Supplementary Material For:

U-2012: An improved Lowry protein assay, insensitive to sample color, offering reagent stability and enhanced sensitivity

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Materials

Purified PROTEINS
Albumin bovine serum fraction V (BSA), carbonic anhydrase, cytochrome C, isocitrate dehydrogenase, lysozyme and trypsin.

Biological samples
Colored fruits and vegetables (blueberry and beetroot), and red wine

Reagents

Acetonitrile
Copper (II) sulfate 5-hydrate (CuSO₄·5H₂O)
Folin-Ciocalteu’s phenol reagent (Folin’s reagent)
Hydrogen peroxide (H₂O₂)
Perchloric acid (PCA)
Sodium carbonate (Na₂CO₃)
Sodium chloride (NaCl)
Sodium hydroxide (NaOH)
Sodium dodecyl sulfate (SDS)
Sodium phosphate (Na₂HPO₄)
Sodium potassium tartrate (Na-K-tartrate)
Sodium pyruvate
Trichloroacetic acid (TCA)
Triton X-100

Proteins and above reagents except perchloric acid (PCA) were obtained from Sigma (St Louis, MO, USA). PCA was obtained from BDH (England).

Recipes

Solution-1: PROTEIN SOLUTIONS FOR STANDARD CURVES
Recipes described below for BSA were also followed for other proteins for the generation of standard curves for unprocessed and processed proteins.

Solution-1a (Stock)
BSA stock solutions for standard curves (10 mL)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mass or Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>400 mg</td>
<td>40 mg BSA/mL</td>
</tr>
<tr>
<td>0.2% Triton X-100 in 0.15 M NaCl (Triton-NaCl)</td>
<td>Made up to 10 mL</td>
<td></td>
</tr>
</tbody>
</table>

Instructions:
- Make three independent 10 mL BSA stock solutions as replicates.
- Store each replicate as 1 mL aliquots in 2 mL Eppendorf tubes in freezer.
- Thaw 1 tube from each replicate stock solution on the day of assay. Each tube is sufficient to make 5 mL of BSA working solution (1B).

Solution-1b (Working)
BSA working solution (Solution-1B) for standard curves (5 mL) was for unprocessed samples

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mass or Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA stock solution 40 mg/mL (thawed &amp; vortexed)</td>
<td>1 mL</td>
<td>8 mg BSA/mL</td>
</tr>
<tr>
<td>(Triton-NaCl)</td>
<td>4 mL</td>
<td></td>
</tr>
</tbody>
</table>

Instructions
- Thaw 1 tube from each replicate BSA stock solution as needed.
- Keep BSA working solution (Solution-1B; 8 mg BSA/mL) at room temperature and use on the day. Discard any unused solution.
- These three 5 mL BSA working solutions were enough to generate the standard curves with triplicate measurements (see Procedure 3, Hint 1).

Solution-1C (Working)
BSA working solution (Solution-1C) for standard curves (20 mL) was for processed samples

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mass or Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA stock solution 40 mg/ mL (thawed &amp; vortexed)</td>
<td>1 mL</td>
<td>2 mg BSA/mL</td>
</tr>
<tr>
<td>(Triton-NaCl)</td>
<td>19 mL</td>
<td></td>
</tr>
</tbody>
</table>
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**Instructions**
- Thaw 1 tube from each replicate BSA stock solution as needed.
- Keep BSA working solution (Solution-1C; 2 mg BSA/mL) at room temperature and use on the day. Discard any unused solution.
- These three 20 mL BSA working solutions were enough to generate the standard curves with triplicate measurements (see Procedure 3, Hint 1).

**Solution-2**
For developing protein color complex (100 mL)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mass or Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.42% (100 mM) Na$_2$HPO$_4$ buffer (pH 12.0)</td>
<td>73 mL</td>
<td>73 mM</td>
</tr>
<tr>
<td>1% (40 mM) CuSO$_4$. 5H$_2$O</td>
<td>1 mL</td>
<td>0.40 mM</td>
</tr>
<tr>
<td>2.5% (88.6 mM) Na-K-tartrate</td>
<td>1 mL</td>
<td>0.886 mM</td>
</tr>
<tr>
<td>5% (173.4 mM) SDS</td>
<td>20 mL</td>
<td>34.68 mM</td>
</tr>
<tr>
<td>Acetonitrile (MW= 41.05; density= 0.777 g/mL)</td>
<td>5 mL (= 3.88 g)</td>
<td>946 mM</td>
</tr>
</tbody>
</table>

**Instructions:**
- Add various ingredients in the order listed.
- Add the next ingredient only after the previous ingredient is completely mixed.
- Avoid vigorous mixing after the addition of SDS.
- Store all the ingredient solutions and Solution-2 at room temperature.
- Na$_2$HPO$_4$ buffer is prone to develop microbial contamination, so examine the buffer solution for presence of microbial contamination before use. Other ingredients are stable for months.
- Solution-2 is stable for at least 2 weeks.

