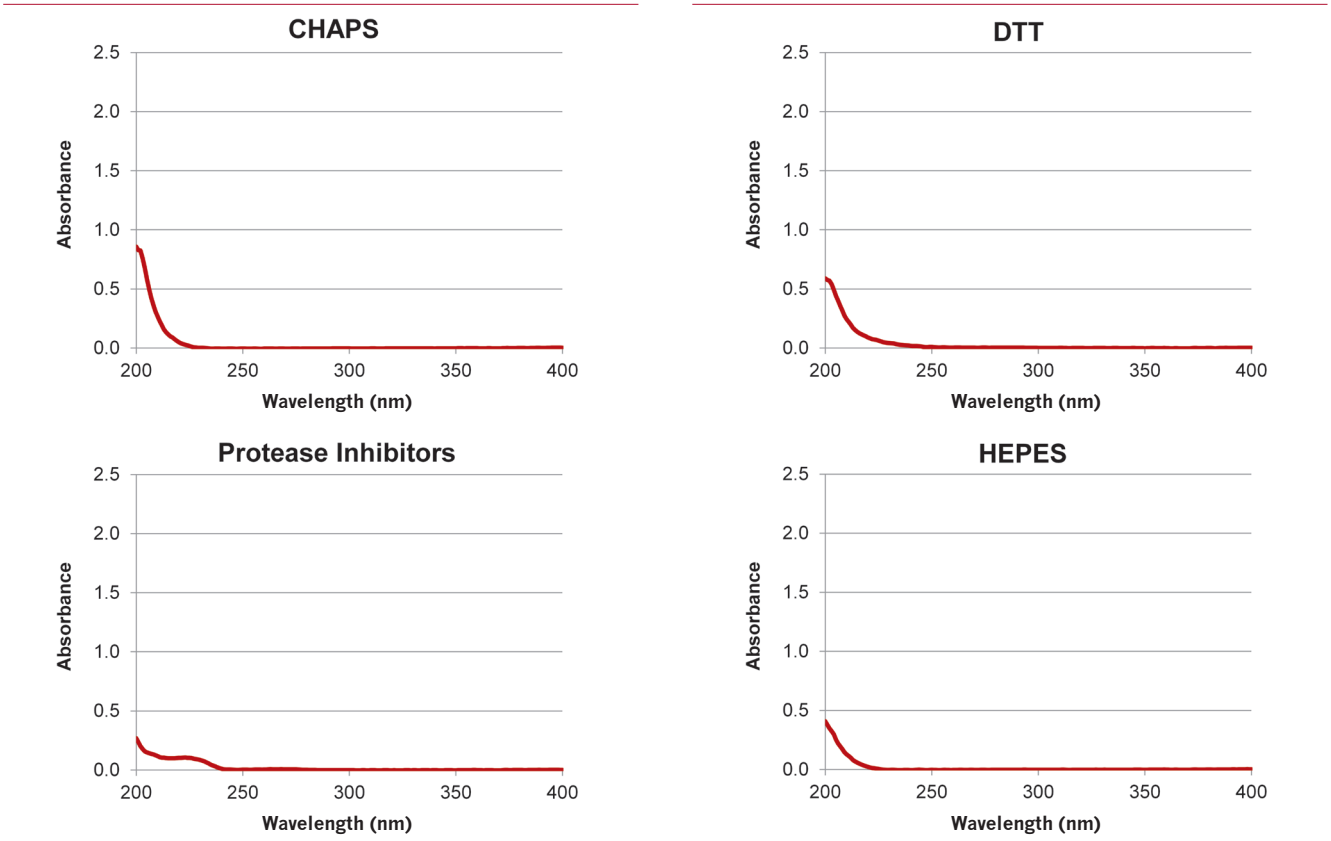


Figure 1: **Spectral analysis of common extraction reagents and buffers for cell lysis.** The absorbance profile of a variety of reagents and extraction buffers commonly used for preparing total cell lysates was recorded by performing a scan in the 200 to 400 nm range using 0.3 µl samples of each solution at typical final working concentrations (1X for RIPA, MPER, T-PER, CHAPS, protease inhibitors and NDSB, 1 mM DTT, 25 mM Hepes). Results are shown on the same absorbance scale (0-2.5).

