RT-PCR – Hiroki Kuroda/Hojoon Lee

Most Common Technique for detecting molecular markers

RNA extraction with RNA-STAT

1. Lyse six embryos by pipetting with 800 µl of RNA-Stat60.
Note: Amount of total RNA is 3-4 µg/embryo and 300-400 ng/AC.
2. Add 200 µl of chloroform and vortex well.
3. Centrifuge at maximum speed (e.g. 15k rpm) for 10 min at 4 ºC.
4. Recover the supernatant and repeat chloroform extraction.
5. Recover 380 µl of the supernatant and add 380 µl of isopropanol.
6. Mix well and incubate in dry ice until frozen or in -80ºC for 30 min.
Note: You can store the sample in -80ºC forever.
7. Centrifuge at maximum speed for 15 min at 4 ºC.
8. Wash the pellet with 70% EtOH and dissolve pellet in 20 µl of water.
Note. Use 4 µl for the Reverse transcription reaction.

(Loading Sample)

Components	20 µl final	Master Mix.
RNA	4 µl	---
Water	6 µl	300 µl
5 x RT buffer	6 µl	300 µl
d[N]$_6$ (100 mM)	3 µl	150 µl
dNTP (5 mM)	3 µl	150 µl
DTT (0.1M)	3 µl	150 µl
RNA guard	0.6 µl	--- µl
BSA (1 mg/ml)	3 µl	150 µl
MMLV RT enzyme	1.5 µl	---

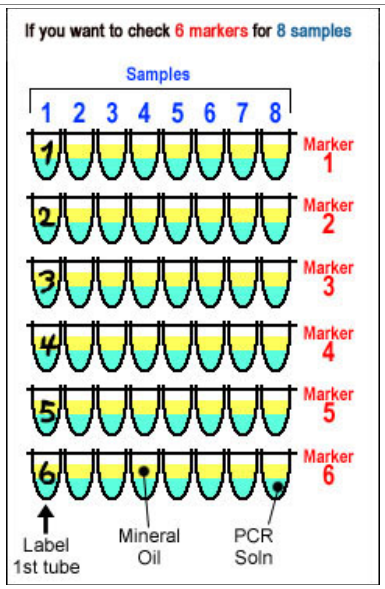
If you have 8 samples----
1. Mix 24 x 8.5 µl of Master Mix, 0.6 x 8.5 µl of RNA guard, and 1.5 x 8.5 µl of MMLV RT enzyme very well (Called Master Enzyme Mix).
2. Mix 4 µl of RNA solution and 26 µl of Master Enzyme Mix very well.
3. Incubate at 42 ºC for 1 h.
4. Add 30 µl of water.
5. Store at -20 ºC (Use as soon as possible. Do not keep for more than 3 months).

(PCR)

Components	20.4 µl final	Master Mix.
cDNA solution	3 µl	---
10 x PCR buffer (with Mg)	2 µl	480 µl
dNTP (5 mM)	0.8 µl	192 µl
α-^{32}P-dCTP	0.1 µl	---
Fw-Rv-Mixed primers (each 20 mM)	2 µl	---
Water	12.2 µl	2928 µl
Taq polymerase	0.3 µl	---

Major markers:

Stage 9	
Wnt target	*Chordin* *Noggin* *Xnr3* *Siamois* *Twin* *Pintallavis* *ADMP*
Nodal target	*Cerberus* *Xnr1* *Xnr2* *Xnr5* *Xnr6* *Sox17α* *Mix1* *Milk* *Mixer*
BMP target	*Vent1* *Vent2* *Msx1* *Msx2* *ID*
BCNE center	*Chordin* *Noggin* *Xnr3* *Siamois* *Twin* *Pintallavis* *ADMP*
Nieuwkoop center	*Cerberus* *Xnr1* *Xnr2* *Xnr5* *Xnr6*
Stage 13-14	Good for AC & VMZ assay
Cement Gland	*XAG*
Anterior Neural	*Otx2* *Six3*
Pan-neural	*NCAM* *Sox2*
Dorsal Mesodermal	*MyoD* *Myf5* *Xbra*
Epidermal	*Cytokeratin*
Ventral Mesodermal	*Bmp4* *Sizzled* *Crossveinless-2* *Vent1* *Vent2* *Msx1*



In the case that you have 8 different embryo samples and want to check 6 marker genes for each sample.
(Please note that font color scheme from the figure above is used in the following protocol)Set up the following cold reaction)
0-1. Set up PCR machine and prepare six sets of 8-connected PCR tubes.

0-2. Put 3 µl of cDNA soln into PCR tube and keep them in 4 ºC.

0-3. A master mix pool will be prepared for each marker (Six in this example.) The master mix is prepared in 1.5 ml tubes (Hence six 1.5 ml tubes). Put 2 x 8.8 µl of respective primer pair mix into 1.5 ml tubes (total six tubes).

RADIOACTIVITY STARTS HERE!!! BE CAREFUL!!!
1. Mix 15 x 9 x 6 µl of PCR Master Mix, 0.3 x 9 x 6 µl of Taq polymerase, and 0.1 x 9 x 6 µl of α-^{32}P-dCTP (called PCR Master Enzyme Soln).
2. Take 15.4 x 8.8 µl of PCR Master Enzyme Soln, add to 1.5 ml tubes with primers (from 0-3). Mix very well (called PCR Master Enzyme & Primer Soln).
3. Add 17.4 µl of PCR Master Enzyme & Primer Soln (from #2 above) into each PCR tube(e.g. 17.4µl each of Marker 1 into the 8 tubes of different samples)
4. Add one drop of mineral oil into each PCR tube.
5. Close tubes and start PCR.
6. Run on a 5 % acrylamide gel after PCR.

Components	Volume
Water	33 ml
40% acrylamide	5 ml
10x TBE	3.3 ml
APS	500 µl
TEMED	30 µl

5% acrylamide gel

	Msx2 *Wnt8*
Stage 18-22	Good for VMZ assay
Ventral	*Sizzled* *β-Globin*
Paraxial	*PAPC*
Neural crest	*Slug*
Axial	*Shh* *Pintallavis*
Floor plate	*F-Spondin*
Dorsal	*α-Actin*
Cement Gland	*XAG*
Heart	*Nkx2.5*
Stage 22-26	Good for AC assay
Pan-neural	*NCAM* *N-Tubulin*
Anterior Neural	*Six3* *Otx2* *Pax6* *RX2a*
Mid-Hind-brain boundary	*Engrailed*
Hindbrain	*Krox20*
Posterior Neural	*Xlhbox6* (=*HoxB9*)
Mesoderm	*α-Actin* *β-Globin*
Cement Gland	*XAG*

Loading Control:
ODC: Always a good loading control marker
EF1α: Much stronger than *ODC* but not good before gastrula.