RNA isolation and RT-PCR

Disclosure : The following protocol is based on the suggested protocols provided by Life Technologies for the different products used.

I. RNA isolation (ArcturusPicopure RNA isolation Kit – Life technologies)

1- RNA extraction:

- Pipette 50ul Extraction Buffer (XB) into a 0.5 ml tube with GV oocytes
- Incubate for 30min at 42°C.

2- RNA isolation:

- Pipette 250ul Conditioning Buffer (CB) onto the purification column filter membrane
- Incubate the column with Conditioning Buffer for 5min at RT
- Centrifuge the column in the provided collection tube at 13000rpm for 1min
- Pipette 50ul of 70% ethanol into the cell extract. Mix well by pipetting up and down. DO NOT CENTRIFUGE.
- Pipette the mixture of approximately 100ul into the column
- Centrifuge for 2min at 3000rpm to bind RNA, immediately followed by a centrifugation at 13000rpm for 30sec to remove flowthrough
- Pipette 100ul Wash Buffer (W1) into the column and centrifuge for 1min at 8000rpm
- **(optional)** DNA may be removed by DNase treatment for the preparation at this point. Mix 5ul DNaseI (Qiagen RNase-free DNase Kit ca:79254) and 35ul RDD buffer by gently inverting. Pipette 40ul DNase I mixture into the comumn. Incubate for 15min at RT. Pipette 40ul W1 buffer into column and spin at 8000rpm for 15sec.
- Pipette 100ul Wash Buffer (W2) into the column and centrifuge for 1min at 8000rpm
- Pipette another 100ul Wash Buffer (W1) and centrifuge for 2min at 13000rpm
- Transfer the column to a new 0.5ml tube provided in the kit
- Pipette Elution Buffer (EB) directly onto the membrane of the column

  - recommended volume 11ul
  - maximum volume 30ul

- Incubate the column for 1min at RT
- Centrifuge the column for 1min at 2000rpm to distribute EB and then for 1min at 13000rpm to elute RNA

II. Reverse Transcription (Using SuperScript™ II RT - Invitrogen)

A 20-µl reaction volume can be used for 1 ng–5 µg of total RNA or 1–500 ng of mRNA.

1- Add the following components to a nuclease-free microcentrifuge tube:

- 1µL 50–250 ng random primers
- xµL 1 ng to 5 µg total RNA
- 1µL dNTP Mix (10 mM each)

Add sterile, distilled water to bring total to12 µL

2- Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add (note for >4 samples, prepare a master mix):

- 4 µL 5X First-Strand Buffer
- 2 µL 0.1 M DTT
- 1 µL RNaseOUT™ (40 units/µl) (optional)*

*RNaseOUT™ (Cat. No. 10777-019) is required if using <50 ng starting RNA.

3- Mix contents of the tube gently and incubate at 25°C for 2 min.

4- Add 1 µL (200 units) of SuperScript™ II RT and mix by pipetting gently up and down. And then incubate tube at 25°C for 10 min.

5- Incubate at 42°C for 50 min.

6- Inactivate the reaction by heating at 70°C for 15 min.

7- Store at -20°C.

III. Polymerase Chain Reaction (Using Taq DNA Polymerase - Invitrogen)

1. Add the following to the PCR tube (for every reaction)

- 5 µL 10x PCR Buffer
- 1.5 µL 50 mM $MgCl_2$
- 1 µL 10 mM dNTP mix
- 1 µL 10 mM Forward and Reverse primer mix
- 0.5 µL Taq DNA Polymerase (~5 units/µL)
- 2 µL cDNA product from RT reaction
- to 50 µL autoclaved, distilled water

2. Enter the Following program on the thermocycler:

- 95°C 5 min
- 95°C 30 sec
- 50-56°C 30 sec
- 72°C 45 sec

Repeat 35-40 times

- 72°C 7 min
- 4°C pause

3. Perform 15-40 cycles of PCR. Use the recommended annealing (may vary with specific primers) and extension conditions for your Taq polymerase.

IV. Gel Electrophoresis

Note: for a 1.5% agarose

1. Add 1.5g agarose per 100 mL of TAE buffer.

2. Heat the solution for ~2 minutes in the microwave, stopping halfway through to mix by swirling. After the agarose has fully dissolved, let the solution cool.

3. Assemble the gel apparatus in the holder with combs.

4. Add 20 µL of EtBr per 100 mL of agarose solution in the hood and mix by swirling.

5. Pour the gel into the mold and let sit for ~15 minutes.

6. Remove the comb from the gel and then the gel from the mold. Place the gel into the gel electrophoresis chamber, filled with TAE buffer so that it is fully submerged.

7. Pipette the ladder and samples into the wells.

8. Run at 120 V for around 30 minutes.

Note: if run time is unknown, make sure to check on the dye front of the gel to determine how far the samples have run.

9. Visualize gel.