When compared to M-PER and T-PER, the interference by RIPA in the 280 nm region is considerably higher (Figure 2A). This interference created by RIPA when measuring protein concentration by direct A280 readings is particularly evident when mixed with a soluble protein.

As shown in Figure 2B, the addition of RIPA to a solution of BSA interferes significantly with its inherent absorbance signal in the 280 nm region, which is otherwise observed when the protein is dissolved in water. In contrast to RIPA, other detergents such as M-PER or T-PER do not exhibit any interference in this region (Figure 2B).

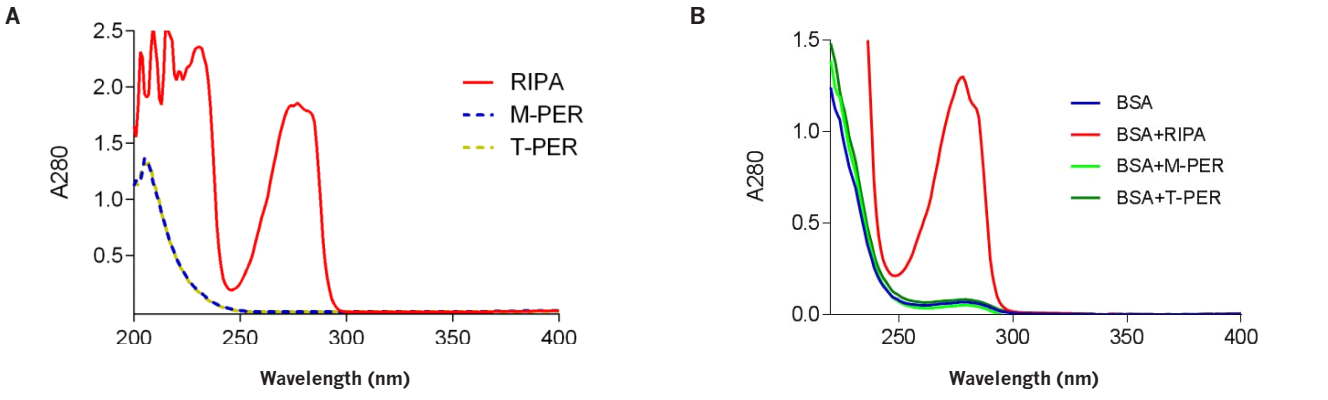


Figure 2: **Interference of A280 readings by RIPA and other extraction buffers.** A scan from 200 to 400 nm was performed using samples of the indicated extraction buffers at typical final working concentrations (1X). A, Overlaid profiles for RIPA, M-PER and T-PER; B, Profile of a 2 mg/ml BSA solution mixed with one volume of water (BSA) or mixed with 1X RIPA, M-PER or T-PER. Note the strong absorbance peak by RIPA in the 280 nm region.

Direct A280 readings in cell lysates

To measure protein concentrations in complex soluble cell extracts, total lysates were prepared from HEK293T fibroblasts in culture using different extraction buffers. Protein concentration was then determined by direct A280 measurements. As expected, direct A280 readings were markedly variable in lysates prepared with RIPA, which is reflected in inconsistent mean values and wide deviations showing as much as 21% error (Figure 3A). In contrast, the signal observed with the T-PER and M-PER lysates was highly reproducible, with only a small level of experimental error of 1% or less regardless of the presence or absence of protease inhibitors, which gave comparable results.

This apparent difference in protein yield between RIPA and all other lysates may suggest substantial variations in extraction efficiency among these samples. However, this possibility is unlikely because immunoblot analysis of equivalent lysate extracts indicates that the relative abundance of a protein expressed in these cells (Fpn-GFP) did not vary significantly based on extraction buffer used see Figure 3B). In this case, lysis extracts are prepared from stable HEK293T fibroblast transfectants expressing the human iron transporter ferroportin (Fpn) fused to green fluorescent protein (GFP). Thus, the relative intensity of the protein band corresponding to the Fpn-GFP fusion protein is similar in all lysates (and in fact appears to be lower in the RIPA samples), indicating that the extraction efficiency of Fpn-GFP in cells lysed with different buffers was comparable (Figure 3B). This is consistent with the similar intensity of the GAPDH protein band across all samples, which is used here as a sample loading control. The equivalent A280 readings in the T-PER and M-PER lysates are paralleled by the similar extraction efficiency of Fpn-GFP in T-PER and M-PER lysates observed in the immunoblot (Figure 3B).

