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L1 - Tissue Extraction I

- *Select tissue previously archived in ultra-cold freezer*
- *Start RNA extractions (add Tri-Reagent to samples and re-freeze)*
- *Extract protein from complementary sample*
- *Design / order Primers to use in Lab 3*

List of Supplies and Equipment

- razor blades
- pipettes (range from 1-1000uL)
- sterile filter tips
- weighboats
- scale (mg)
- mortar/pestles
- fine forceps (sterile)
- Tri-Reagent
- ice buckets
- microcentrifuge (refrigerated) or in fridge
- Sterile (RNase free) 1.5 ml microcentrifuge tubes
- gloves
- paper towels
- liquid waste container for phenol/chloroform
- solid waste container for phenol/chloroform
- pastuer pipets (1ml, 2ml)
- pastuer pipet controllers (bulb)
- lab pens
- Kim wipes
- spectrophotometer
- cuvettes
- CelLytic MT Cell Lysis Reagent
- Protease Inhibitor Cocktail
- Coomassie Protein Assay Reagent
- Water bath or heating block
- lab tape
- biohazardous waste bags (autoclavable)
- 500mL beakers (for used pipette tips)

Background - RNA Extraction

You will isolate RNA from whole tissue using TriReagent. TriReagent allows for separation of RNA from other cellular components, including DNA. There are three primary components of TriReagent that allow this to happen. The first is guanidine isothiocyanate which is a potent protein denaturant, the second is phenol, and the third is pH.

Guanidine isothiocyanate denatures proteins, such as the highly abundant histones that coat DNA. Even more importantly, RNases are denatured. This denaturing action allows for better access of phenol (an organic solvent) to cellular proteins and improves its ability to keep the proteins insoluble. The pH of TriReagent is acidic. The low pH keeps DNA out of solution while RNA remains soluble.

After homogenizing/lysing your tissue in TriReagent, chloroform (another organic solvent) will be added to your sample to allow for separation of the phenol and insoluble cellular components (DNA, proteins) from soluble cellular components (RNA). This will result in

three distinct layers: the organic phase (the bottom portion), the interphase (layer of cell debris) and the aqueous phase (the top portion). The aqueous phase (the RNA) can then be easily isolated.

The RNA can be precipitated and washed to remove residual phenol and salt carryover. Then the RNA can be resuspended in a suitable solution and quantitated.

RNA is quantitated using a spectrophotometer and measuring the absorbance of your RNA sample at 260nm (A260). The concentration of your sample is calculated with the following equation:
[RNA] = 40ug/mL x A260 x Dilution Factor

In addition to the A260, absorbance at 230nm and 280nm should be taken. The ratio of A260 to each of these absorbances can be used to assess the purity of your RNA. Various substances will absorb at 230nm, which will indicate carryover of phenol, ethanol or high salt in your sample. Proteins generally absorb light at 280nm. For clean RNA, A260/A280 should range between 1.8-2.0. The A260/A230 should range between 1.5-2.0 for clean RNA.

IMPORTANT NOTES

1. Wear clean gloves - For your own safety as well as the integrity of your RNA samples, you must wear gloves throughout this week's lab. Phenol and chloroform are nasty, cuastic chemicals, so gloves are necessary when handling anything that comes in contact with either reagent. Additionally, RNases are constantly secreted from your skin and can easily enter, and subsequently degrade, your RNA sample.

2. Phenol/Chloroform Handling and Disposal -
A. Handling - You must wear gloves, safety glasses and lab coats at all times! These chemicals have potential to do damage to clothing and exposed body parts. TriReagent may be used on the benchtop, but be aware that it is caustic, very volatile and has a very strong odor. Chloroform must only be used in a fume hood! It is extremely caustic, volatile, and inhalation of fumes can be dangerous.
B. Disposal - All tips/tubes/gloves that come in contact with phenol/chloroform must be disposed of in the "Solid Phenol/Chloroform Waste" container found in the fume hood. None of this type of waste should be discarded in regular trash! Any liquids that have phenol/chloroform must be disposed of in the "Liquid Phenol/Chloroform Waste" container found in the fume hood. None of this type of waste should be disposed of down the drain or in the regular trash!

3. RNA Handling - Due to the prevalence of RNases, gloves should be worn at all times when handling your samples. Samples should also be stored on ice at all times (to reduce the activity of any contaminating RNases remaining in your sample), unless otherwise noted.

