

Real Time PCR for Gene Expression Analysis

Isolate RNA using hot acid phenol protocol.

Make cDNA

Reagents:

- DEPC water
- Filter tips
- Recombinant DNase I (*ABgene, catalog # AB-1154/a*)
- 10X DNase I reaction buffer
 - 100mM Tris-HCl pH 7.5
 - 25 mM MgCl₂
 - 5 mM CaCl₂
- EDTA
- 10mM dNTPs
- 10uM reverse primer
- SuperScript II Reverse Transcriptase (*Invitrogen, catalog # 18064-014*)
- 5X FS Buffer (included with RT)
- 0.1M DTT (included with RT)
- RNaseOUT (*Invitrogen, catalog # 10777-019*)
- RNase H (*Invitrogen, catalog # 18021-071*)

Reaction:

You will need reactions for each sample with each primer set. Also you can do an additional reaction with each primer set for just one sample to serve as a negative control. Treat this sample as the rest, but leave out the reverse transcriptase.

1. Set up reactions in 1.5 ml eppendorf tubes w/...

- 5 ug RNA
- H₂O to 8 ul total volume
- 1 ul 10X DNase I buffer
- 1 ul DNase I

2. Incubate at room temperature for 15 minutes.
3. Add...

- 1 ul 25mM EDTA

4. Incubate at 65°C for 10 minutes.
5. Add...

- 1 ul 10mM dNTPs
- 1 ul 10uM reverse primer

6. Incubate at 65°C for 5 minutes.
7. Put on ice for 1 minute.
8. Add...

4 ul 5X FS Buffer
2 ul 0.1M DTT
1 ul RNaseOUT

9. Incubate at 42°C for 2 minutes.
10. Add...

1 ul Superscript II reverse transcriptase

11. Incubate at 42°C for 50 minutes.
12. Incubate at 70°C for 15 minutes.
13. Add...

1 ul RNase H

14. Incubate at 37°C for 20 minutes.
15. Store cDNA at -20°C.

Standard curve for new primers and cDNA

Reagents:

- DEPC water
- 5 uM forward and reverse primers
- SYBR Green PCR Master Mix (*Applied Biosystems, catalog # 4364344 for 2 pack*)
- MicroAmp Optical 96-Well Reaction Plates (*Applied Biosystems, catalog # N8010560*)
- MicroAmp Optical Caps, 8 caps/strip (*Applied Biosystems, catalog # 4323032*)

Reaction:

1. Make 6 5-fold serial dilutions of WT sample. Designated 125X, 25X, 5X, 1X, 0.2X and 0.4X.
2. Make 4 different master mixes, enough for 6 reactions each.

For 200 nM primers...

<u>1 rxn</u>	<u>6 rxn</u>	
0.8	4.8	5 uM forward primer
0.8	4.8	5 uM reverse primer
7.4	44.4	DEPC water
10	60	SYBR Green PCR Master Mix

For 400 nM primers...

	<u>1 rxn</u>	<u>6 rxn</u>	
	1.6	9.6	5 uM forward primer
	1.6	9.6	5 uM reverse primer
	5.8	34.8	DEPC water
	10	60	SYBR Green PCR Master Mix

3. Set up reactions in the 96 well plate...

19 ul master mix
1 ul cDNA

4. Record layout of 96 well plate with cDNA concentration, primer concentration and primer name. Example...

125 200 nM gene1	25 200 nM gene1	5 200 nM gene1	1 200 nM gene1	125 400 nM gene1	25 400 nM gene1	5 400 nM gene1	1 400 nM gene1				
5 200 nM actin	1 200 nM actin	0.2 200 nM actin	0.04 200 nM actin	5 400 nM actin	1 400 nM actin	0.2 400 nM actin	0.04 400 nM actin				

5. Cap the wells containing reactions and wrap the plate in saran wrap. Vortex for a few seconds.
6. Remove any bubbles by flicking the wells before spinning.
7. Spin the plate at 300 rpm for about 5 minutes.
8. Repeat flicking and spinning until all bubbles are gone.
9. Put the plate in the ABI 7300 machine.
10. Set up the program as follows...
 - Open 7300 System Software.
 - File – New
 - In the new document wizard chose your primer pairs or add new ones – Next.
 - Assign primers to wells by highlighting wells and selecting primer set.
 - Assign standards by highlighting wells and selecting standard from pull down menu.
 - Assign quantity by highlighting well and typing in quantity (i.e. 125, 25, 5, 1).
 - Next – Change reaction volume to 20 ul and add dissociation stage.
 - Save the file and then start run.

Experiment

If the standard curve run showed a slope between -3.0 and -3.5 for both primer concentrations (i.e. the cDNA and primer concentrations are good to be in the linear part of the reaction) and the dissociation plot showed one peak (i.e. the primer pair is giving only one product), continue to the experiment using one of the middle cDNA concentrations used for the standard curve.

Set up the reactions and everything the same as before. Set up each sample in triplicate using 200 nM primers the same as with the standard curve. Also rerun the standard curve. Leave the wells with unknown samples (i.e. not the standard curve) marked as unknown in the program set up.