Small Scale total RNA extractions

Hexadecyltrimethilammonium bromide (CTAB) extraction protocol, based on Chang et al. 1993 with some modifications

Before Starting:

A) Make sure all reagents are prepared, and that liquid nitrogen is available.

B) Turn on water bath, add distilled water if necessary, and adjust temperature to 65ºC.

C) Transfer the required volume of CTAB buffer to a Falcon tube (about 1 mL/sample), and add β-mercaptoethanol to a final concentration of 2% (v/v) (20 µL/mL CTAB buffer). Heat to 65°C in water bath.

D) It is convenient to extract RNA of 12 samples and run a chip on Bioanalyzer per day.

E) Have your tissue ready in 2 ml tubes at -80ºC and take them out of the supercooler to liquid nitrogen.

F) Prepare 1.5 mL Eppendorf tubes. Two sets of tubes will be needed if samples will be ground and extracted in the original tubes. If ground tissue needs to be transferred to separate tubes, then three sets of tubes will be needed (1st one for tissue must be 2 ml MCT) . The set used for the LiCl precipitations should be well labeled with the sample number, the extraction date, and "Total RNA".

Extraction Protocol:

Perform following steps 1-8 inside hood; collect liquid and solid waste into respective containers.

1. Add 1000 µL CTAB + β-ME to ground tissue in 2 mL Eppendorf tubes. Immediately vortex or shake well to mix. (If tissue has already been ground, then you might choose to add tissue to buffer. Store tissue tubes in liquid N with a 2 inch cardboard box before adding extraction buffer)
2. Incubate tubes in water bath at 65°C for 10 minutes, vortexing periodically every minute.
3. Add 500 µL chloroform : isoamyl alcohol (24:1) to each tube, vortexing each tube vigorously. Weight the tubes and make pairs with same weight for the following centrifugation step. Add chloroform : isoamyl alcohol (24:1) to tubes for making the pairs if needed.
4. Centrifuge at 14000 rpm for 15 min.
5. Remove the aqueous phase and transfer to a new tube. Take care to remove as much of this aqueous phase as possible in order to have better yields, but avoid the interphase.
6. Repeat steps 3 to 5 a second time. The aqueous phase should be transferred to the tubes with the full labels.
7. Determine the weight of the aqueous phase recovered after the second partitioning with chloroform : isoamyl alcohol. Add 2/3 of the volume of 10 M LiCl. Mix gently by inverting the tubes several times and place the tubes at -20°C freezer for 60 minutes to two hours. Be consistent with your time of incubation! Turn on refrigerated centrifuge and adjust temperature to 4°C at least 30 minutes prior to use, or centrifuge at cold room.

8. Centrifuge for 15 minutes at 14000 rpm at 4ºC. Discard the supernatant.
9. Wash the pellet by adding 800-1000 µL of 80% ethanol. Vortex briefly, then centrifuge at 14000 rpm at room temperature for 5 minutes. Remove the supernatant. Do a quick spin to collect the supernatant at the bottom of the tubes. Remove remaining liquid using a pipette with a 200 µL tip.
10. Let the pellets dry with the tube on a piece of clean paper towel until the edges begin to look a little transparent. Do not let the pellet dry too long or it will be very difficult to be re-suspended.
11. Re-suspend the pellet in RNase free water – use between 10 and 40 µl, depending on the size of the pellet. Pipetting water to completely re-suspend RNA and put tubes on ice (pipetting up and down each sample at least for two minutes).
12. Determine the relative quality and quantity of RNA using the spectrophotometer. Treat cuvette specific for RNA with Rnase away for 1 minute and rinse with Milli Q water twice. Use RNase free water as blank.

    Record readings of A220, A280, A320, ratio between A260 and A280, and concentration.

    For the spectrophotometer:

    i. A_{260}: measures nucleic acids
    ii. A_{280}: measures principally proteins and phenolics.
    iii. A_{230}: measures carbohydrates
    iv. A_{320}: measures any film or dirt on the cuvette and is often used to adjust the other calculations accordingly

Nucleic Acid Quantification (NAQ)

· Nucleic acids can be quantified at 260 nm because it is well established that a solution of dsDNA in a 10 mm pathlength cell with an optical density of 1.0 has a concentration of 50 µg/ml, ssDNA of 37 µg/ml or 40 µg/ml in the case of RNA. Oligonucleotides have a corresponding factor of 33 µg/ml, although this does vary with base composition; this can be calculated if the base sequence is known. Please refer to 10.1 Nucleic acid quantification for further details.

· The instrument uses factors 50, 37, 40 and 33 as default settings for dsDNA, ssDNA, RNA and oligonucleotides, respectively, and compensation factors for dilution and use of cells which do not have 10 mm pathlength. Dilution factor and cell pathlength can be entered.

Nucleic Acid Purity Checks

· Nucleic acids extracted from cells are accompanied by protein, and extensive purification is required to separate the protein impurity. The 260/280 ratio gives an indication of purity; it is only an indication, however, and not a definitive assessment. Pure DNA and RNA preparations have expected ratios of 1.8 and 2.0, respectively; deviations from this indicate the presence of impurity in the sample, but care must be taken in interpretation of results.

· The 260 nm reading is taken near the top of a broad peak in the absorbance spectrum for

nucleic acids, whereas the 280 nm reading is taken on a steep slope (i.e. small changes in wavelength cause large changes in absorbance). Consequently, small variations in wavelength at 280 nm will have a greater effect on the 260/280 ratio than variations will at 260 nm. Thus different instruments of the same and different types may give slightly different ratios due to variations in wavelength accuracy. But each instrument will give consistent results within itself.

· Concentration also affects 260/280 readings. If a solution is too dilute, the readings will be at the instrument's detection limit, and results may vary as there is less distinction of the 260 peak and the 280 slope from the background absorbance. This is one reason why the Abs 260 value should be greater than 0.1 for accurate measurements.
· An elevated absorbance at 230 nm can indicate the presence of impurities as well; 230 nm is near the absorbance maximum of peptide bonds and also indicates buffer contamination since TRIS, EDTA and other buffer salts absorb at this wavelength. When measuring RNA samples, the 260/230 ratio should be > 2.0; a ratio lower than this is generally indicative of contamination with guanidinium thiocyanate, a reagent commonly used in RNA purification and which absorbs over the 230 - 260 nm range. A wavelength scan of the nucleic acid is particularly useful for RNA samples.

· The instrument can display 260/280 and 260/230 ratios, and compensates for dilution and use of cells that do not have 10 mm pathlength; dilution factor and cell pathlength can be entered.

13. Using the concentrations obtained from the spectrophotometer, prepare dilutions of the extracts in order to carry out analyses using the Bioanalyzer or gel electrophoresis on agarose (1%). The ideal sample concentration should be 100-200 ng/µL, but the practical detection range of the instrument is between 25 and 500 ng/µL. Run 1 ug RNA on 1% ethidium bromide stained agarose gel at gel room and take photos.