# Overview

The Lowry Protein Assay is used to determine the protein content of a homogenized solution. This lab uses Lowry Protein Assays to allow for the generation of 1 mg/ml homogenized solutions in preparation for a western blot or other form of quantitative analysis. This protocol involves first creating the homogenized solution, then performing the LPA with 10 µL of the homogenized solution, and finally diluting the original homogenized solution to the desired concentration.

# Additional resources

# Need more help?

Check the resources, and then see Ken

# Main content

**Materials**

* Ice bucket/dry ice bucket
* Eppendorf Tubes, 2 per sample
* Razors/scissors
* Urea thiourea aliquots
* Glass Homogenizer set
* DI H2O
* BSA (2 mg/mL solution –Thermo - Prod#: 23210)
* [RC DC Protein Assay] RC Reagent I, Bio-Rad cat#500-0117
* [RC DC Protein Assay] RC Reagent II, Bio-Rad cat#500-0118
* [DC Protein Assay] Reagent A, Bio-Rad cat#500
* [DC Protein Assay] Reagent B, Bio-Rad cat#500-0114
* Falcon tubes (15 mL), one per sample
* Glycerol – aliquot in 100 mL tubes
* Bromophenol blue- 1% aliquot in falcon tube next to heating block, stock (solid)
* Pipetters (1-10µL, 30-300µL, 100-1000µL, 1-10 mL), pipette tips
* Insulin syringe 9A

**Part 1: Sample Homogenization**

1. Obtain urea thiourea aliquots and place out to thaw. Usually, one 2-mL eppi will be enough for 2 samples.
2. Fill an ice bucket in prep for homogenization.
3. Obtain samples and label 2.0 mL eppendorf tubes.
   1. If needing to transport samples, obtain separate dry ice container and fill.
   2. For each sample, label 2 eppendorf tubes.
      1. Label one tube with the surgery harvest date or identifying code, tissue type, and the words “sample #”.
      2. For the other, label with only the surgery date (or identifying code) and tissue type.
4. Weigh out sample (0.015-0.02 g).
   1. Cut the frozen tissue with a razor to the appropriate size. Small surgical scissors are appropriate for thawed tissue but attempt to keep the tissue frozen to prevent further degradation.
   2. Nest the tissue into the bottom of the Dounce Homogenizer.
5. Add appropriate amount of Urea-thiourea (UTU) buffer to Dounce Homogenizer. This will be a 1:30 ratio (assuming the tissue has a 1 g / mL density)
6. Homogenize sample and transfer to clean 2.0 mL eppendorf tube.
   1. Homogenize with the homogenizer in the ice. Be careful not to cause too many bubbles while homogenizing, or else transfer will be more difficult. Homogenization is complete once the solution is clear of identifiable tissue. The grinder may get tissue stuck on the tip and a pair of forceps can be used to move it to the side. Note: white tissue fragments, which appear insoluble, are likely collagen and should not be considered viable tissue. If there is more tissue, make another sample; otherwise make a note of the collagen in the sample.
   2. Pipette the solution into the “sample” tube for the appropriate date or identifier. Make sure that everything in the homogenizer (including bubbles and homogenized tissue fragments) is transferred to the eppendorf tube.
7. Vortex the sample (30-60 seconds). Keep on ice until all samples are homogenized.
8. If doing multiple homogenizations, place recently homogenized samples on ice as they wait to move forward in the LPA process. Wash out the homogenizer with soap and rinse thoroughly with DI water before beginning moving on to step 9. Dry the homogenizer with air flow.
9. Centrifuge the samples at 4000 rpm for 30 seconds. This pulls the entire sample down to the bottom of the tube and gets rid of most of the bubbles. When you centrifuge, make sure and balance the sample holder (usually means in opposition)
10. Add 10 µL of the homogenized supernatant to the other eppendorf tube (the one **without** the “sample” label) with 90 µL DI H2O. These will be used for the assay, and is the 1:10 dilution that is corrected at the end of the assay.
11. These tubes will be the unknowns for the protein assay.
12. To dilute, it will be easier to have an aliquotted eppendorf tube of DI H2O.
13. Place the homogenized samples (undiluted) into the -20 freezer. Dilutions of these samples (to 1 µg/µL) will follow the protein assay.
14. It is important to keep all samples on ice in order to preserve the current phosphorylation state. However, the actual protein assay does not require this. In order to ensure phosphorylation/protein integrity preservation, be sure to keep all raw tissue and any eppendorf tubes with the label “sample” on ice at all times.