4. Razor Blades Handling and Disposal -
A. Handling - Obviously, these are extremely sharp. Use them with extreme caution. Pay careful attention to what you are cutting. Only cut tissue that is on a flat, stable surface. Do NOT attempt to cut anything with a razor blade while holding the object in your hand!
B. Disposal - Razor blades MUST be disposed of in the available "Sharps" container! The "Sharps" container is bright red and easily visible. If you cannot find it, ask the TA. Under no circumstances are razor blades to be disposed of in the regular trash!

RNA ISOLATION 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. Using a clean razor blade, 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*

Background - Protein Extraction

You will isolate cellular protein from whole tissue using CelLytic MT. This is a proprietary reagent that contains a mixture of salts and detergents to effectively disrupt lipid membranes, lyse cells, and buffer the cellular proteins at the appropriate pH. This solution will also be supplemented with a cocktail of protease inhibitors to minimize the impact of the numerous proteases that are ubiquitous within all cells.

After extraction, you will determine the concentration of proteins in your sample. Due to the high variability of protein structures, these molecules do not uniformly absorb light at any specific wavelength like nucleic acids. We will use the Bradford Assay to determine the concentration of proteins in your sample. This is a colorimetric assay that uses a reagent (Coomassie Blue) that interacts with proteins. When the reagent is mixed with a solution containing proteins, the solution will turn different intensities of blue depending on the amount of proteins present in the sample. This blue dye absorbs at 595nm and the absorbance can be directly correlated to a specific amount of protein present in the sample when compared to a standard curve (already determined for you).

 **IMPORTANT NOTES**

1. Wear clean gloves - Proteases are present on you skin and are detrimental to the integrity of your samples.
2. Mixing - The Bradford assay works best when samples are mixed well. Invert tubes frequently during incubations, and immediately before measuring absorbance to ensure accurate absorbance readings.

PROTEIN EXTRACTION PROTOCOL

1. Add 0.5mL of CelLytic MT solution to a 1.5mL snap cap tube.2. Add 25mg of your tissue to the tube.3. Homogenize the tissue with a disposable pestle.4. Close the tube and invert the tube several times.5. Spin the tube in a refrigerated microfuge for 10mins. @ max speed.6. While spinning, label a fresh tube with the word "Protein", source organism/tissue, your initials, and today's date.7. Carefully transfer supernatant to labeled tube and store tube on ice.8. To a fresh tube, add 1.5mL of Bradford reagent.9. To this same tube, add 30uL of your protein extract.10. Invert the tube several times and then incubate at RT for 10mins.11. Mix the tube several times and transfer 1mL to a plastic, disposable cuvette.12. Measure the absorbance at 595nm and record the value.13. Remove the cuvette from the spectrophotometer. Using a P1000 set to 1mL, carefully pipette the solution in the cuvette up and down a couple of times to mix.14. Measure the absorbance at 595nm and record the value.15. Repeat steps 13 and 14.16. Average the three absorbance values you recorded.17. Plug your average absorbance that you just calculated into the following equation to determine the concentration of protein in your sample:

18. Write the concentration on your tube and place tube in TA's ice bucket. Your sample will be stored @ -80C.

Background - Primer Design

Primers, or oligonucleotides (oligos), are short stretches of DNA that are used to direct DNA polymerases to amplify specific regions of larger DNA molecules. Oligos are synthesized by various manufacturer's to contain the precise sequence requested by the customer.

Here is a list of things to take into consideration when designing primers. Although none of these are absolute, they will help ensure your primers will hybridize to your target sequence with the best efficiency.

1. 18-30 bases in length.
2. Melting temp. (Tm) of primers should be within 2C of each other.
3. Avoid primer dimers and primer hairpins
4. Avoid high G/C stretches, particularly at the 3' end
5. G/C clamp at 3' end of primers.

Primer design is most commonly done via computerized means and the algorithms used take the above rules into consideration. Of course, the user always has the opportunity to adjust the parameters that define how primers are designed by the software. There is a great deal of software available for primer design. Integrated DNA Technologies (IDT), a manufacturer of oligos, provides an excellent, free suite of oligo design and anlysis tools. It is a web-based software called SciTools and can be found at their website: www.idtdna.com

The software will allow you to enter a full DNA sequence and then define what region(s) you would like to amplify, the ideal size of the amplicon (PCR product), the ideal length of the oligos, etc. Additionally, after you have selected some proposed primers, you can compare melting temps, G/C content, primer dimer/hairpin probabilitites, etc.