

TAQMAN

Materials and Methods

Kelly Lab, revised Aug 8, 2007

RT Reaction:

- Isolate total cellular RNA by standard methods.
- Quantitate by using absorbance at 260 nm.

Use filtered pipet tips in all subsequent steps to reduce contamination.

- 1.) Begin to thaw RT reagents (except RNase inhibitor and Reverse Transcriptase) on ice right away because it may take some time.
- 2.) In a new tube, dilute an aliquot of each RNA sample to 5 µg/50µl (0.1 µg/µl) using RNase-free water.
- 3.) Supplied below is a sample recipe for 0.5 µg RNA RT reaction (20 µl final volume). The volume may be increased or decreased according to your needs (If you plan to perform many TAQMAN runs with this RT product, you may need to increase the volume).

<u>20 µl Master Mix RT RXN (all reagents are in Perkin Elmer RT kit)</u>	
10 X RT buffer	2µl
25 mM MgCl ₂	4.4 µl
dNTP	4µl
Random Hexamers	1µl
RNase inhibitor	0.4 µl
Reverse Transcriptase	0.5 µl
RNase-free water	2.7 µl (amount of water should be adjusted if RNA is less concentrated)
Total:	15 µl

Take your number of samples + 1 (to account for pipeting error) and multiply by each of these amounts to find out how much you need for your master mix of reagents.

- 4.) Divide the master mix into 15 µl aliquots in PCR tubes, one for each of your samples.
- 5.) Add 5 µl of your diluted RNA to its respective tube containing the master mix. Pipet up and down briefly to mix.
- 6.) Incubate the reaction at 42°C for 45-60 min. Then heat to 72°C for 10 min to inactivate the RT and stop the reaction.

Use the RT product fresh or if storing for longer periods than 2-3 days, store at -20°C.

TAQMAN PCR **Materials and Methods**

PCR Reaction:

Use filtered pipet tips in all steps to reduce contamination.

- 1.) Begin to thaw probe and primer sets on ice. If necessary, thaw RT products on ice as well.

- 2.) While PCR reagents are thawing, label four sterile microfuge tubes with one of the labels listed: 50X, 20X, 10X, 2X. These tubes will contain your standard curve.

Into the tube labeled 50X, pipet 5 μ l from each unknown RT RXN (undiluted), and vortex to mix. Serially dilute the mixture as follows:

-move an appropriate* amount of 50X (so that enough remains for PCR \rightarrow 2 μ l per probe used) into the 20X tube and dilute 1:2.5 (1 in 2.5) with H₂O. Mix.

-move an appropriate amount of 20X into the 10X tube and dilute 1:2 (1 in 2) with water. Mix.

-move an appropriate amount of 10X into the 2X tube and dilute 1:5 (1 in 5) with water. Mix.

**It's worth calculating the amounts for each transfer beforehand to generate equivalent volumes. This preparation can be stored and used with these samples in another run with a different probe.*

Undiluted RT can be returned to the fridge or -20 ° now. Store long-term at -20.

Label another sterile 1.5 mL microfuge tube for each unknown and all four standards. If you are using more than one experimental probe, you must make a tube for each sample and standard for each probe. These will be your **final rxn** mixture tubes. Set them aside until later.

* To calculate the number of wells and, as a result, the amount of master mix needed to run your samples, consider these factors. A full set of four serially-diluted standards (in triplicate) (i.e., 12 wells) should be used for each probe (not counting the internal control probe (GAPDH)) used in each TAQMAN run. All unknowns (your samples) should be run in triplicate. For instance, if one probe is used, 96 wells-12 wells standards=84 wells/3 (triplicate)=28 unknowns on one plate. If this is a new probe, negative and positive control RNAs need to be employed.

- 1.) Supplied below is a sample recipe for each reaction (25 μ l final volume) and for a full 96 well plate (110 RXNs to account for pipeting error). Adjust the

volume according to the number of samples assayed. Combine the following in a sterile 15 mL conical tube.

