RT qPCR

Protocol for qRT-PCR
(Quantitative reverse-transcription PCR)

This protocol is based on manufacturer recommendations, Nathan’s notes, and Benjy’s notes.

DNase treatment:

1. Following isolation of RNA using our standard protocol, calculate the volume of sample needed to obtain 5-8ug of total RNA. If you are working with RNA from cells grown in nutrient limiting conditions, you may have low yields of RNA to begin with. If this is the case, then use whatever you have.
2. Set up a reaction with RNA, 1/10 volume of RQ1 DNase buffer, and 1 ul RQ1 DNase for each ug of RNA. The manufacturer recommendation is to use 1ul RQ1 DNase per ug of input RNA, but less should suffice if necessary. The volume of the reaction is not terribly important, but must be greater than 30ul and should be less than 100ul. Make sure buffer is added in the correct proportion.
3. Incubate at 37C for 30 minutes in water bath.
   a. Optional: add nuclease free water to dilute reaction to a more reasonable working volume, ie just under 400ul. Benjy raises the volume of a 30ul reaction up to 100ul.
4. Add one volume of acid phenol. Vortex to mix. Spin down for 2 min at 12,000 rcf.
5. Pipette out the aqueous layer into a new Eppendorf. Add one volume of chloroform. Vortex to mix. Spin down for 2 min at 12000 rcf.
6. Pipette out the aqueous layer into a new Eppendorf. Precipitate with either method:
   a. Add 1/10 volume 5M NaCl, 1 ul glycooin or glycolblue, and 1.1 volume Isopropanol. Mix. Let sit at room temperature for 10 minutes. Spin down at full speed in cold room centrifuge for 30 min. This is the method Benjy uses for the protocol.
   b. Alternatively, do an ethanol precipitation with 1/10 volume 3M sodium acetate, 1ul glycooin or glycolblue, and two volumes ethanol. Put in -20 or -80 for at least 20 min. Spin down in coldroom centrifuge for 30 min.
7. Discard the supernatant. Add 400ul of 70% EIOH. Spin 3-5 min fullspeed.
8. Discard the supernatant. Make sure the pellet is dry before resuspending. Resuspend the pellet in 10 ul of water and quantify using the Qubit (or nanodrop). The nanodrop cannot distinguish between DNA and RNA, but can be more accurate for larger concentrations. When Benjy measured a reaction that began with ~8ug (based on nanodrop), the yield was ~450ng/ul in 10ul, or about 4.5ug (based on Qubit).

cDNA synthesis

Notes: Make sure you make the correct working solution concentration of each reagent. Make sure to use nuclease free water that has not been DEPC treated, in all parts of the protocol (ie Hyclone).

1. Create working solutions for random hexamers and dNTP. The working concentration of dNTP (which is at 10mM of each dNTP) should be made to 10mM total (2.5mM each dNTP). Benjy makes a working stock of 25 ng/ul of hexamers.
2. In a single reaction tube, add 2ul hexamers, 6ul of dNTP and 16ul of RNA sample +water. The total reaction volume should be 24 ul.
3. Heat at 80C for 5min. Put on ice for 5 min. Note: This is a good point to heat the 10xRT buffer to bring the DTT back into solution. The buffer shouldn’t be used until it has cooled down, otherwise it could inactivate the RT. Keep in mind that the buffer and random hexamers to use for the RT reaction depends on the reverse transcriptase you are using. This protocol is written for M-Mulv RT.
4. Pipette out 16ul for a positive reaction, and the other 8ul will be for the negative reaction.
5. To each positive reaction, add 2ul of 10xRT buffer, 1ul of RNase out (10Units / ul), and 1 ul of M-Mulv RT, for a total reaction volume of 20ul.
6. To each negative reaction, add 1ul of 10xRT buffer, 0.5ul of RNase out (10units / ul), and 0.5 ul of water, for a total reaction volume of 10 ul.
7. Incubate at 42C for 1hr.
8. Incubate at 90C for 10 min to heat inactivate the RT.

qPCR:

Because the volume is so small, slight pipetting error will cause big problems in the final qPCR values. Try to use volumes on the high end of the pipette’s range (ie 2ul on a p2 instead of 3ul on a p20), and pipette as little as possible into the well to save effort and error.

Before using primers for an experiment, a dilution series should be performed to ensure that primers amplify with the expected efficiency.

Make sure samples are diluted appropriately from the RT reaction. Benjy usually dilutes his samples 1:1 (i.e. 8ul sample to 8ul water), although this depends on the amount of input RNA into the RT reaction, as well as the number of genes you wish to investigate. Once all samples are loaded, make sure to load a sample with water. Another negative control, if you have space, is the RT negative controls to make sure there was no contamination.

1. A single well on the qPCR plate should have the following:

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>a. Sybregreen</td>
<td>5µl</td>
</tr>
<tr>
<td>Primer1</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Primer2</td>
<td>0.5µl</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Water</td>
<td>2µl</td>
</tr>
<tr>
<td>Sample</td>
<td>2µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>10µl</strong></td>
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</tbody>
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It is preferable to make a master mixes. One option is to master mix Sybergreen and each primer set, you could also master mix Sybergreen and sample (seems to work, check this a few more times).

2. Load the wells by pipetting onto the side of the well. The samples will mix when they are spun down, so there is no need to pipette up and down in the bottom of the well. Also, make sure to use different tips for each sample.
3. Cover the plate with the clear sealing membrane and seal it on with a roller.
4. Spin the plate for 2 min at 2000 rpm.
5. Load the Gresham Lab SYBERGREEN protocol on the qPCR machine.
6. Run.
7. When done, use max 2nd derivative to find Cp values.