**Part 2: Protein Assay**

1. Label 5 (2.0 mL) eppendorf tubes for the standards.
   1. Label the tubes as the following: 2.0, 1.0, 0.5, 0.25, and 0.0.
2. Perform a serial dilution of the 2.0 µg/µL BSA standard in order to prepare the 5 standards.
   1. Add 100 µL BSA to the ‘2.0’ tube.
   2. Add 100 µL DI H2O to the ‘1.0’, ‘0.5’, ‘0.25’, and ‘0.0’ tube.
   3. Add 100 µL BSA to the ‘1.0’ tube and lavage the solution 10 times, making sure to not create any bubbles or shoot water up the side of the eppi.
   4. Extract 100 µL from the ‘1.0’ tube, add it to the ‘0.5’ tube, and lavage 10 times.
   5. Extract 100 µL from the ‘0.5’ tube, add it to the ‘0.25’ tube, and lavage 10 times.
   6. Extract 100 µL from the ‘0.25’ and dispose of it in order to make all of the tubes volumes consistent at 100 µL.
3. Add 500 µL RC Reagent I to each standard tube and to each sample tube.
4. Vortex pulse each tube four <5 seconds and let them incubate for 1 minute at room temperature.
5. Add 500 µL RC Reagent II to each standard tube and to each sample tube.
6. Vortex each tube. Let the samples incubate at room temp for about 5 minutes.
7. Centrifuge standard and sample tubes at 15,000 rpm for 5 minutes.
   1. Be sure to keep the joint of the tube (where the lid attaches) facing upwards (to the outside of the circle) when orienting the tubes inside of the centrifuge. This ensures that the pellet will form in a predictable area and will make the subsequent pipetting easier to manage.
8. Drain tubes and set them aside to dry.
   1. You should see globs of protein bound to the tube after the centrifuge. When draining, monitor the precipitate closely. You do not want any of the precipitate to get flushed out with the solution. If this occurs, stop immediately and re-centrifuge the sample for a few minutes (same rpm).
   2. The waste goes into the hazardous waste container.
   3. Dry the tubes checking on them in 30 minute intervals. If there is a large amount of solution in the tube cap, it may be possible to use a Kim wipe too speed up the process, as long as none of the protein is in contact with the Kim wipe. The tubes generally take ~1 hour total to dry, depending on lab’s local environment. When dried, there will be only solid precipitate in the eppis.
9. After the tubes are fully dried, add 510 µL Reagent A’ to each tube. Vortex each tube, let them incubate for 5 minutes, and then vortex the tubes again.
10. Acquire an appropriate number of 15 mL falcon tubes and label them as the standards (2.0, 1.0, 0.5, 0.25, and 0.0) and the unknowns.
    1. Add 4 mL Reagent B to each falcon tube.
11. Add the entirety of each eppendorf tube’s contents (including bubbles) into the falcon tube.
12. Vortex each falcon tube and allow them to incubate at room temperature for 15 minutes.
13. The spectrophotometer is located in the Esser lab. Make sure and bring all of the materials needed to load the samples into the lab. You will need the long 200 μL pipette tips, the 30-300 μL pipette, a 96-well plate (wrap in kim wipes to prevent scratching and contamination on the outside!), gloves, an insulin syringe, and a flash drive. **Don’t forget the flashdrive**!
14. Load the standards and samples in triplicates into a 96-well plate.
    1. 200 µL for each sample should be used. Use an insulin syringe to pop any bubbles that appear in the wells.
15. Run the protein assay on the spectrophotometer in the Esser Lab.
    1. Turn on the spectrophotometer.
    2. Open up the SoftMax Pro program.
    3. Click on “Assays” and select “Lowry”.
    4. Click on “Template” and enter the standards and unknowns.
       1. Both the standards and the unknowns are measured in mg/mL, but the unknowns have a dilution factor of 10.
    5. Gently wipe down the 96-well plate with a kim wipe to remove any contaminants and insert it into the spectrophotometer.
    6. Save the file and also export it as a text file.
    7. Take out the 96-well plate and shut down the spectrophotometer.
16. Waste disposal: if not too much volume, pour falcon tubes out in the sink, dilute with running water for a few minutes, and clean the falcon tubes for reuse. Eppendorf tubes and pipet tips are placed into the nonhazardous waste cardboard boxes.

**Part 3: Sample**

1. Take the samples out of the freezer and heat the samples at 60° C for 10 minutes. Place glycerol aliquot(s) in the heating block for later use to allow for it to be less viscous. While the samples are heating, label a new set of eppendorf tubes for each sample.
2. Determine the amounts of individual volume available in the thawed sample tubes and add the measured volumes into the newly labeled eppendorf tubes. Note: if the sample is under 1 g/L, it would be best to perform a new homogenization, with more tissue needed per buffer solution and the protein assay performed again.
   1. Determine a single volume of each sample to be diluted. This will generally be roughly 50-75 µL less than the UTU buffer added in the initial homogenized step.
   2. Enter this value into the “Homogenized Volume Used” cell on the protein assay spreadsheet template found in Microsoft Excel. Save you work as a different file name to maintain a clean template.
3. According to the protein assay spreadsheet, add the appropriate amount of 30% glycerol, bromophenol blue, and UTU buffer to each tube in order to adjust each concentration to 1 µg/µL.
   1. Use the adjusted concentration (from the protein assay) as the value for the “[protein] after assay” cell on the protein assay spreadsheet.
   2. The spreadsheet should now contain all the necessary volumes of 30% glycerol, bromophenol blue (BPB), and UTU in order to perform the correct dilutions.
   3. It is possible that a dilution will require a higher volume than 2.0 mL. In this case, use a larger 5 mL cryogenic vial. Make sure that all the homogenized tissue that has settled to the bottom is transferred into the cryogenic vial. **If this step is necessary**, aliquot the solution evenly into 2.0 mL eppendorf tubes, as the 5 mL cryogenic vials will not fit into the centrifuge.
4. Vortex each tube.
5. Centrifuge the tubes at 13,200 rpm for10 minutes. While centrifuging, label final sample eppendorf tubes. This step allows the contents to mix much easier due to the density differences of the compounds.
6. Divide up each tube into at least two aliquots of greater than 100 μL. Creating multiple aliquots may minimize freeze-thaw issues. (Most samples shouldn’t be re-used after ~3-4 thaw cycles)
7. Keep samples frozen in the -80°C freezer until it is time to load them.
8. If there is any waste, it is placed in the hazardous waste can.