

Ribosomal Proteomics Protocol

This supplement supports research published in AJUR, Volume 12 | Issue 1 | August 2014, entitled: Proteomic Study of Ribosomal Proteins from *Escherichia coli*, *Saccharomyces cerevisiae*, *Bos taurus*, *Gallus gallus*, and *Oncorhynchus tshawytscha*: Application in a Teaching Laboratory Setting.

Text in red indicate things the instructor might have to do to save time in the laboratory.

Backup samples should be made for all steps of this procedure, particularly: homogenization, ultra-centrifugation, and SDS-PAGE.

Day 1

HOMOGENIZATION:

The instructor will provide you with the frozen samples of livers and grown and precipitated *E.coli* and yeast. Make sure to keep samples on ice as much as possible.

For *E. coli* (Strain BL21)

Grow BL21 in LB media overnight and harvest at 4,000 x g. It can be stored frozen until needed. Wash out the *E. coli* contained in the mini-centrifuge tube into a 15 mL Fischer centrifuge tube by adding Solution A and vortexing. *Use 1 mL of Solution A for every 0.3 g of E. coli.*

- Sonication:
 1. With the sample on ice, place the sonicator probe in the sample.
 2. Turn on the sonicator and sonicate for a total of 2 minutes at 30% cycle, setting 3. Be sure to move the tube up and down to ensure the entire sample is coming into contact with the probe.
 3. Turn off sonicator and keep the sample on ice. The probe should be cleaned with 70% ethanol between sample preparations.

For Yeast

Grow baker's yeast overnight in LB media supplemented with 0.85 g/ml sucrose. Centrifuge at 4,000 x g's and store in the freezer till needed. Wash out the yeast contained in the mini-centrifuge tube into a 15 mL Fischer centrifuge tube by adding Solution A and vortexing. *Use 1 mL of Solution A for every 0.3 g of yeast.*

- French Press: Your instructor will show you how to use this machine. (Yes, it is a machine, not an instrument.)
- Once you have French Pressed your sample, run it through the machine one more time.

For Beef, Chicken, or Salmon Liver

Purchase livers at local grocery store. Using clean mortar and pestle, grind 1 gram of liver in 4.5 mL of Solution A and 0.5 g of sea sand for 5 minutes.

Filter homogenate through eight layers of cheese cloth to remove fats and cellular debris into a 15 mL Fischer centrifuge tube as shown in **Figure 1**. Use gloves when handling the cheesecloth. The size of the cheesecloth should be approximately 2 x 2 inches when folded.



Figure 1. Recommended set up for Beef/Chicken/Salmon extraction through cheesecloth.

FILTRATION AND EXTRACTION:

1. Make certain all tubes are labeled properly, and draw a notch on the tube and cap of the Fischer centrifuge tube. Place the tubes in the centrifuge so the notch is facing upward. This way, it will be known on which side the pellet will form in the tube. While the pellet will be clearly visible in this step, it will become essential when using the ultracentrifuge.
2. Balance the tube within 50 mg of another tube by adding additional volumes of Solution A.
3. Centrifuge for 10 minutes at 29,000 x g (10,000 rpm in Sorvall RC6 centrifuge) to remove large cellular debris.
4. Remove visible fats from the top of the solution using a pipette tip (*only for beef, chicken, and salmon samples*). **Do not pipette up the fats.** Simply holding the tip in your fingers and gently moving it across the surface of the solution should make the fat stick to the plastic tip. Use multiple tips if necessary.
5. Place supernatant in a new 15 mL centrifuge tube and repeat steps 1 through 4.
6. Obtain a 10 ml ultracentrifuge tube and make a notch on the side near the top with using a marker as before. From the bottom, *carefully* make the following layers: 2 mL of Solution C, 2 mL of Solution B, then 2.5 mL of supernatant from step 3. Ensure tubes are balanced to the **milligram** by adding Solution A or removing sample.
7. Spin tubes at 180,000 x g for 4 hours (Sorvall T-890 rotor at 45,900 rpm) to precipitate ribosomes.

End of Day 1

BEFORE CLASS

8. Discard supernatant and remove as much liquid as possible from the tube by inverting the tube onto a paper towel—your pellet will not fall out. (Tapping the inverted tube against the towel works well too; this step might be done for you by the instructor after the run is done.)