**Solution-3**
For developing protein color complex (10mL)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mass or Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folin’s reagent</td>
<td>5 mL</td>
<td>50%</td>
</tr>
<tr>
<td>Deionized water</td>
<td>5 mL</td>
<td>-</td>
</tr>
</tbody>
</table>

**Instructions:**
- Store Folin’s reagent in refrigerator (~4°C).
- Dilute (1:1; v/v) with water just before use. Discard the unused Solution-3.
- 10 mL of Solution-3 is sufficient for 100 assays. Adjust the amount of Folin’s reagent as needed.

**Troubleshooting**

**Problem 1: Microbial growth in Na$_2$HPO$_4$ buffer**

*Solution*
Discard (preferably after autoclaving) and prepare a new batch of the Na$_2$HPO$_4$ buffer.

**Problem 2: Solution-2 becoming turbid**

*Cause*
Drop in room temperature to a point where detergent (SDS) makes micelles. This is quite common in winter.

*Solution*
Transfer the bottle containing Solution-2 to a 30°C water bath. When solution becomes clear, equilibrate to ~ 20°C before use.

**Equipment:**
- Knives and grating device (for chopping biological samples)
- Food processor (500 mL capacity; high speed)
- Cheese cloth and funnel (for filtration)
- Homogenizer - T 25 digital Ultra-Turrax® (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany or equivalent)
- Centrifuge - Eppendorf AG. minispinplus Hamburg, Germany (for 2 mL tubes) and Rotanta 460 R, Hettich Zentrifugen, UK (for 50 mL tubes).
- Multiwell plate reader (PowerWave XS, BioTek) with software (KC4 v3.1)

**Procedures**
There are four procedures in the U-2012 assay:

**Procedure 1-Preparation of homogenates.**

* HINT
1. Proteins are assayed in an aliquot of the homogenate without adding glycerol. If enzymatic studies are to be carried out, glycerol should be added to a final concentration of 10% of the homogenate (see Step 4 below).
2. Choose containers for Step 6 which can be tightly capped and can withstand freeze-thaw conditions.
3. Maintain ice-cold conditions during Steps 3 to 6.

**Steps:**
1. Fresh or frozen blueberries were washed. Beetroot was peeled then finely grated.
2. For a 50% homogenate, homogenize 100 g of blueberry (or beetroot) in 40 mL of 1% Triton X-100 in 0.15M NaCl for 2 min. Make up to a final volume of 200 mL with 0.15M NaCl (the final Triton X-100 concentration is 0.2%).
3. Remove fibrous tissue by filtration through two layers of cheese cloth.
4. Further homogenize the filtrate using the Ultra-Turrax® homogenizer at 13,500 rpm for 1 min. If the homogenates are to be used for enzyme assay, glycerol can be added. Glycerol should not be in samples for protein assay.
5. Distribute and freeze (if necessary) the above homogenates in appropriate containers in 1 to 10 mL aliquots. On the day of protein assay, thaw the homogenate sample and vortex prior to protein assay.

**Procedure 2- Processing homogenates.**

* HINT
1. A preliminary protein assay in samples with low protein content (e.g. fruits and vegetables) should be carried out with the stated protocol. The volumes of colored biological sample and PCA can be proportionally adjusted later so as to get the sample absorbance close to the absorbance corresponding to C$_{50}$ (see main paper) after dissolving the pellet.
2. Use NaOH (rather than KOH) and 30% H$_2$O$_2$ (rather than sodium hypochlorite). KOH (used for neutralization of...
acid and solubilization of homogenate protein) and sodium hypochlorite (used for decolorization of homogenates) result in precipitation in U-2012 assay and therefore are incompatible.

3. For improved protein recovery at Step 5 (see below), the PCA supernatant should be poured off carefully without disturbing the pellet. Excess acid from the sidewall and the mouth of the centrifuge tube can be removed by touching the mouth of the tube to a paper towel. When handling large number of samples, the acid precipitated pellets can be stored in refrigerator for processing next day (step 6; see below).

Steps:
1. In a 2 mL Eppendorf tube add 1.2 mL of ice-cold 50% homogenate or 2 mg protein/mL.
2. Add 0.4 mL of ice-cold 20% PCA, incubate for at least 1 hr under ice-cold condition.
3. For red wine 12 mL was mixed with 4 mL of PCA in a 50 mL centrifuge tube.
4. Centrifuge red wine samples at 1000 x g for 30 min at 4 °C other samples for 5 min at maximum speed (about 7000 x g) in mini-centrifuge.
5. Discard supernatant. To the pellet add 0.05 mL 5% Na₂CO₃ (in 0.15M NaCl). Vortex to ensure residual acid has been neutralized.
6. Add 0.09 mL of 1.5M NaOH (in 0.15 M NaCl), 0.06 mL 1% Triton X-100 (in 0.15 M NaCl) then 0.02 mL 30% H₂O₂. Vortex thoroughly after each addition.
7. Incubate capped tubes at 50°C for 1 h. Dark colored samples will lose their intense color and become either colorless or pale yellow. The heating at 50°C is critical for the destruction of the color as well as the interfering substances like sugars which are bound to the precipitated proteins.
8. Unused H₂O₂ was destroyed by the addition of 0.09 mL of sodium pyruvate solution (330 mg/mL in water). Incubate at room temperature for additional 30 mins. Protein is now is in a total volume of 0.3 mL, giving a 4x concentration of BSA to a final concentration of 8 mg/mL, 4x concentration of the original homogenates of beetroot and blueberry and a 40x concentration of red wine proteins.
9. Pool 4 of the above tubes into 1 tube (total volume 1.2 mL) which was sufficient for generating the standard curves for BSA (and other proteins) and to determine the quantity of protein in the colored biological samples.

