## mRNA Extraction

### Materials:

* Mortar & pestle
* Liquid nitrogen
* Skin/blubber sample
* 1.5ml Eppendorf tubes (>3 per sample)
* Chloroform
* 70% ethanol
* RNeasy universal extraction kit
  + Lysis buffer (phenol)
  + RWT buffer
  + RWE buffer
  + gDNA eliminator solution
  + RNase-Free Water
  + RNeasy mini spin columns (pink)
  + Spare 2ml Collection Tubes (at least 3 per sample)
* RNase free DNase set
  + DNase 1, RNase-Free
  + Buffer RDD
  + RNase-Free water
* Refrigerated centrifuge
* Regular centrifuge (easiest for room temp, otherwise just heat refrigerated)
* P1000,P200,P100,P20, P10 pipettes preferred

### Preparation

* Sterilize mortar & pestle with thorough clean (or autoclave) and then overnight bake at 180° C or higher.
* Dilute buffer RWT with 2 parts ethanol as written on the label. Mark container to indicate dilution
* Dilute buffer RPE with 4 parts ethanol as written on the label. Mark container to indicate dilution
* Mix detergent (Triton X100) into lysis buffer at a 2% solution (900ul lysis buffer per sample).
* Dissolve DNase 1 in 550ul of RNase-free water. Mix gently by inverting. DO NOT VORTEX.
* Add 10ul (per sample) DNase stock solution to 70ul (per sample) Buffer RDD in an Eppendorf tube. Refrigerate (fridge)

### Methods:

1. Place the sample in the mortar and freeze with liquid nitrogen. Keeping liquid nitrogen in the mortar, break up the sample as thoroughly as possible.
2. After nitrogen has evaporated, immediately pipette 900ul lysis buffer into the Mortar. Continue grinding and stirring the tissue into the buffer. At this point, the mRNA is generally stable if anything else (samples moved to freezer) must be done.
3. Transfer all of the lysis buffer into a 1.5ml Eppendorf tube
4. Repeat steps 1-3 for all samples. Gently invert the completed tubes to stir in any settled tissue as you progress.
5. Leave the final tube at room temperature for a minimum of 5 minutes before proceeding. For blubber with detergent, 30 min+ is preferable.
6. Add 100ul gDNA eliminator solution. Secure cap and shake or vortex for 15s.
7. Add 180ul chloroform. Securely cap the tube and shake or vortex vigorously for 15s
8. Rest at room temperature for 2-3 min.
9. Centrifuge at 12,000g (RCF) for 15 min @ 4° C. The volume of the aqueous phase should be approximately 600ul.
10. Transfer the aqueous phase into a new 1.5ml Eppendorf tube. Ensure that none of the pink layer is included in the transfer. It is preferable to leave a small amount of the aqueous phase behind than include contaminants
11. Add 1 volume (usually 600ul) of 70% ethanol and mix by pipetting up and down. Immediately proceed to the next step.
12. Transfer up to 700ul of the sample into an RNeasy mini spin column with a collection tube (pink cap). Close lid and centrifuge for 15 seconds at >8000g (10,000 rpm) at room temp. Discard flow-through then re-attach the collection tube.
13. Repeat step 12 using the remainder of the sample discard flow through.
14. Add 350 ul RWT buffer to the RNeasy spin column centrifuge for 15s at >8,000g (10,000 rpm). Discard the flow-through and re-attach tube.
15. Gently invert DNase solution (premixed and stored in fridge) to mix. Centrifuge briefly at low speed to collect liquid at the bottom.
16. Pipette 80ul the DNase solution directly onto the membrane at bottom of the spin column.
17. Rest at room temperature for 15 minutes.
18. Add 350 ul RWT buffer to the RNeasy spin column centrifuge for 15s at >8,000g (10,000)rpm. Discard the flow-through and re-attach tube.
19. Add 500 ul RPE buffer to the RNeasy spin column. Centrifuge for 15s at >8,000g (10,000 rpm). Discard the flow-through and re-attach tube.
20. Add another 500 ul RPE buffer to the RNeasy spin column. Close the lid gently and centrifuge for 2 min at >8,000g (10,000 rpm).
21. Place the spin column in a new 2ml collection tube. Close the lid and centrifuge for another minute at >8,000g (10,000 rpm).
22. Place the spin column in a new 2ml collection tube.
23. Add 50ul RNase-free water directly to the spin column membrane. Close the lid gently to elute RNA, centrifuge for 1 minute at >8,000g (10,000 rpm).
24. Transfer RNA water to 1.5ml Eppendorf tube. Place on ice and measure yield using nanodrop. Store for use at -80 or proceed to cDNA synthesis.

