RT-PCR stands for “**Reverse Transcriptase - Polymerase Chain Reaction**”. In this procedure, we use Reverse transcriptase to make cDNA, then PCR to amplify a specific gene.

The Reverse transcriptase converts RNA into DNA. It needs a “primer” to do this. The primer is a short DNA sequence that is complementary to part of the RNA. We will not be making the second strand of DNA, as we don’t need it for our experiments. To make DNA, the Reverse transcriptase needs dNTPs and an appropriate salt solution.

After making the cDNA strand, we will use Ribonuclease H (RNase H) to remove the RNA. The H stands for hybrids, so the RNase digests the RNA of an RNA-DNA hybrid. After the RNase, we will have single-strands of DNA that are complementary to our mRNA.

Last, we perform PCR on the sample. In this case, we use primers from specific genes. After the PCR, if we see bands on gel electrophoresis, it suggests that the gene was transcribed and therefore the mRNA was present in your mRNA sample.

**Materials**

SuperScript™ First-strand Synthesis System for RT-PCR
Primers for the specific genes you wish to amplify

**Methods**

**Notes:** Wear gloves and use autoclaved tips for the first few steps (until the RT step is started)

**RT procedure**

1) Use the following amounts to prepare samples in 0.2 ml PCR tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>No RT control*</th>
<th>Control RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Your mRNA</td>
<td>Up to 8 µl</td>
<td>Up to 8 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>Control RNA</td>
<td>0 µl</td>
<td>0 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer</td>
<td>1 µl</td>
<td>1 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>To 10 µl total volume</td>
<td>To 10 µl total</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

**Notes:** The No RT control tests for DNA contamination. The control sample is a positive control. These controls are often not necessary.

* Only run these samples if instructed to do so.

2) Use the iCycler to incubate the samples at 70°C for 5 minutes, and then place them on ice for 1 minute.

3) Prepare the following reaction mixture. Be sure to add each item in the order that they are listed. The standard way to do this is to make a “cocktail” of these ingredients, then add an
RT-PCR

aliquot of it into each tube. Adjust this step for how many samples you are making. The reason for the cocktail is to minimize pipetting errors. Normally you make a cocktail with enough volume for an extra half or whole reaction so that you aren’t a couple of microliters short.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volumes to add</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One sample</td>
</tr>
<tr>
<td>10X RT buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>4 μl</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNaseOUT recombinant RNase inhibitor</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

4) Add 9 μl of the mix from the previous step to each of your three samples. Mix gently and centrifuge for a few seconds.

5) Add 1 μl SUPERSCRIPT II RT (50 units) of to the samples and controls tubes but not to the “No RT” tubes. Mix.

Note: Steps 6 through 8 will be done automatically in the iCycler PCR machine.

6) Incubate the samples at 42°C for 50 minutes.

7) Terminate the reactions by incubating at 70°C for 15 minutes.

8) Incubate at 6°C for 5 minutes. [The samples can be left in the machine overnight.]

9) Collect the samples by centrifuging for a few seconds.

10) Add 0.5 μl of RNase H to each tube and incubate for 20 minutes at 37°C. [This step is optional]

Note: The samples can be frozen at this step.

PCR procedure

11) In a 0.2 ml PCR tube, mix together the following (one for each primer):
Component | Volume
--- | ---
cDNA (from step 10) | Up to 8 μl
Primer (two for each gene or two primers for the control) | 1 μl each
Deionized water | To 10 μl total volume
Fidelitaq PCR mix | 10 μl

Note: 2 μl of cDNA is the normal amount to use.

12) Put the samples into the PCR machine and incubate them at:
   a) One cycle of 95°C for 4 minutes
   b) Thirty cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 4 minutes
   c) One cycle of 68°C for 4 minutes

   Notes: The 50°C temperature varies with the primers, and the 4 minute elongation time at 68°C varies with the expected length of the fragment.

Note: If necessary, the samples can be frozen at this step.

13) If you are performing nested PCR, repeat steps 11 and 12 with the nested primers.

14) To see if the procedure worked, electrophorese your samples on 1% agarose gels. Use the 1 kb plus or 100 bp ladders as a control. If you are trying to amplify short fragments, increase the agarose to 1.5 or 1.8% and use the 100 bp ladder. Otherwise use 1% agarose and the 1 kb plus ladder.

References

