Actions

New Page
Recent Changes
Manage Wiki

Search

Navigation

HOME

Courses
Mol Techniques
Environ Physiology

Notebooks
Sam's Notebook
Steven's Notebook
Rachel's Notebook
Tatyana's Notebook
Mac's Notebook
Christin's Notebook
Leslie's Notebook
Kevin's Notebook

Gadgets powered by Google

edit navigation

L2 - Tissue Extraction II

- *re-spec dilution of protein sample*
- *Continue with RNA extraction protocol*
- *Run SDS-PAGE protein gel*
- *Order Primers*

List of Supplies and Equipment:

- protein gel box (SR provided)
- SDS/PAGE gels
- trays for staining gels
- protein ladder marker
- digital camera
- power supply
- microfuge tube racks
- running buffer (SR provided)
- coomassie stain (SR)
- platform rocker/shaker
- plastic wrap
- sandwich bags
- gel loading tips
- pipettors (1ul-1000ul)
- sterile, 1.5mL screw cap tubes
- pipet tips (filter - RNAse free)
- RNase free water
- hot plate
- tube "floatie" (8 tube capacity)
- glass container for boiling water that can accommodate "floatie"
- 2X SDS reducing sample buffer (SR provided)
- heating block/water bath
- acetic acid
- chloroform
- isopropanol
- ethanol
- ice buckets
- phenol/chloroform waste containers (liquid/solid)
- cuvettes
- filter tips
- methanol
- safety glasses
- gloves
- timers
- 50 ml Falcon tubes / holders
- DEPC treated water (SR provided)----
- Normal light box (SR provided)

RNA ISOLATION PROTOCOL (see also Lab 1 Tissue Extraction I and Manufacturer Protocol)

1. Turn on heating block to 55C. Also turn on spectrophotometer.
2. Add 500uL of TriReagent to a 1.5mL snap cap tube. Store on ice.
3. Cut a piece of frozen tissue weighing between 50-100mg and add to tube containing TriReagent.

4. Carefully homogenize the tissue using a disposable pestle.
5. Add an additional 500uL of TriReagent to the tube and close the tube.
6. Vortex vigorously for 15s.

----- Stop here for Lab 1 and freeze sample at -80
Turn on heating block to 55C.

7. Incubate tube at room temperature (RT) for 5 mins.
8. In the fume hood, add 200uL of chloroform to your sample and close the tube. NOTE: Due to the high volatility of chloroform, pipetting needs to be done carefully and quickly. Have your tube open and close to the container of chloroform before drawing and chloroform into your pipette tip.
9. Vortex vigorously for 30s. You are vortexing correctly if the solution becomes a milky emulsion.
10. Incubate tube at RT for 5 mins.
11. Spin tube in refrigerated microfuge for 15 mins. @ max speed.
12. Gently remove tube from microfuge. Be sure not to disturb the tube.
13. Slowly and carefully transfer most of the aqueous phase (the top, clear portion) to a fresh microfuge tube. Do NOT transfer ANY of the interphase (the white, cell debris between the aqueous and organic phase).
14. Close the tube containing the organic and interphase and properly dispose of the liquid inside the tube as well as the tube itself at the end of the lab.
15. Add 500uL isopropanol to the new tube containing your RNA and close the tube.
16. Mix by inverting the tube numerous times until the solution appears uniform. Pay particular attention to the appearance of the solution along the edge of the tube. If mixed properly, it should no longer appear viscous/"lumpy".
17. Incubate at RT for 10 mins.
18. Spin in refrigerated microfuge at max speed for 8 mins.
19. A small, white pellet (RNA and salts) should be present. If not, do not fret. Continue with procedure.
20. Remove supernatant.
21. Add 1mL of 75% EtOH to pellet. Close tube and vortex briefly to dislodge pellet from the side of the tube. If the pellet does not become dislodged, that is OK.
22. Spin in refrigerated microfuge at 7500g for 5mins.
23. Carefully remove supernatant. Pellet may be very loose. Make sure not to remove pellet!
24. Briefly spin tube (~15s) to pool residual EtOH.
25. Using a small bore pipette tip (P20 or P200 tips), remove remaining EtOH.
26. Leave tube open and allow pellet to dry at RT for no more than 5mins.
27. Resuspend pellet in 100uL of 0.1%DEPC-H2O by pipetting up and down until pellet is dissolved.
28. Incubated tube at 55C for 5mins. to help solubilize RNA.
29. Remove tube from heat, flick a few times to mix and place sample on ice. This will be your stock RNA sample.
30. Quantitate RNA yield using spectrophotometer.

RNA QUANTIFICATION

1. Obtain two disposable plastic cuvettes: one for a blank and another for your RNA sample.
2. Label both cuvettes at the very TOP of the cuvettes.
3. Add 1mL 0.1%DEPC-H2O to a fresh 1.5mL snap cap tube. This will be your blank.
4. To a fresh 1.5mL snap cap tube, add 990uL 0.1%DEPC-H2O and 10uL of your RNA sample. Mix well by inverting tube multiple times.
5. Transfer these two samples to their respective cuvettes.
6. Ensure wavelength of spectrophotometer is set to 260nm.
7. Insert blank cuvette as demonstrated and zero this sample.
8. Remove blank cuvette from spectrophotometer and insert your RNA sample cuvette.
9. Record the A260 value displayed.
10. Adjust the wavelength and record the values at 230nm and 280nm.

11. Calculate RNA concentration (conversion: 1 A260 unit = 40 ng/uL single-stranded RNA), total RNA yield, A260:A280, and A260:A230.
12. Clearly label your stock RNA sample with the word "RNA", source organism/tissue, your initials, today's date and the concentration in ug/uL.
13. Place tube in ice bucket at the front of the lab. Store your samples at -80C.