Day 2

9. Re-suspend ribosomal pellet in 100 μ L H₂O by pipetting up and down. Note: the pellet, especially for the single-celled organisms will be transparent and extremely difficult to see. Make use of the notch you made on the tube to know where to direct the water when dissolving the pellet.
10. Use 5 μ L of solution made in the previous step to prepare 1:25 and 1:50 dilutions serially to run on NanoDrop (or equivalent) for UV spectroscopy and confirm presence of ribosomes using A_{260/280} value. Note: the readings are unreliable when the peak absorbance is greater than 2.0. Take a screen shot of the curve and save the image to use in the laboratory report. Purified ribosomes should have a value between $1.7 < A_{260/280} < 2.0$.
11. Calculate protein concentration using the following equation:

$$\text{Protein concentration (mg/mL)} = (1.55 \times A_{280}) - (0.76 \times A_{260})$$

Determine what volume of which dilution would be required to obtain a total of 20 μ g of protein while keeping below 20 μ L. (Having less volume will make it significantly easier to load the gel.)

1D SDS PAGE – GEL:

12. Add appropriate volume of diluted sample from the previous step to a 0.5 mL plastic tube along with an equal volume of 2x SDS sample buffer. Place tube in a thermocycler and run program PRTDNAT (Run \rightarrow Directory A \rightarrow 23: PRTDNAT \rightarrow Hot lid: Auto \rightarrow Tube) which will heat the sample at 95° C for 3 minutes. An alternative heating method is acceptable. Load sample onto premade 1D SDS PAGE gel. Avoid using right and left-most lanes of the gel (not including MW marker lanes). *Make sure to write down which lane contains your protein.*
13. Run gel at 200V for 50-55 minutes.

Participate in the “Total protein MALDI” training while waiting:

14. Retrieve Sinapinic acid (SA) from the freezer and prepare as follows:
 - a. Add 100 μ L of 50% ACN, 0.1% TFA solution per mg of SA.
 - b. Vortex for 2 minutes.
 - c. Centrifuge at 14,000 rpm for 5 minutes.
15. Prepare sample in 1:4 sample to SA matrix ratio (2 μ L : 8 μ L).
16. Spot one 2.0 μ L sample onto the target plate. Make sure you do not touch the plate with a tip when spotting.
17. If target plate has space, spot another 2.0 μ L sample for backup; record the spots for your samples.
18. Prepare protein standards by obtaining 2.0 μ L from one of the tubes labeled protein standard, placing it into a new lo-bind tube, and making a 1:4 dilution with the SA solution.
19. Prepare two spots of 2 μ L of peptide/matrix solution on the plate.
20. Dry for approximately 15 minutes by placing plate on the lip of the fume hood.
21. Load plate into MALDI-TOF. Place the plate in the instrument with A1 well oriented left and towards you.

Note: At this point, you *may* take a break and leave the sample inside the instrument. *However*, the proteins will degrade over time so it would be best to analyze them within 24 hours at the latest. Keep in mind though the fresher the sample is the better.

22. Start FlexControl. Choose the linear LP_12kDa.par or ProteomicsLab1method. Perform calibration for a protein mixture, if needed. Collect spectra (while varying the spot location and the intensity of the laser) in the sum buffer, label peaks, save data in the class folder, and print the spectrum for the full protein. Note that you might find $m+H^+$, $(m+2H^+)/2$, and $2m+H^+$ peaks on the spectrum.
23. Disassemble PAGE apparatus, extract gel, and then perform three washes of water for 10 minutes each; discarding water after every wash. Place in Bio-safe Coomassie overnight.

End of Day 2

BEFORE CLASS

24. Discard staining solution, perform two 5 minute washes with water, and scan/photograph gel.

Day 3

DIGESTION:

