

# Two-step Quantitative Real-Time PCR Protocol



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## I. Reverse Transcription

1. Prepare a reverse transcription reaction in a microfuge tube containing the following in total volume 10  $\mu$ l. (ABI TaqMan Reverse Transcription Reagents or equivalent):

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10 x RT Buffer	1 $\mu$ l	
25mM MgCl <sub>2</sub>	2.2 $\mu$ l	(final 5.5 mM)
dNTP Mix	2 $\mu$ l	(final 500 $\mu$ M each)
Random Hexamer or Oligo-dT	0.5 $\mu$ l	(final 2.5 $\mu$ M)
RNase Inhibitor	0.2 $\mu$ l	(final 0.4 U/ $\mu$ l)
RT enzyme	0.25 $\mu$ l	(final 1.25 U/ $\mu$ l)
Total RNA	1-2 $\mu$ g	

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The RT volume may vary from 10  $\mu$ l to 100  $\mu$ l. Increase the volume proportionally.

2. Load the reaction mix into a thermal cycler with following conditions;

Hold 25°C 10 min

Hold 42°C 1 hr

Hold 95°C 5 min

After thermal cycling, store all cDNA samples at -20 °C.

## II. PCR

1. Prepare the reactions in a microAmp Optical 96-well reaction plate. A housekeeping gene should be included for normalizing the quantification of mRNA samples (Note 1). The plate wells should contain no template control (NTC) (Note 2). NTC is performed by adding 5  $\mu$ L of water instead of template.
2. A standard curve can be created for a relative quantification with a housekeeping gene, making serial dilutions of RT reactions, or for an absolute quantification, known concentrations of plasmid DNA can be used to make a standard curve.



3. Prepare a master mix of reagents, primer and probe set for a total volume of 25  $\mu$ l PCR reaction. Be sure to make enough master mix for 1.2 times the number of samples that you actually have. (ABI TaqMan Universal PCR Master mix or equivalent):

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2 x Taq Man PCR Master Mix	12.5 $\mu$ l
primer set	final 200 nM each
Taq Man Probe	final 200 nM
Template RT-Rxn	1-3 $\mu$ l

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The final concentration of primers and probe needs to be optimized in a range of 50 to 900 nM.

4. Cap the wells using optical adhesive covers. Mix well by inverting the plate a few times and spin the plate briefly in a centrifuge to collect all volumes at the bottom of the wells.
5. Load the plate onto the ABI Prism® 7700 Sequence Detection System(or equivalent system). Make sure the plate is aligned properly, then run the reaction with following cycling conditions;

Hold	50 °C 2 min
Hold	95 °C 10 min
40 cycles	
	95 °C 15 sec
	60 °C 1 min

**NOTE:**

1. A group of endogenous control genes should be evaluated to select one that does not alter its expression upon treatments, and can be used to normalize the quantification of an mRNA target for differences in the amount of total RNA added to each reaction. Commonly used endogenous control: genes encoding acidic ribosomal protein, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase,  $\alpha$ -glucuronidase, and hypoxanthine ribosyl transferase.
2. NTC must be included in each plate every time for each tested gene. This is a good control for checking for any contamination in primer/probe mix or formation of primer dimer.

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**GenScript Corporation**

120 Centennial Ave., Piscataway, NJ 08854

Tel: 732-885-9188, 732-357-3839

Fax: 732-210-0262, 732-885-5878

Email: [info@genscript.com](mailto:info@genscript.com)Web: <http://www.Genscript.com>