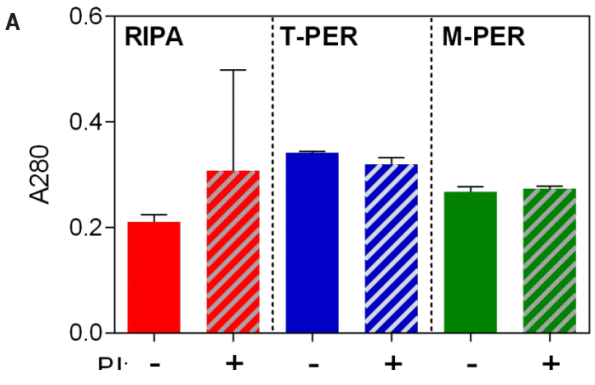


Figure 3A: **Comparison of protein yield in cell lysates prepared with different extraction buffers.** Direct A280 measurement values were obtained in undiluted samples of cell lysates prepared in each of the indicated extraction buffers (with and without protease inhibitors).

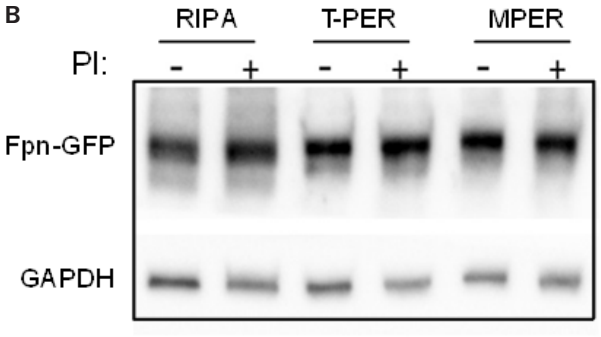


Figure 3B: **Comparison of protein yield in cell lysates prepared with different extraction buffers.** Total cell lysates from HEK293T stable transfectants fibroblasts expressing an Fpn-GFP fusion protein were prepared in the indicated extraction buffer, supplemented or not with protease inhibitors. Proteins were resolved by electrophoresis, transferred to immunoblot membranes and developed with anti-GFP polyclonal antibodies followed by HRP-conjugated secondary antibodies. Detection of resulting immune complexes was accomplished by chemiluminescence. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping protein loading control for each lane.

Calculating protein concentration in crude cell lysates by A280

For calculation of protein concentration in these samples by direct A280 measurements, only lysates prepared with M-PER and T-PER were used in view of the wide variation observed in RIPA extracts. For A280 data interpretation, three different reference standards were used and compared to calculate the concentration of protein in these lysates (Figure 4).

As expected, the result in each case was different because the extinction coefficient for each reference standards was different[5]. Thus, the calculated protein concentration in T-PER lysates was 4.72 ± 0.02 mg/ml when BSA was used as the standard (Figure 4A). However, the result is 3.31 ± 0.03 mg/ml for the same samples if the assumption is made that the overall extinction coefficient of a 1 mg/ml solution containing a crude mixture of proteins is approximately 1 (OD1), which is commonly used to provide a rough estimate of protein concentration[5].

Yet a different result is obtained if calculations are based on a method that uses the average extinction coefficient (Avg ε) of 116 proteins with a wide range of amino acid compositions and molecular weights, which is approximately 1.3 for a 1 mg/ml solution[5,9]. Applying this parameter, the calculated concentration in the same T-PER lysates was 2.49 ± 0.03 mg/ml (Figure 4A). Thus, regardless of which method is correct or the most appropriate, it is clear that the result will be different depending on which reference standard is used for calculation. Although a somewhat lower protein concentration was found in M-PER lysates, the relative

difference between the three reference standards was the same as with T-PER (Figure 4A). The extremely small variation (± 1%) and consistency of results for each set is noteworthy in both types of lysates extracted with either M-PER or T-PER buffer.