Procedure 3- Protein assay by the U-2012 assay

*HINT*
1. In this study the standard curves were generated using 19 concentrations between 0 to 4 mg/mL. A 12 point graph would be sufficient by limiting standard curves to 1.6 mg/mL (closer to C₅₀ values for BSA, see Figure 2).

Steps:
1. Place in 2 mL Eppendorf tubes 0 to 100 µL of 8 mg/mL proteins for generating standard curves or 0 to 200 µL of processed homogenates of colored biological samples (see HINT 1 for future implementation).
2. Top up with Triton-NaCl to a final volume of 200 µL when required.
3. Add 60 µL of 1.5 M NaOH.
4. Incubate for 10 min at room temperature.
5. Add 140 µL 0.47 mg mL sodium pyruvate in water (final volume 400 µL).
6. Add 1 mL of Solution-2.
7. Incubate for 10 min at room temperature.
8. Add 100 µL of Solution-3. Mix and incubate for at least 1 h at room temperature.
9. Transfer 200 µL to 96-well plate in duplicates for absorbance measurements.
10. Read absorbance (650 nm) using the multi-well plate reader.
11. Plot standard curves: absorbance (Y-axis) versus BSA concentration (X-axis).
12. Calculate amount of protein in the homogenates using appropriate standard curve (Procedure 4 below).

Procedure 4- Calculation of the amount of protein

*HINT*
1. May need to download and install the Solver module into Microsoft Excel if it is not already there. Instructions for this are provided in Excel’s Help.
2. The Excel workbook can be optimized to extract replicate standards and unknowns from a region of the worksheet arranged to visually correspond to the layout of the multi-well plates to make data entry and cross checks easier.
3. If preferred the absorbance zero point (A₀) can be calculated directly from the blank replicates and be deposited directly into the A₀ parameter location. In this case Solver is asked to minimize the residual standard deviation by adjusting just C₅₀ and A₀ alone. If this approach is used the analyst should check the graphical output for consistency of the zero point at low absorbance.

Initial values are required for the three parameters, A₀ and A₅₀, may be guessed from minima and maxima values from the measured array of absorbency. C₅₀ is an intermediate concentration giving an absorbance midway between A₀ and A₅₀. It is not critical to obtain exact values.

On the same sheet the parameters of the model can be used directly to compute the concentrations of the unknown replicate means, or alternatively can compute the individual replicate concentrations before computing the mean and standard deviations of these concentrations.

Steps:
1. Enter the values of standards concentrations and replicated absorbency into parallel columns of the worksheet. These may be copied down from the data region of the sheet using a formula if Hint 2 is adopted.
2. Compute average absorbance at each concentration in a new column.
3. Plot replicate and mean absorbency against concentration using an X-Y scatter plot.
4. Check for outliers and anomalies.
5. Create a three row by two-column table for the names and values of the three parameters (A₀, C₅₀, and A₅₀) and enter sensible guesses as to their values.
6. Create a column parallel to the average absorbency to take the calculated model absorbency and enter them into the equation

\[
A = A_0 + \frac{(A_M - A_0)\text{Conc}}{(C_{50} + \text{Conc})}
\]

to calculate absorbance using concentrations from Step 1 and parameters from Step 5.
7. Add the resulting values from this column as a smoothed line to the X-Y plot in Step 3.
8. In a new cell create a formula to calculate the residual standard deviation between the measured and modeled absorbency i.e. $\text{RES} = \text{SQRT} \left( \frac{\text{SUMXMY2(Model.Absorb.Array, Meas.Absorb.Array)/(N-1)}}{\text{N}} \right)$ where the two arrays are the spreadsheet coordinates of the N modeled and measured absorbency.

9. Command Excel’s Solver to set the residual standard deviation to a minimum by adjusting the values of the three (or two if $A_0$ has been pre-defined) parameters.

10. Inspect the plot for consistency of fitted and measured results.

11. The parameter values obtained by Solver can now be used to compute the concentrations of the unknown samples using the equation

$$\text{Conc} = \frac{C_{40} (A - A_0)}{(A_M - A)}$$

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