## cDNA Synthesis

### Materials:

* RNA extract
* Tetro cDNA Synthesis Kit
  + Random Hexamer
  + 10mM dNTP mix
  + 5x RT Buffer
  + RiboSafe RNase Inhibitor
  + Tetro Reverse Transcriptase (200 u/ul)
  + DEPC-treated water (may not be necessary)
* 1.5ml Eppendorf tubes
* Mini PCR tubes
* PCR machine

### Preparation:

* Keep all RNA and DNA synthesis kit ingredients on ice while preparing cDNA

### Methods:

1. Vortex cDNA kit solutions and briefly centrifuge before use.
2. Prepare priming master mix in a 1.5ml Eppendorf tube. Prepare 10% more solution than needed to ensure adequate supply.
3. Add the following to the master mix tube:

|  |  |  |
| --- | --- | --- |
| Item | Per sample | For 10 samples (x11 for extra 10%) |
| Random Hexamer | 1ul | 11ul |
| 10mM dNTP mix | 1ul | 11ul |
| 5xRT buffer | 4ul | 44ul |
| RiboSafe RNase Inhibitor | 1ul | 11ul |
| Tetro Reverse Transcriptase | 1ul | 11ul |

1. Add 8ul of master mix to mini PCR tubes (one for each sample)
2. Add 12ul mRNA to mini PCR tubes (or mRNA and DEPC water to dilute if needed). Mixing gently by pipetting. The final volume should be 20ul per tube.
3. Run PCR cycle with following settings:

Stage 1: 10 minutes @ 25° C

Stage 2: 30 minutes @ 45° C

Stage 3: 5 minutes @ 85° C

Stage 4: infinite @ 4°C

1. Plunge into ice immediately following Stage 3. Stage 4 is only a safety in the event you are not present with ice at the end of stage 3.
2. Check DNA yield using nanodrop (ss) Store mini PCR tubes at -20 or proceed to PCR.

## qPCR

### Materials:

* Sample cDNA
* 1.5 ml Eppendorf tubes
* Syber Green Power-up
* DNase RNase free sterile water.
* Forward and Reverse Primers
* 96 plate well
* Plate covers
* qPCR machine

### Methods:

1. Dissolve primers into sterile water according to primer-specific manufacture instructions.
2. In a separate Eppendorf tube dilute the 100uM primer to 10uM (1:9 ratio 1/10 dilution).
3. Prepare Syber mix for each primer pair in the Eppendorf tube being careful not to contaminate with any sample DNA:

|  |  |  |
| --- | --- | --- |
| Item | Per well | For 12 wells (one row) |
| Syber | 10ul | 130ul |
| Sterile H20 | 8ul | 104ul |
| Forward primer | .5ul | 6.5ul |
| Reverse primer | .5ul | 6.5ul |

1. Dilute sample DNA 1:9 (1/10 dilution) with sterile H20 (typical 2ul cDNA and 18ulH20).
2. Add 19ul of Syber mix to each well, noting the primer pair used.
3. Add 1ul of diluted cDNA to each well, noting the sample used.
4. Apply plate cover, being careful to only touch the edges.
5. Vortex (or gently shake) the plate and flick (“centrifuge”) so that all of the contents in each well are collected at the bottom.
6. Load into qPCR or store in the refrigerator for use.
7. Run qPCR on cycle:

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temp (°C) | Duration | Cycles |
| UDG activation | 50 | 2 minutes | 1 |
| Dual-Lock DNA polymerase | 95 | 2 minutes | 1 |
| Denature | 95 | 15 seconds | 50 |
| Anneal | 64 | 15 seconds | 50 |
| Extend | 72 | 1 minute | 50 |
| Melt curve | .5 intervals |  | 1 |

1. Following qPCR store refrigerated in case of product sequencing.