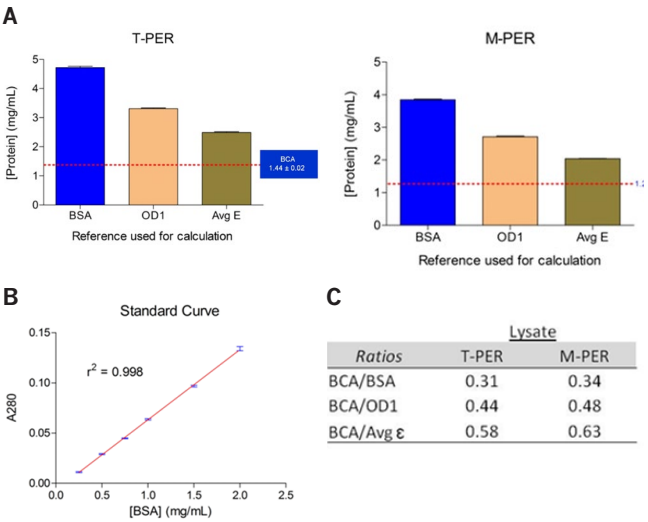


Figure 4: **Protein concentration in cell lysates by direct A280 measurement.**
A. Protein concentration was determined in T-PER and M-PER lysates by direct A280 readings using three different reference standards to calculate concentration values (BSA, bovine serum albumin; OD1, assumed extinction coefficient of 1 for a 1 mg/ml stock; Avg ε, average extinction coefficient of 1.3 for a 1 mg/ml stock). Protein was also measured by a colorimetric BCA assay in same lysates (dashed red line and blue boxes).
B. Calibration curve generated by serial dilution of a 2 mg/ml stock solution of BSA and used for calculation of values shown in A (BSA blue bars).
C. For each lysate, the ratios shown were calculated as the protein concentration values determined by BCA colorimetric divided by each of the A280 methods in A (BSA, OD1 and Avg ε).

Colorimetric assays

Protein concentration was also determined in parallel in the same T-PER and M-PER cell lysates using a colorimetric method (BCA assay). In these samples, the concentration of protein in T-PER and M-PER lysates determined by the BCA assay was 1.44 ± 0.02 and 1.29 ± 0.02 mg/ml, respectively (Figure 4A). These values are lower than those estimated by direct A280 measurements. Notwithstanding the reason for the difference between the BCA and direct A280 results, it is important to bear in mind that certain reagents typically added to lysis buffers are well-known to interfere with the BCA assay (e.g., detergents or chelating agents, including EDTA and EGTA)[8]. In addition, the BCA assay uses BSA as a standard reference protein to calculate protein concentrations in the samples, which implies the assumption that proteins in a crude lysate behave similarly to BSA in terms of their bicinchoninic acid-Cu^{+} complexation. The color reaction in the BCA assay is known to be predominantly influenced by the presence of certain amino acid residues in a protein (cysteine, cystine, tyrosine and tryptophan), which are not equally represented in every protein[10]. Regardless of these considerations, and of which method is more suitable or accurate, it is possible to derive a correction factor using the ratio of BCA to any of the A280-based methods described here. In the case of T-PER and M-PER lysate samples, the average ratios of BCA colorimetric to A280 readings were approximately 0.32, 0.46 and 0.6, based on BSA, OD1 and Avg ε, respectively (Figure 4C).