Background - SDS - Polyacrylamide Gel Electorophoresis (SDS-PAGE)

SDS-PAGE is the process of separating proteins from one another on the basis of molecular weight. A mixture of proteins is subjected to an electric field and pulled through a polyacrylamide matrix towards the cathode. However, proteins must be treated prior to separating them in this manner. This is because the charge on any given protein is dependent upon the amino acid sequence as well as the pH of the solution. Thus, a crude protein extract from cells or tissue contains a hetergenous mixture of proteins with varying charges. Without treating them in some manner, the proteins will migrate independent of their molecular weight. Additionally, proteins have various tertiary or quarternary structure that can influence the rate at which they migrate in an electric field. In order to address these issues, protein samples are prepared in a specific fashion to linearize the proteins and impart the same charge to all proteins in the sample to ensure that they become homogenous and their migration rate during SDS-PAGE is solely due to molecular weight.

Protein samples are combined with a reducing sample buffer. This reducing sample buffer contains sodium dodecyl sulfate (SDS), B-mercaptoethanol, glycerol, Bromophenol blue and a buffer. SDS imparts an overall negative charge to all the proteins in a sample. This ensures that all of the proteins will migrate in the same direction (towards the cathode) when placed in an electric field. B-mercaptoethanol is a reducing agent. It accepts electrons from disulfide bonds formed between two cysteine residues. It serves as one step to help break any tertiary or quartenary structure of proteins in the sample. Gycerol simply serves as a sinking agent for your sample. It has a greater density and will allow your sample to sink into the buffer contained in the wells of the gel. Bromophenol blue is a negatively charged dye that allows one to visually track the migration of your samples through the polyacrylamide gel. Bromophenol blue migrates at the same rate of proteins ~5-7kDa. Finally, the buffer is present to maintain the appropriate pH for your sample. Once samples have the appropriate amount of reducing sample buffer, they are boiled. Boiling causes the proteins to fully denature, eliminating any tertiary or quartenary structure and leads to linear chains of amino acids.

Samples are then run through polyacrylamide gels. Traditionally, a single polyacrylamide gel is actually comprised of two gels with different percentages of polyacrylamide, pH and buffer. The top portion of the gel, relative to the bottom portion of the gel, has a lower percentage of acrylamide, a lower pH and a lower concentration of buffer. This gel is referred to as the stacking gel. The bottom gel has higher mounts of all three components listed above and is called the running gel.

The low percentage of polyacrylamide in stacking gels allows all the proteins in the sample, regardless of molecular weight, to quickly and easiy migrate through the gel. When the samples begin to enter the lower gel containing a higher percentage of polyacrylamide (running gel), the proteins are now all "stacked" upon one another. This allows all of the proteins in a sample to enter the running gel at essentially the same time. Additionally, the differences in pH and buffer content between the stacking and running gels leads to a local increase in voltage around the sample, which helps drive the sample from solution in the well into the polyacrylamide matrix of the stacking gel.

After SDS-PAGE is complete (when the dye front has reached the bottom of the gel), the gel is either set up for Western blotting (see Lab #4) or is stained to reveal the proteins. There are a number of stains that can be used, depending on the sensitivity needed to visualize proteins of interest, but we will use Coomassie Brilliant Blue. This is a non-selective stain,

meaning it binds all proteins regardless of their amino acid makeup. Additionally, it is cheap and rather sensitive. The dye will initially turn the entire gel blue and even a short exposure (5 minutes) often results in over staining. Thus, it is necessary to destain the gel to wash the dye out of the areas of the gel where no protein is present. After destaining, the proteins should appear as blue/purple bands and the rest of the gel should remain relatively clear.

PROTEIN GEL PROTOCOL - See also Manufacturers Protocol / Manual: Precise™ Protein Gels

1. *Begin boiling water on hot plate.*
2. Thaw you protein extract from last week. Mix well by inverting tube several times.
3. In a fresh, 1.5mL SCREW CAP tube add 15uL of your protein sample and 15uL of 2X Reducing Sample Buffer.
4. Mix sample by flicking. Briefly centrifuge (10s) to pool liquid in bottom of tube.
5. Boil sample for 5 mins.
6. While sample is boiling, observe assembly of gel box and gels. Rinse gel wells thoroughly as demonstrated.
7. When sample is finished boiling, immediately centrifuge for 1min. to pool liquid.
8. Slowly load your entire sample into the appropriate well using a gel loading tip.
9. Put lid on gel box and plug electrodes into appropriate receptacles on the power supply.
10. Turn power supply on and set voltage to 150V. Run for 45mins.
11. Add ~150mL (does not have to be measured - just need enough to cover the gel) of Coomassie Stain to a designated container.
11. Turn off power supply and disconnect gel box from power supply.
12. Remove lid from gel box.
13. Disengage the tension wedge.
14. Remove gel from gel box.
15. Carefully crack open cassette to expose gel.
16. Trim wells at top of gel.
17. Notch a designated corner of the gel to help you remember the correct orientation of the gel (i.e. which is the top/bottom of the gel, which is the right/left side(s) of the gel)
18. Place gel into container with Coomassie Stain.
19. Incubate on shaker/rocker for 5 mins.
20. Carefully pour stain back into original container. Be careful not to dump out gel!
21. Rinse gel briefly with 10% acetic acid and pour this wash down the drain.
22. Add ~250mL (no need to measure) 10% acetic acid to container with gel. Incubate on shaker/rockers for 15mins. Change out buffer and repeat until bands become clearly visible. This may need to incubate O/N. If so, cover container with plastic wrap and leave on shaker/rocker.