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Technical note

Coomassie does it (better): A Robin Hood approach to total protein quantification

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ABSTRACT

Quantitative comparative proteomics require accurate and reproducible assessments of total protein concentration. We report a straightforward, cost-effective adaptation of an established commercial method for total protein quantification (EZQ™), utilising non-proprietary materials and colloidal Coomassie Brilliant Blue (cCBB) staining to achieve greater reproducibility, equal sensitivity, and optimal linearity of signal within a practical concentration range for proteins in common solubilisation buffers (i.e. for isoelectric focussing and/or SDS-PAGE). This method provided more accurate and precise determinations of total protein concentration in human serum prepared for two-dimensional gel electrophoresis, indicating it may be better suited as the lead-in to most quantitative proteomic analyses.

Protein extraction and solubilisation of samples destined for quantitative proteomics may involve any effective combinations and concentrations of buffering, chaotropic, reducing, alkylating, and chelating agents, as well as detergents, ampholytes, salts, inhibitors (peptides and small molecules), sugars, alcohols, and dyes. Such components may impede reliable protein quantification, the accuracy of which is paramount for meaningful comparative analyses.

Samples prepared for top-down gel-based analyses, i.e. two-dimensional gel electrophoresis (2DE; isoelectric focussing (IEF) followed by SDS-PAGE), are routinely solubilised in buffer containing high concentrations of urea and thiourea, and detergent(s) such as CHAPS. With or without the addition of ampholytes, 2DE lysis buffer (2DB) is incompatible with most approaches to total protein quantification [1], thus methods which are preceded by protein precipitation to remove interfering substances are commonly utilised, e.g. the 2-D Quant™ Kit (GE Healthcare, Buckinghamshire, UK). However, such methods require microgram protein amounts for accuracy, and result in protein losses that can variably alter the content of resolubilised proteomes and influence subsequent quantitative analyses; assaying several samples in parallel is also challenging due to multiple components and procedural steps. Therefore, more practical alternatives, which account for or remove interfering substances by simpler means, are desirable.

Solid-phase protein quantification assays thus appear well-suited, as samples are dot-blotted onto membrane or filter paper, non-protein buffer components washed away, and immobilised proteins are quantified either directly or following elution from the solid support. One adaptation is currently offered commercially as the EZQ™ Protein Quantitation Kit (Invitrogen, Carlsbad, CA) [2]. This convenient high-throughput method claims compatibility with 2DB (± ampholytes), SDS-PAGE 'Laemmli' buffer (1DB) [3] and commonly used solvents. The ~40-min protocol requires minimal sample (1–3 µl, depending on dilution factor), with apparent high sensitivity (≥10-fold greater than 2-D Quant™ [2,4]), a 500-fold dynamic range (linear between 0.01 and 1 µg), high reproducibility, and low inter-protein variability (IPV) – albeit at a relatively high and recurrent cost (though this is substantially lower than that of 2-D Quant™). Furthermore, it has been shown to more accurately estimate total protein content relative to 2-D Quant™, representing an overall more suitable and practical approach to total protein quantitation for 2D gel-based proteomics [4].

One EZQ™ kit consists of proprietary assay paper and fluorescent protein stain, two aliquots of chicken egg albumin (CEA), and a bottomless 96-well cassette to assist blotting and detection. A minimum of five protein standard dilutions are suggested, each spotted in triplicate, allowing for another 27 sample dilutions per assay sheet, including buffer-blanks. As dilutions are routinely necessary to estimate protein concentration in native samples, one assay sheet realistically accommodates a maximum of eight individual samples. Cost per sample is therefore ~2.4 USD, ~10% of the total cost of triplicate mini-format Coomassie-stained 2DE gels.

We report an improved and more reproducible method in which

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Non-standard abbreviations

1DB	SDS-PAGE lysis buffer
2DB	2DE lysis buffer
AU	Arbitrary units
cCBB	colloidal Coomassie Brilliant Blue
CEA	Chicken egg albumin
IPV	Inter-protein variability

proprietary EZQ™ components are substituted with chromatography paper and colloidal Coomassie Brilliant Blue (cCBB) stain [5,6]. The protocol is cost- and time-efficient, maintaining the advantages of the EZQ™ method while reducing cost per sample by > 75%. The protocol is simpler than previously reported solid-phase CBB methods [7–12], essentially following the commercialised EZQ™ procedure and completed in ~60 min.

All chemicals other than those supplied with two EZQ™ kits were of ultra-pure or analytical grade. Aliquots of CEA supplied with the EZQ™ kits were solubilised in either 1DB (25 mM TRIS [pH 8.8], 2% (w/v) SDS, 12.5 mM DTT, 7.5% (w/v) glycerol, and 0.001% (w/v) bromophenol blue) [3,5,6] or 2DB (8 M urea, 2 M thiourea, and 4% (w/v) CHAPS) [13] with and without 0.5% or 1% (v/v) pH3-10 ampholytes (Bio-Rad Laboratories, Hercules, CA). Protein concentrations of 5–0.01 $\mu g \mu l^{-1}$ were prepared by dilution and used immediately without freeze-thawing. 1DB preparations were boiled (5 min, 100 °C) and cooled to room temperature prior to spotting.

One microliter volumes of each dilution as well as buffer-blanks were spotted in triplicate onto either EZQ™ assay paper or Whatman 3 MM cellulose chromatography paper (GE Healthcare, Buckinghamshire, UK), for EZQ™ and cCBB staining, respectively. Triplicate blots were prepared per assay condition. Assay paper could be secured between a 96-well plate and an empty pipette-tip rack in lieu of the EZQ™ cassette. Following spot drying, blots were washed with methanol for 5 min (to remove buffer components), and dried again under ambient conditions (~10 min) prior to staining.