25. Excise a single band using a razor blade and cut the band into smaller fragments. Place the fragments in a lo-bind tube. (Try not to get parts of the gel without any protein in it i.e. the area surrounding the band.)
26. Add 200 μ L of destaining solution, incubate at 40°C for 10 minutes, shake every 5 minutes (make sure all gel pieces are submerged in solution before returning tube to incubator). Discard destaining solution; repeat this step until no more dye present in gel.
27. Add 40 μ L of reducing buffer (add more if not all gel pieces are covered) and incubate at 60°C for 10 minutes. Cool to room temperature and discard buffer.
28. Add 40 μ L of alkylating buffer (add more if not all gel pieces covered) and incubate in the dark at room temperature for 30 minutes. Discard buffer.
29. Wash sample by adding 200 μ L destaining buffer to tube. Incubate sample at 37°C for 15 minutes, shake once midway through the incubation and make sure all gel pieces are submerged in solution before returning tube to incubator. Discard buffer, repeat.
30. Add 50 μ L of acetonitrile and incubate at room temperature for 15 minutes to shrink the gel pieces. Discard acetonitrile and allow sample to air dry for 5-10 minutes.
31. Add 15 μ L activated trypsin (add more if all pieces are not covered) and incubate at room temperature for 15 minutes. **DO NOT REMOVE TRYPSIN**
32. Add 25 μ L of digestion buffer to each tube; incubate at 37°C with shaking overnight.

End of Day 3

BEFORE CLASS

33. Sonicate samples for 30 minutes.

Day 4

MALDI: DIGESTED SAMPLE PREPARATION

34. Place liquid sample into clean lo-bind tube. Leave the gel pieces behind.
35. Vacuum centrifuge sample to dryness at 60°C. Samples should be dry in 1 hour.
36. Re-suspend sample in 10 µl of 0.1% TFA.
37. Sonicate for 30 minutes in water bath sonicator.
38. Zip-tip sample using C4 tips:

Note: Make certain you read the following section extremely thoroughly and refer to the list of reagents at the end of the protocol (starting on page vii of this document).

Making a mistake here could result in complete loss of your sample.

- a. Equilibrate the tip:
 - i. Aspirate 10 µL **wetting solution** into tip and discard wetting solution. Repeat.
 - ii. Aspirate 10 µL **equilibration solution** and discard equilibration solution. Repeat.
- b. Bind and wash sample:
 - i. Aspirate and dispense your **sample** 3-7 cycles.
 - ii. Aspirate **washing solution** and discard washing solution. Repeat wash.
- c. Elute sample:
 - i. Dispense 4 µL of **elution solution** into clean lo-bind tube using a *standard pipette tip*.
 - ii. Aspirate and dispense **elution solution** through zip tip at least 3 times without introducing air.

End of Day 4

Day 5

39. Retrieve a-cyano-4-hydroxycinnamic acid (CCA or ACC) from freezer.
40. Prepare CCA solution:
 - a. Add 100 µL of 30% ACN, 0.1% TFA solution per mg of CCA.
 - b. Vortex for 2 minutes.
 - c. Centrifuge at 14,000 rpm for 5 minutes.
41. Prepare sample in 1:4 sample to CCA matrix ratio (2µL : 8 µl).
42. Spot one 2.0 µl sample onto target plate. Make sure you do not touch the plate with a tip when spotting.
43. If target plate has space, spot another 2.0 µL sample for backup; record the spots for your samples.
44. Prepare peptide standards by taking 2.0 µL from one of the tubes labeled pep std in the white box in the freezer into a new lo-bind tube, and make a 1:4 dilution with CCA solution.
45. Prepare two spots of 2 µL of peptide/matrix solution on the plate.
46. Dry for approximately 15 minutes by placing plate on the **lip** of the fume hood.

47. Load plate into MALDI. Place the plate in the instrument with A1 well oriented left and towards you.

Note: At this point, you *may* take a break and leave the sample inside the instrument. *However*, the proteins will degrade over time so it would be best to analyze them within 24 hours at the latest. Keep in mind though the fresher the sample is the better.