PCR RXN (using Applied Bio's Taqman and Sybrgreen 2X PCR MM)

TAQMAN (Standard):

	<u>1RXN</u>	<u>110RXNs</u>
2X PCR Taqman Master Mix	12.5 λ	1375 λ
H2O	11.38 λ	1252 λ
Target gene FWD primer (20μM)	0.15 λ	16.5 λ
Target gene REV primer (20μM)	0.15 λ	16.5 λ
Target gene PROBE (10μM)	0.15 λ	16.5 λ
Use 2 ul of 1:1 diluted RT per triplicate		

TAQMAN (FAST):

	<u>1RXN</u>	<u>110RXNs</u>
2X PCR Taqman FAST Mix	5 λ	550λ
H2O	4.48 λ	492.8λ
Target gene FWD primer (20μM)	0.06 λ	6.6λ
Target gene REV primer (20μM)	0.06 λ	6.6λ
Target gene PROBE (10μM)	0.06 λ	6.6λ
Use 1 ul of 1:1 diluted RT per triplicate		

SYBR:

	<u>1RXN</u>	<u>110RXNs</u>
2X PCR Sybrgreen Master Mix	12.5 λ	1375 λ
H2O	11.53 λ	1268 λ
Target gene FWD primer (20μM)	.15 λ	16.5 λ
Target gene REV primer (20μM)	.15 λ	16.5 λ
Use 2 ul of 1:1 diluted RT per triplicate		

*Use 36b4 as your control → set it up on its own the same way you would set up any other Sybrgreen run except use half as much primer (*can be done on a plate with another primer set if you have enough room*).

- 2.) TAQMAN PCR RXN mixture thoroughly by vortexing.
- 3.) Aliquot the TAQMAN PCR RXN mixture into the **final rxn** mix tubes (from page 2) at 80 μl per tube (**33ul for FAST**).
- 4.) To the 80 μl mixture in the **final rxn** tube, add 2 μl from the corresponding sample **RT rxn** tube from page two. Then add 2 μl from the corresponding standard tubes into the appropriate standard **final rxn** tube. Mix all by pipeting
- 5.) Split the **final rxn** into three wells of the 96 well PCR plate (Microamp

Optical Plate) at 25 μ l/well (10ul/well for FAST). ***Be sure to make a map of which sample goes in which well of the plate using pre-made 96 well forms.***

6.) Just before you place the cover on the plate, look at each well to be sure that no air bubbles are present in the bottom of the well. If there are bubbles, tap the well to make them float to the surface and pop with a .1-10ul pipet tip. Place the Optical Adhesive Cover over your wells and use the included scrapper to apply the cover.

7) At the machine, open the 7500 software. Go to File-New

8) Change the Plate name to your file name, click next

9) Choose the detectors that you used, and click add. After you have chosen all the detectors, click next. Highlight the corresponding wells for each detector and then click on the detector. It will put a color on the well if done correctly. Click finish

10) Click Instrument

11) If you are using SYBR or Taqman standard, under the Run Mode, change from FAST to 9600 emulation. When it prompts you if you want to change the default parameters, click YES. Change the volume of the sample. For SYBR, click to add the dissociation stage. Save the file. Start. Before walking away, ensure that the program has started...will take a minute or so.

12) At the end, it will say reaction complete. Click OK

13) Go to the Amplification Plot and highlight a group of wells containing the same primer set.

14) Right click on the y-axis and change the post-run axis to Linear, click ok

15) Decide which cycle the curve begins to rise and change the end cycle to that number

16) CHANGE BACK TO LOG on the y-axis and move the threshold bar up or down in order for the blank wells to read Undet. But the rest of your samples to read values. Make sure the threshold bar is kept in the portion of the curve where your triplicates are the tightest and the slope is still increasing. After moving the bar, click analyze.

17) Click on the Report tab.

18) Check the numbers to make sure the blank reads Undet as in #16 above. If the numbers are correct, go to File, Export, results, and save to a CD.

19) Do this for each detector separately from #13-18. When saving to the CD, change the name to highlight the detector that you have optimized the threshold for so it will be easy to go back and figure out which is which.

20) Once they are all exported, burn to the CD.

21) Export into Excel.

22) Graph the standard curve (see someone's template)

23) Calculate the log input based on the line from the standard curve.

24) Convert to input by taking the inverse log.

25) Average the triplicates. Divide this number by the average of your control probe. Graph data.

Addendum:

- RT can be diluted 1:1 before use. This generally does not affect the results.
- The entire process can be modified to utilize the multi-channel pipettor if desired. Use 4 PCR strip tubes per plate to hold the **final rxn** and aliquot to the PCR plate accordingly.
- Before doing calculations, be sure your sample CTs fall within the range of the standard curve.