

RT-PCR Protocol Using Qiagen One-Step RT-PCR

Notes:

- Set up reactions on ice: maintain RNase-free environment when isolating RNA & when setting up the reaction, but prepare reaction mixtures away from this area and also away from bench where PCR product analysis occurs
- Make sure the thermal cycler is preheated to 50°C before placing samples in it

1.) Thaw template RNA, primer solutions, dNTP Mix, 5X QIAGEN One-Step RT-PCR Buffer, Q-Solution, and RNase-free water, and place on ice.

2.) Prepare a Master Mix:

- Prepare a master mix volume that is 10% greater than what you plan to use
- Prepare a negative control (without template) for each experiment
 - Contamination Control= lacks the template RNA in order to detect possible contamination of the reaction components
 - Control for DNA contamination= normal PCR analysis without the reverse transcription (if using this control, perform separately from RT-PCR)
 - **MIX: Per 1 Reaction (@50λ):**
 - **10 λ:** 5X Qiagen One-step RT-PCR Buffer
 - **2.0 λ:** dNTP Mix (containing 10 mM of each dNTP)
 - **1.0 λM** Forward Primer *
 - **1.0 λM** Reverse Primer *
 - **2.0 λ:** Qiagen One-Step RT-PCR Enzyme Mix
 - **1pg-2λg:** template RNA **
 - **Add amount of RNase-free that will bring final volume to 50 λ**

*: This can range from 0.5-1.0 μM, but recommend using 1.0 μM for a 50 λ reaction. Determine concentration of primers, and calculate how many λ this equivocates to before beginning experiment.

** : Use RNA concentration to determine how many λ you need. Make sure you include this value before beginning experiment,

^Calculating these 2 values prior to beginning enables you to determine the quantity of RNase-free water you will need to bring the final volume to 50 λ.

NOTE: This reaction can be cut in half. You can divide everything by 2 to make a 25 λ reaction and make the kit last longer.

3.) Mix the master mix thoroughly, and dispense correct volumes into PCR tubes

- Mix gently by pipetting

4.) Add template RNA (<u>2 λg/reaction</u>) to the individual PCR tubes

5.) When using a thermal cycler with a heated lid, do not use mineral oil. Go to step 6. If lid is not heated, overlay with ~ 50 λ mineral oil

6.) Program thermal cycler:

- Reverse Transcription – 30 min @ 50°C
- Initial PCR activation step- 15 min @ 95°C (activates Taq Polymerase. Reverse Transcriptases are inactivated and cDNA template is denatured)
- 3-step cycling:
 - Denaturation – 0.5-1 min @ 94°C
 - Annealing -0.5-1 min @ optimized annealing temperature
 - Extension- 1 min @ 72°C
 - Number of cycles: 25-40
 - Final Extension-10 min @ 72°C
 - Keep thermal cycler at 10°C if samples aren't being taken out right away

7. Keep samples on ice until thermal cycler reaches 50°C. Then, add tubes and begin RT-PCR program.