EZQ™ staining/destaining was according to manufacturer's instructions, with handling and storage in a darkroom. cCBB stain was prepared fresh as previously described [5,6] and blots were stained for 10 min (optimised, not shown) with continuous agitation. These were destained with water 5 × 5 min (optimised, not shown), turning once to ensure release of residual cCBB. Although fully dried in ~2 h, for convenience all blots were dried under ambient conditions overnight prior to imaging. cCBB blots could be dried in ≤10 min under compressed air without compromising signal quality (not shown).

EZQ™ blots were imaged with the Synergy™ HT microplate reader (BioTek, Winooski, VT) using 485 ± 10 nm/590 ± 18 nm excitation/emission. Readings were acquired by top excitation, with spot application side facing up. Scans were repeated to ensure precision of

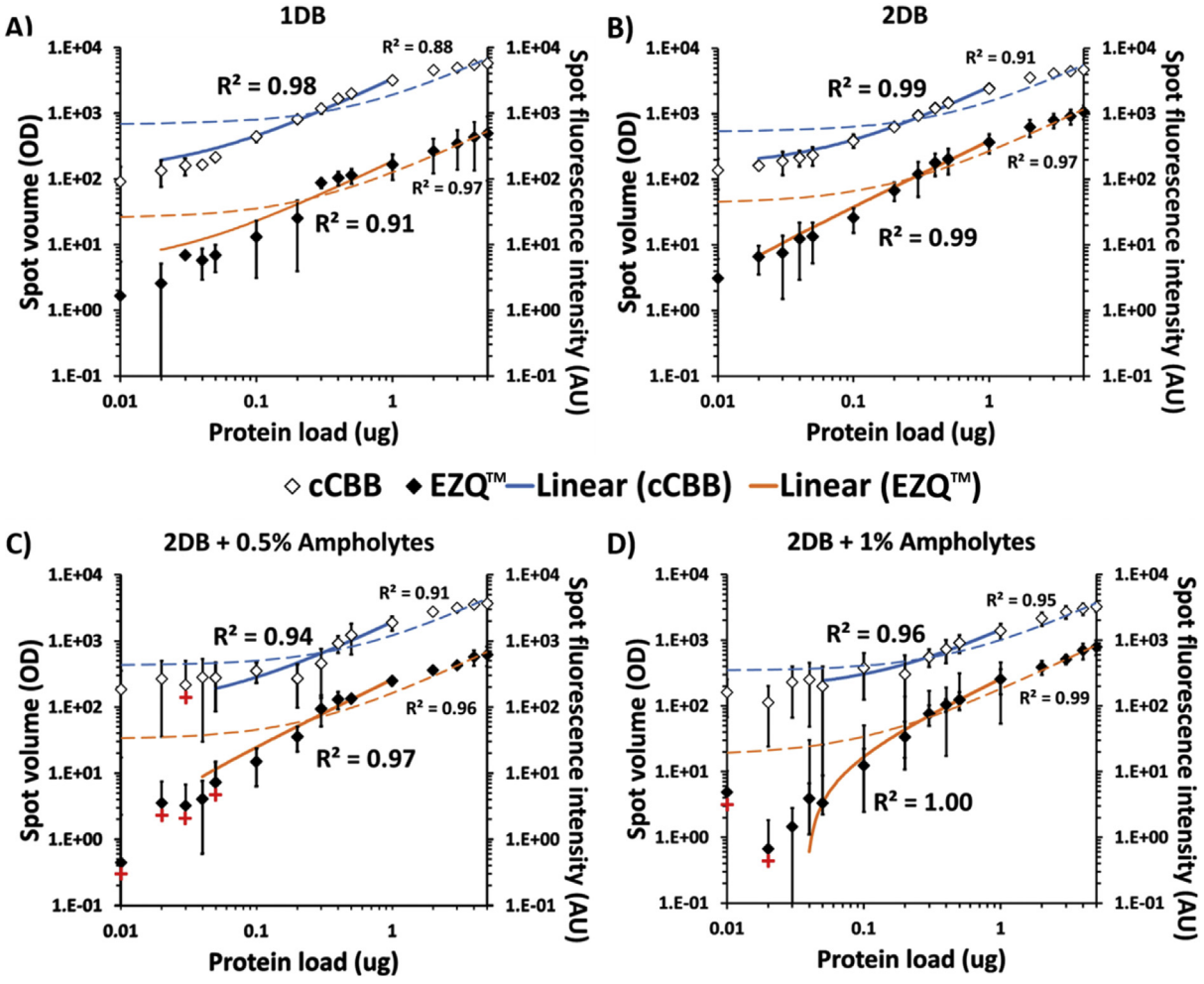

Fig. 1. Average spot signal from cCBB (OD) and EZQ™ (AU) vs. CEA load (μg) prepared in A) 1DB, B) ampholyte-free 2DB, C) 2DB with 0.5% (v/v) ampholytes, and D) 2DB with 1% (v/v) ampholytes. Linear fits for EZQ™ (orange) and cCBB (blue) are shown. R-squared values are shown for analyses of 1 μg to respective LODs (solid lines, large text) and for full data ranges (dashed lines, small text). All axes are log-scale. Error is STDEV (blot n = 3). '+' (red): unidirectional error-bars indicate error ≥ mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)