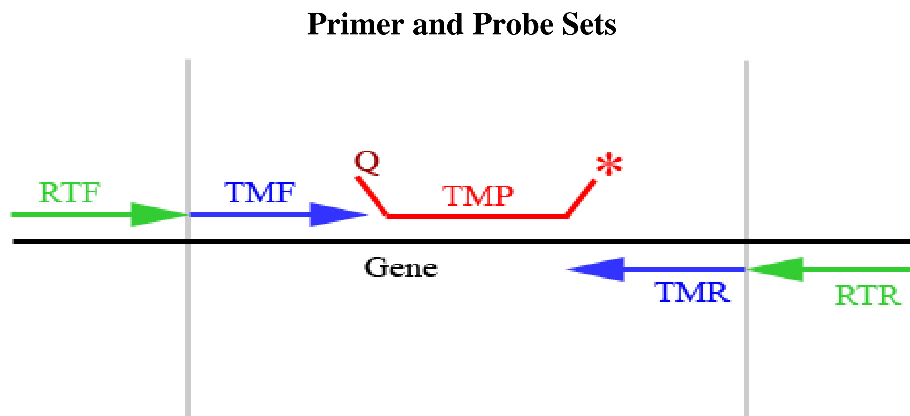
Protocol:
Genome Expression Profiling of MTB using Two-Step Multiplex qRT-PCR

The protocol below consists of three parts:
(1) Preparation of Primer and Probe Sets
(2) cDNA synthesis and Pre-Amplification
(3) Taqman RT-PCR

1. Preparation of Primer and Probe Sets

This protocol details the steps in primers and probe preparation. Primers and Probe sets are prepared as the first step in the qRT-PCR assay.

Primer and Probe Sets



RTF – RT Forward (Amplification or "outflanking" primers)
RTF – RT Reverse (Amplification or "outflanking" primers)
TMF – Taqman Forward (TM Mix)
TMP – Taqman Probe (TM Mix) - Has fluorescent label and a quencher
TMR – Taqman Reverse (TM Mix)

A. Prepare TM Mix for qRT-PCR:

1. Thaw TMF, TMP, and TMR (from 20°C), can store at 4°C while thawing/using for a few days.
2. Quick spin down all primers.
3. Vortex each primer for 10-20s on a vortexer before using, especially TMP, as it is heavier.

Primers and probe are at 100uM stock concentration. Ideally, forward and reverse primers are diluted to 1500nM and the probe to 500nM. Using 2ul of this mix in a 10ul qPCR reaction, final primer concentration is 300nM, while the probe is at 100nM.

In a 2ml, screw top, RNase-free tube combine:

TMF	27 µl
TMP	9 µl
TMR	27 µl
H_2O	1737 µl (for pipetting, 868.5 µl x2)
	1800 µl

B. Prepare RT Primer Mix for Multiplex Pre-amplification:

Determine how many µl of mix you will need of each gene and add equal amounts of each RTF and RTR in a 2ul, screw top, RNase-free tube. To determine amount, use: 1/10th of total genes per reaction, so 5 genes needs 0.5µl/rxn (See further explanation in pre-amplification section). If you have 10 samples, you will need 0.5 * 10 = 5 µl total. It is OK to make more, especially if you will be using this gene set again. A good general amount is at least 5 µl each. For example, if you have 5 genes, that's 10 total primers to add (RTF+RTR), and will be 50 µl total.

2. cDNA Synthesis and Pre-Amplification

This protocol details the cDNA synthesis and pre-amplification steps before qPCR RT-PCR. Currently, RT amplification primers are prepared in 3 sets of RT mixes containing approximately 740 genes each designated as Mix 1, Mix 2, and Mix 3.

A. First Strand cDNA Synthesis:

- Before beginning, normalize all RNA samples to the same concentration if possible.
- If concentration is unknown, such as in in-vitro samples, use the same volume instead.
- Do not exceed 2ug per cDNA reaction.

I) <u>**Random Hexamer Mix**</u> (for + and -)

- Exo-resistant Random Hexamers (Fermentas)
- 10 mM dNTPs (Fermentas)

	(+)	(-)
RH (random hexamers)	0.5ul	0.5ul
10mM dNTP's	1.0ul	1.0ul
RNA (X ng)	**X**	**X**
H2O (RNase free)	10.0-X	10.0-X
	10.0ul	10.0ul

- **X** = volume of RNA added to mix, will vary depending on concentration.
- **Pipette or flick to mix, do not vortex.**
- Centrifuge for 2 min. @1200rpm

II) Incubation #1

3 min. at 70°C in thermocycler
1 min. at 45°C in thermocycler

- While incubating, make RT (+/-) mixes or have them ready ahead (minus enzymes) if you don't think you'll have enough time
- Add enzymes right before use

III) RT Reagent Mixes (RT+ / RT-)

- Fermentas Maxima 5X Buffer and Reverse Transcriptase
- Ribolock RNase-Inhibitor (Fermentas)

	(+)	(-)
5X buffer	4.0ul	4.0ul
Ribolock	0.5ul	0.5ul
Maxima RT	0.5ul	------
H2O (Rnase free)	3.1ul	3.5ul
	10.0ul	10.0ul

- **Pipette or flick to mix, do not vortex.**
- Set up mixes on ice, quick spin 1-2 seconds.
- Add 10ul of RT reagent mix to treated samples from steps (I & II) quickly, preferably at 45°C. If it will take some time, add reagents on ice.
- Quick spin to avoid too much of a temperature change. Continue to step IV.

