Techniques and Protocol(s)

RNA Isolation:

Cut embryos out of capsules and pipette into RNAse free 1.5ml eppendorf tube. For 15 embryos, 10ul of FASW is sufficient.

1. Add 300ul to 500ul Trizol to the tube with embryos, in two steps. First, add 150ul, homogenize the embryos with a pestle, and then add the remainder of the Trizol (500ul). Keep at room temperature for ~5 minutes.

2. Transfer lysate solution to Phase Lock Gel (PLG) Heavy tubes (PLG tubes must be pre-spun for 30 seconds before adding lysate).

3. Add RNAse free chloroform to the PLG tube (200ul chloroform per 1ml Trizol). Mix and spin for 10 minutes at high speed in cold room (4 degrees).

4. Transfer aqueous phase to a new tube and add RNAse free 2-propanol (isopropanol) – add ½ volume of the initial amount of Trizol used in step 2. Place at -20 for 10 minutes.

5. Centrifuge in cold room at high speed for ~10 minutes.

6. Decant the supernatant and wash with 500ul 70-75% RNAse free EtOH. Centrifuge for 10 minutes (room temperature or cold room are both okay).

7. Decant the supernatant and reconstitute the pellet in 150ul RNAse free H_20. Do not allow the pellet to completely dry as it will be hard to reconstitute.

8. Transfer the solution to a new PLG tube. Add 150ul RNAse free phenol/chloroform and spin for 5 minutes. Add 150ul RNAse free chloroform and spin for another 5 minutes.

9. Transfer the aqueous layer to a new tube and add 1/10th volume ammonium acetate and an equal volume of RNAse free 2-propanol. Place at -20 degrees for 10 minutes and centrifuge in the cold room for 10 minutes.

10. Decant the supernatant and wash the pellet with 500ul 70-75% RNAse free EtOH. Spin for 10 minutes (cold room or room temp).
11. Decant the supernatant and reconstitute the pellet in the desired amount of RNAse free water (5-20ul).

First-Strand cDNA Synthesis

1) Preheat the thermal cycler to 65°C.
2) Combine the following in a separate 0.2-ml thin-walled PCR tube or plate well at room temperature or on ice:

Template RNA 5 µl
Primer (50 µM oligo(dT), or
50 ng/µl random hexamers) 1 µl
Annealing Buffer 1 µl
RNase/DNase-free water to 20 µl

3) Incubate in a thermal cycler at 65°C for 5 minutes, and then cool down at room temperature or on ice for at least 1 minute. Collect the contents by brief centrifugation.
4) Remove the seal/caps from the RTS plate/strip wells. Add the RNA/primer mix to the pellet in a well at room temperature.
5) Seal the plate/strip wells (see page 2 for sealing instructions) and vortex for 5–10 seconds. Note: Vortexing is crucial to ensure complete dissolution of the pellet. Centrifuge briefly to collect the contents, and then incubate as follows:
Oligo(dT)20 or GSP primed: 50 minutes at 50°C Random hexamer primed: 5–10 minutes at 25°C, followed by
50 minutes at 50°C
6. Terminate the reactions at 85°C for 5 minutes, and then cool down at room temperature or on ice.
Store the cDNA synthesis reaction at -20°C, or proceed directly to PCR. Better results are achived when combining both random primers with oligo (dT) in separate reactions.

Probes Primer Design

1. Open BatchPrimer3 and input your sequences using the instructions.
2. Set Product Min to about 300, Optimum to 700, Max to 1000;
4. Click "Select Primers"
5. Paste the T7 sequence TAATACGACTCACTATAGGG to the 5' end of the forward primer

   for example, 5' TAATACGACTCACTATAGGGtcacgacccatttggactgac 3'

5. Paste the T3 sequence AATTAACCCTCACTAAAGGG to the 5' end of the reverse primer

   for example, 5' AATTAACCCTCACTAAAGGGgaagaagagaacgcgtgacc 3'

Some sets worked best with a standard run, other primer pairs worked best in a step up run.

The PCR products will have T7 and T3 sites on each end so that you can use your PCR product as template for both sense (T7) and antisense (T3) probes.

Probe making

PCR recipe:

- 2.5 µl 10x Buffer (labeled 1)
- 0.4 µl 10 µM dNTP mix
- 1 µl cDNA
- 19.98 µl ddH2O
- 0.125 µl TAQ (DNA Pol. Mix)

Multiply by number of genes + 0.5 to make master mix. Make sure master mix is thoroughly mixed (vortex and or pipette up and down). Add 24 µl of master mix to each PCR tube with 0.5 µl F primer and 0.5 µl R primer. Use a step up protocol on the PCR machine.

Visualize on 1% agarose gel using 5 µl of the reaction + 2 dye. Run at 80-85 volts for at least an hour with a 1 Kb ladder or similar DNA ladders.