48. Open FlexControl, use “proteomics_course_digest.psm” method. (If the program ever asks if you want to save the **METHOD**, say “No.”) Collect good quality sum spectra for your **standards** (zooming, low power, and patience are your friends). Go to the calibration tab, choose appropriate mono_pep mix and choose 5% zooming. Click left from each available peak to adjust standards, check that they are not very different from the current settings, and accept them. Make sure to use the linear fit if working with a wider range of MW in your samples as compared to the standards, and use the quadratic fit if working within the range of the standards. Take a few shots to check on standards, save, print, zoom in on one peak to show quality, print.
49. Collect PMF spectra for your sample. **Save the spectra (not the method)**, go to Flex Analysis. (**Make sure the “Sum” box is checked when you save.**) To prepare spectrum, go to Process, smooth, then subtract background. To get masses of fragments, go to Mass list, find. To remove trypsin and keratin peaks, go to Calibrate, internal, select Trypsin Promega-porcine-Robert, click on each name, compare to the spectrum, and delete from the list if within 0.5 Da.
50. For further processing of PMF, go to Tools, Biotools, click on MS button to submit PMF to the database. Make sure to select for appropriate settings (size – blank, tolerance – start at 0.5 Da and adjust to try different parameters search fails; permanent modifications – carbamidomethyl (C) and variable modifications Oxidation (M), missing cleavages 2, select taxonomy and a data base). **Uncheck Results: Overview** to make search significantly faster. Start. Repeat search by changing variables. Adjust the number of cleaves and tolerance to get higher MOWSE score. Check the best hits and get the RMS data. Report both the MOWSE score and the difference between that score and the next closest hit Δ MOWSE. When hits are significant, import the best one into Flex Analysis by clicking on hit button. Close the top window. Report % coverage and % intensity covered. Save file, print the spectrum and the overlap data. Do not forget that you are looking for ribosomal proteins of your particular species.
51. **The training for the digest analysis will be done in the class.** You may analyze your total protein and both of your digests on your own time. Please sign in on the time sheet on the door.

Reagents

Day 1

Solution A

- 25 mM Tris-HCl
- 50 mM KCl
- 5 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$
- 250 mM sucrose
- 0.1 mM EDTA
- 7 mM BME
- pH 7.6

Solution B

- Same as *Solution A* but 1.5 M sucrose

Solution C

- Same as *Solution A* but 2.0 M sucrose

Preparation: Measure out appropriate masses of Tris-HCl, KCl, MgSO_4 , and sucrose. Dissolve solutes in volume of water less than final desired volume with heating and stirring. **Be cautious when preparing high molarity sucrose solutions, for displacement of water can make the final volume much greater than the desired final volume.** Prepare 0.5 M EDTA stock solution and add appropriate amount of stock to solution. Allow solution to cool before adding HCl to adjust pH. Store in refrigerator until use. Before use, add appropriate amount of BME to make solution 7 mM.

Day 2

2x SDS Sample Buffer

- 3.55 mL DI H_2O
 - 1.25 mL 0.5 M Tris-HCl, pH 6.8
 - 2.5 mL glycerol
 - 2.0 mL 10% (w/v) SDS
 - 0.2 mL 0.5% (w/v) bromophenol blue
- Add 50 μL β -mercaptoethanol prior to use.

Electrode Buffer (SDS PAGE)

- 3.03 g Tris (25 mM)
- 14.4 g glycine (192 mM)
- 1g SDS (3.47 mM)
- Bring volume up to 1L with DI H_2O

Biosafe Coomassie

50% acetonitrile, 0.1% trifluoroacetic acid solution

Reagents continued

Day 3

Destaining Solution

- 50 mL acetonitrile
- 50 mL DI H₂O
- 200 mg ammonium bicarbonate

Should be sufficient for both sections

Reducing Buffer (100 mM)

- 15.4 mg dithiothreitol
- 1 mL DI H₂O

Prepare 1 tube for each section (maybe 2nd section only needs ½ a tube?)

Alkylating Buffer (100 mM)

- 19 mg iodoacetamide
- 1 mL DI H₂O

Prepare 1 tube for each section (maybe 2nd section only needs ½ a tube?)

Digestion Buffer

- 20 mg ammonium bicarbonate
- 5 mL DI H₂O

Activated Trypsin

- Dilute trypsin stock in 1:5 dilution with digestion buffer
- Prepare 1.5 µL of dilution + 13 µL of digestion buffer per sample; the recommended amount of protease is 1:100 to 1:20 of the amount of target protein present.

Day 4

Zip-Tip Solutions

Equilibration & Washing Solution (0.1% TFA in DI H₂O)

- 100 µL 1% trifluoroacetic acid
- 900 µL DI H₂O

Wetting & Elution Solution (50% ACN in 0.1% TFA)

- 500 µL acetonitrile
- 500 µL 0.1% trifluoroacetic acid

Day 5

30% acetonitrile, 0.1% trifluoroacetic acid solution