IV) Incubation #2

50°C for 60 min.
95°C for 2 min.
4°C HOLD

- When final incubation is complete, use immediately for direct qPCR or proceed to pre-amplification.
- Can store at 4°C overnight. Storage at -20 ^{o}C is stable for a few months.

B. RT Primer Mix Volume Calculation:

Calculate volume of oligo mix to add. Oligo mix volume per reaction tube is dependent on number of genes being screened (# of oligos = 2x # of genes). All stock oligos are standardized to 100µM and each reaction requires final oligo concentration to be 5.0 pMol or 100nM. Example for 184 oligos (92 genes) in a 50ul reaction:

$$\frac{100\mu Mol}{184} / 1000ml \rightarrow \frac{100nMol}{184} / 1000\mu l \rightarrow \frac{100pMol}{184} / 1\mu l$$

$$\rightarrow \frac{0.54pMol}{1ul} = \frac{5.0pMol}{X} \rightarrow \mathbf{X} = \frac{5.0}{0.54} = 9.259\mu l.$$

9.259 µl of oligo mix must be added for each reaction (sample) in the set.

or simply....# of genes / 10. For 30 µl reactions: (# genes / 10) * 0.6

C. Multiplex Pre-amplification:

- Advantage 2 Polymerase Buffer (Clontech/Takara)
- 10mM dNTPs (Fermentas)
- RT Primer Mix (Biosearch)

- Make enough for RT+ and RT- reactions. Scale up depending on how many samples you have. Make extra to account for pipetting.
- Since our large-scale qRT-PCR profiling of the MTB genome currently consists of three mixes of ~740 genes each, three separate amplification reactions are required for each cDNA sample. (PA1, PA2, and PA3)
- To use our standard concentration listed above would require ~45ul of RT Mix which does not fit in a 30ul reaction. Instead, we use ~23ul of the primer mix and eliminate Water. This reduces each primer to a concentration of ~52nM.

I) Amplification Reagent Mix for 1 Reaction (1X):

10X Adv. 2 Buffer	3.0 µl
dNTP's	0.6 µl 1
RT Primers Mix	**X** (from step 1)
Advantage 2 Polymerase	0.6 µl
cDNA	**Y** - up to 1/5th of reaction (2 – 6 µl)
Rnase-free H2O	(30 µl – sum of above reagents)
	30.0 µl

- Aliquot 24–28 µl (depending on how much cDNA you will add) per well into a 96 well plate for RT (+/-), add cDNA's mixing with pipette.
- Centrifuge @ 1200 RPM for 2 min
- Proceed to Step II – Amplification.

II) Amplification

- Typically, for in vitro samples, 15 cycle amplification is sufficient for qRT-PCR.
- In vivo tissue samples and sputa require 2 rounds of amplification totaling 25 cycles.

95°C 5 min.

95°C 30 sec. 60°C 20 sec. 68°C 1min.	**15** cycles

4°C HOLD

If a second amplification is needed, take 1/5th (6 ul) of the 1st pre-amp reaction into a 2nd reaction per Step I, adjust water, run another **10** cycles.

3. Quantitative RT-PCR

- Direct Taqman RT-PCR is used for assays that do not require amplification. For MTB. Use 100pg to 1 ng of cDNA (based on starting RNA concentration) / gene.
- For Amplified samples, use 0.05ul to 0.1ul per reaction.

Reagents:

- Roche LightCycler 480, 384-well plate
- Roche Probes Master (2X Master Mix)
- PCR Grade Water

A. Prepare qRT-PCR Master Mix:

- Scale the 1X reaction up depending upon how many samples you have.
- Make extra to account for pipetting.

Basic 1X Reaction:

2X Probes Master Mix	5.0	µl
cDNA (Direct or Amp.)	X	µl
PCR H2O	8 µl - sum of above -	µl
	8.0	µl

- Aliquot 8 µl of this master mix into each well on a 384 plate. The number of wells is equal to the number of genes that will be assayed.
- Add 2 µl of the gene-specific TM Probe Mix to each well using a Matrix pippette.
- Seal the plate with the provided cover and centrifuge for 2 min. at 1200 RPM.
- Run on the LightCycler 480. (Proceed to Step B)

B. PCR Profile for Quantitative RT-PCR:

95 °C	5 min	Ramp Rate: 4.8°C/sec.	
95 °C	**30 sec**	**Ramp Rate: 4.8°C/sec.**	**40 Cycles**
60 °C	**30 sec**	**Ramp Rate: 2.5°C/sec.**	
40 °C	30 sec	Ramp Rate: 4.8°C/sec.	1 cycle - cool down

Analyze Second Derivative, High Confidence on Roche Lightcycler Software.