Probe Synthesis:

- 6 µl ddH2O
- 4 µl 5x transcript buffer
- 2 µl 0.1 M DTT
- 2 µl digNTPs
- 1 µl T3 or T7 RNA polymerase (T7 for Sense T3 for Anti-sense)

Multiply by the number of genes plus 0.5 to make master mix. If making sense control probes (T7) as well as anti-sense T3 probes, make a second master mix. Add 15 ul of master mix to each 1.5 ml eppi tube with 5 µl of PCR product. Mix.

1. Incubate tubes in 37 °C water bath for at least 2 hours
2. Add 0.5 µl DNAase 1 to each tube, mix
3. Incubate at 37 °C for 15-30 minutes
4. Add 80 µl ddH2O, 10 µl LiCl , 200 µl ice cold 100% EtOH, and 0.5 µl LPA. Mix well (by inverting)
5. Incubate at least 15 minutes (or overnight) at -20° C

6. Centrifuge 10 minutes at 4°C (pre-cool the centrifuge)
7. Locate pelted RNA, remove supernatant
8. Wash with 750 µl 75% EtOH
9. Centrifuge 10 minutes at room temperature
10. Locate pellet, remove supernatant
11. Air dry 1-2 minutes (not more than 2 minutes)
12. Re-suspend RNA pellet in 50 Hyb solution without blockers

Ilyanassa in situ protocol

Rehydration:
-Add RNAse-free PBTw to a clean 9-well plate. Pipet embryos from tubes into the PBTw.

-Wash 3X 5' with 400 µl RNAse-free PBTw

-Transfer embryos to RNAse-free 1.5 ml tube

-Wash 5' with 400 µl 0.1M TEA

-Wash 10' with 500 µl 0.1M TEA +10 µl/ml acetic anhydride added right before use

-Add 500 ul 0.1M TEA +20 µl/ml acetic anhydride (added right before use); incubate 10'

-Wash 3X with 500 µl PBTw RNAse-free

Pre-hybridization:
-Wash 10' with 400 ml Hyb w/ no blockers RNAse-free

-Replace with 400 µl Hyb w/ blockers and incubate for 2-3 hours at 68C

Hybridization:
-Dilute probe in Hyb w/ blockers and heat for 10 minutes at 68 C

-Replace pre-hyb with probe dilution and incubate at 68 C overnight

Washing:
-Preheat Hyb w/ no blockers (not RNAse-free) to hyb temp (~68 C).

-Wash with 1 ml hot Hyb 3X over 2 hours. (~ Every 40 minutes)

-After 3 washes replace with 500 µl PBTw, mix, wait 5-10 minutes

Blocking:
-Wash 3 times for 10 minutes with PBTw2%BSA

Anti-Dig antibody:
-Dilute Roche sheep anti-Dig-AP to 1:1500 in PBTw+2%BSA. Make ~ 150 ul per tube.

-Remove last blocking wash, add antibody dilution, incubate RT overnight.

Antibody Washing:
-Wash 3X fast with PBTw in tubes, move to clean glass 9-well plate, wash every 10 minutes for an hour.

Developing:
-Wash 2 times for 5 minutes with AP developing buffer.

-Make AP developing solution by adding 3.5 µl/ml BCIP and 3.5 µl/ml NBT to developing buffer. Vortex after adding each substrate.

-Add developing solution to all wells. Keep dish in dark at RT.

-Watch for the first ~30 minutes to make sure it is not going to come up immediately, check at 5 minutes, 30 minutes and then 1 h and hourly thereafter.

-Stop reaction by washing with PBTw, counterstain with 500 ng/ml DAPI in PBTw for 15-30 minutes, wash 3 times PBTw, Mount in 80% glycerol. Store at 4C.

Injection

1. Using a glass slide, scratch a groove that is roughly as deep as the diameter of the embryo into the bottom of the 60-mm polystyrene Petri dish. Embryos will eventually be pipetted into this groove for the injections.

2. Filter the injection solution through a spin column. Use 6 µl of 1x injection buffer to wet the filter and remove the flowthrough before applying the injection solution. This prevents the loss of the injection solution into the filter pad.

3. Back-fill the microinjection capillaries with the filtered injection solution using a Microloader tip

4. Attach the needle to microinjector, angle it at 30–45° from the horizontal, and perform a test injection into the seawater solution to verify that the correct puff size is being delivered and adjust pressure accordingly.

5. Immediately after PL2 has fully retracted in the prezygotic embryos or cells have compacted following a cell division in later-stage embryos, pipette the embryos into the groove scratched into the 60-mm Petri dish
6. Use the pneumatic microinjector and fine-scale micromanipulator to inject the embryos with an injection buffer under 80x magnification. For zygotic injections, angle the microinjection needle 10–60° from the animal pole. As a control, some embryos from the same egg capsule should remain uninjected. In addition, for each set of experiments, some embryos should be injected with 1x injection buffer containing 1% FITC-dextran without the molecule of interest.
7. Immediately after the injection, transfer the zygotes to a new 35-mm Petri dish containing FASW.
8. Examine the embryos 3–8 hours postinjection under a stereomicroscope. Identify the embryos that have normal morphology and are at a similar developmental stage as the uninjected control embryos from the same egg capsule. Move these embryos to a new 35-mm Petridish containing FASW for further study.