Conclusions

The simplest and most reliable method to quantify protein concentration is through direct UV measurement at 280 nm. This is true provided that the protein of interest contains aromatic amino acids and does not possess any prosthetic groups with significant UV280 absorbance. Quantitative accuracy of direct A280 measurements is maximized in the ideal situation of purified proteins in solution with known molar absorption coefficients. However, when applied to complex, heterogeneous mixtures of unknown or uncharacterized proteins contained in cell lysates, this approach is often hampered by the presence of interfering reagents and detergents in the extraction buffers commonly used including RIPA. This would also be compounded by the partially denaturing character of RIPA depending on the detergents added and their concentration, which would tend to increase protein intrinsic absorbance[11]. For example, inclusion of an ionic detergent such as SDS would impart its denaturing properties to the buffer, as opposed to non-polar detergents. Although some colorimetric assays offer the advantage of overcoming some of these detergent-related issues, they are much more time and labor intensive, with substantial sample volume requirements. In this application note, cell lysates containing crude protein mixtures were used to determine protein concentration by direct A280 measurements. A practical advantage of the direct A280 approach is the possibility of processing a large number of lysate samples more efficiently using a highly consistent and reproducible method, provided that all samples are prepared with the same extraction protocol. This method is rapid, simple, low cost, does not require addition of any reagents or incubations, and only needs an extremely small sample volume (0.5 µl). An additional advantage is the virtual dilution feature of the NanoPhotometer® determined by the path length used when measuring absorbance, which in most cases allows the use of undiluted protein samples for direct A280 readings.

By definition, colorimetric assays for measuring protein concentration all require setting up a reaction to incorporate an appropriate chromophore into the proteins in the sample (e.g. Bradford, Lowry, BCA). This means that the sample must be mixed with assay reagents at the appropriate dilution, incubated to achieve color development, and the absorbance signal detected in a spectrophotometer recording in the visible range. The results then need to be related to a standard calibration curve, typically generated with a stock solution of BSA using the exact same process

in parallel before protein concentrations in the sample can be calculated. In contrast, using a direct UV A280 method that does not involve any intervening manipulations not only eliminates potential sample handling errors, but also saves the time needed to set up the assay reactions and incubations and the cost of assay reagents.

Using the direct A280 approach, the protein concentration results will depend on the reference standard used for calculation. Although the decision of which reference is the most appropriate lies with each investigator, the Avg ε method has the advantage of using a parameter calculated on the basis of extinction coefficients from known proteins[9]. Comparison with a common colorimetric method (BCA) indicates that direct A280 measurements can be related to the results of a BCA assay through a ratio-based conversion factor for equivalent samples. In the situation where it is necessary to measure protein concentration in a large number of cell lysate samples, the much simpler A280 direct method offers a good option, provided that they are prepared in non-RIPA containing extraction buffers.

Recommendations

The following recommendations involve the much more direct and accurate measurement of protein concentration by direct A280 readings, which is immediately applicable to total cell lysates prepared with non-RIPA extraction lysis buffers:

Prepare total lysates using a non-RIPA extraction buffer for all samples (M-PER and T-PER buffers recommended). Follow appropriate lysis procedure.

- Blank the instrument with water
- Read a sample of the same buffer or diluent used for the lysates
- Blank the instrument again using this buffer before making any measurements to avoid negative peaks
- Always vortex protein samples before applying to the measuring window
- Use the reverse pipetting technique to apply the sample:
    1. Immerse the pipette tip into the undiluted sample and fully press the plunger (to the second stop) before releasing to fill the tip
    2. Apply the sample (0.5-1 µl) by gently placing the tip on the measuring window and then press the plunger to the first stop to dispense the liquid devoid of any foam or bubbles
- Cover with appropriate lid and measure absorbance

For calculating results:

- If desired, prepare a calibration curve using a reference protein standard
- Calculate protein concentration in each series against:
    1. A reference protein with a known extinction coefficient
    2. An average value based on known proteins (e.g. Avg ε)

If a reference comparison to a colorimetric assay is desired:

- Perform the colorimetric assay separately using a small but representative number of equivalent samples (e.g. triplicates), prepared with the same lysis procedure and reagents
- Take the concentration value determined by the colorimetric assay against an appropriate reference standard curve and calculate the colorimetric/A280 ratio as described in this application note

For a given set of lysates or crude protein samples, it is important to perform this procedure at least once to ensure that the resulting ratio falls within a small margin of error (≤5%). This ratio can then be used as a conversion factor between direct A280 and colorimetric measurements for equivalent samples.

Acknowledgements

We thank Drs. Elizabeta Nemeth and Tomas Ganz from the UCLA Division of Pulmonary and Critical Care Medicine (Los Angeles, CA) for kindly providing cultured cells and cell lysates, and for making their laboratory facilities available to perform some of the studies described here. We also thank Dr. Bo Qiao for performing the immunoblot experiments.

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