Using RT qPCR for quantifying *Mycobacteria marinum* from in-vitro and in-vivo samples.

Han Xaio, Stephen H. Gillespie

School of Medicine

University of St Andrews

Summary

Mycobacterium marinum, the causative agent of fish tuberculosis and is rarely a human pathogen causing a chronic skin infection. It is now wildly used as a model system in animal models, especially in zebra fish model, to study the pathology of tuberculosis and as a means of screening new anti-tuberculosis agent. To facilitate such research, quantifying the viable count of *M. marinum* bacteria is a crucial step. The main approach used currently is still by counting the number of colony forming units (cfu), a method that has been in place for almost one hundred years. Though this method well established, understood and relatively easy to perform, it is time consuming and labour intensive. The result can be compromised by failure to grow effectively and the relationship between count and actual numbers is confused by clumping of the bacteria where a single colony is made from multiple organisms. More importantly, this method is not able to detect live but not cultivable bacteria, and there is increasing evidence that mycobacteria readily enter a “dormant” state which confounds the relationship between bacterial number in the host and the number detected in a cfu assay. DNA based PCR methods detect both living and

dead organisms but here we describe a method, which utilizes species specific Taq-Man assay and RT-qPCR technology for quantifying the viable *M.marinum* bacterial load by detecting 16S ribosomal RNA (16S rRNA).

1. Introduction

16s rRNA, which accounts for 82-90% of the total RNA in mycobacteria, is the core structural and functional component present in all bacteria. Its high abundance and critical functional makes it a suitable biomarker for mycobacterial quantification. Methods to detect *M. tuberculosis* have been described previously and applied successfully in clinical trials [1,2]. This important observation has now been expanded as we have developed this assay further to make it more robust in laboratory practice and expanded the range of its use by designing species-specific Taq-man assay allowing the quantitative evaluation of *M. marinum* 16s rRNA. Meanwhile, to take into account the potential loss of RNA during extraction procedure, an internal control (IC) must be included. in this assay we use a fragment of potato RNA, with known concentration is spiked into the sample prior to RNA extraction to normalize such loss[1]. Taq-man assays for *M. marinum* 16s rRNA and the IC are run simultaneously as a duplex qPCR run.

2. Materials

1. *Mycobacterium marinum* M strain (see **Note 1)**
2. Homogenisation of the cell culture or tissues requires a homogenization kit-Micro-organism lysing VK01-2mL (Bertin Instrument), which contains 0.1mm glass beads, is used for homogenization of the cells pellets or tissues.

3. RNA extraction: FastRNA PRO BLUE KIT (MP biomedicals) or Purelink RNA mini kit (Invitrogen), are used for extraction of RNA (see **Note 2**).
4. DNA removal: DNA-free™ kit DNase (Invitrogen) Treatment and removal reagents are used for removing the DNA (see **Note 2**).
5. RT-qPCR: primers and probes should be purchased from a supplier using the sequences noted in Table 1 (see **Note 2**).
6. A QuantiTect-multiplex RT-PCR NR kit can be used to run the PCR (see **Note 2**).
7. A real time PCR machine e.g., Rotor-Gene Q (Qiagen) (see **Note 3**).
8. Internal control: a segment of potato RNA is used as Internal control, the generation of which is described in one of our papers published previously[1].

3. Method

Carry out all procedures at room temperature unless otherwise specified.

3.1 Mycobacterial quantification by colony forming unit (CFU)

Bacteria are quantified by a modified Miles and Misra method as described previously[3].

3.2 RNA extraction and DNAse treatment

3.2.1 *Extraction of RNA from liquid culture of Mycobacterium marinum*

1. Take 2 x 1mL of the liquid culture, spin at 100000*g* for 10 min.

2. Remove the supernatant and re-suspend the cell pellet in 950µl of lysing buffer supplemented with 10% 2-mercaptoethanol for the Purelink RNA mini kit), which is provided by the RNA extraction kit.
3. Spike in 50ng of the internal control
4. Transfer the suspension to the homogenization tube and make sure it is tightly closed.
5. Place the homogenization tubes in the homogenizer and spin it using programme 6.0 for 40 sec if using Fastprep.
6. Transfer the homogenization tubes to a bench-top centrifuge and spin it at 12000*g* for 5min
7. Carefully transfer the supernatant to a clean tube without disturbing the glass beads
8. A: If using Purelink RNA mini kit, follow the manufacturer's instruction by referring to the section of RNA Purification of the quick reference supplied with the kit.
9. B: If using FastRNA Pro, follow the manufacture's instruction by referring to the quick reference protocol starting with step.
10. The extracted RNA could be subjected for DNase treatment immediately or stored at -20°C if the DNase treatment is to be carried out within a month, or stored in -80°C for future use.

3.2.2 *RNA extraction using zebrafish embryos as an example*

1. Pool 10 or more embryos into a micro-centrifuge tube and spin at 3000*g* for 10 min.

2. Remove the supernatant without disturbing the embryo.
3. Add 950uL lysing buffer supplemented with 10% 2-mercaptoethanol for the Purlink RNA mini kit (see **Note 4**)
4. Continue with step 3 and onwards from section 3.1a

3.2.3 *DNAse treatment*

1. Make a master-mix of the Turbo DNase I 10x buffer and DNAse I enzyme for the number of samples plus 10% extra (see **Note 5**)
2. Mix by vortexing and then pipette 11 µL into each tube containing RNA extracted from 3.2.2.
3. Mix again by vortexing and then spin briefly (5–10 s at 13000 *g*).
4. Incubate at 37°C for 30 min in the hot-block or incubator.
5. Add an additional 1 µL of DNase directly into each tube and mix well by vortexing.
6. Incubate at 37°C for a further 30 min (see **Note 6**).
7. Thaw the DNase inactivation reagent 10 min prior the finish of DNase incubation and keep in the fridge. Re-suspend by vortexing.
8. Add 10 µL of DNase inactivation reagent into each RNA extract.
9. Vortex 3 times during the 5-minute incubation step at room temperature.
10. Centrifuge at 13,000 *g* for 2 min.
11. Transfer the supernatant to 1.5 mL RNase free tube without touching any of the inactivation matrix.

3.3 <u>RT-qPCR</u>

1. Prepare 1 in 10 dilution of the RNA extracted from 3.2 in duplicate for RT-qPCR.
2. Prepare stock primer and probe with the final concentration as 10uM.
3. Fluorescence signals are used as the read out of *M. marinum* 16s rRNA and IC assay, are collected on Fam and Hex channel respectively. (see **Note 7**)
4. Programme the thermal cycler and include PCR reaction components as listed in Table 1 and 2 respectively.
5. Make sure a no-RT reaction, for which reverse transcriptase is excluded for the RT-qPCR reaction components, is included for every sample to test if there is any DNA present in the sample.

3.4 **Generation of the correlations of CFU and total RNA detected by *M. marinum* 16s rRNA assay**

1. Use liquid culture at late exponential phase.
2. Prepare seven decimal dilutions of the culture in triplicate.
3. Use one set of the dilutions to carry out a CFU counting and count the colony 5 days after the plating or once the colony is countable.
4. Use the duplicate dilutions prepared at step 2 for RNA extraction, as described previously, and RT-qPCR.
5. A standard curve of M. marinum total RNA comprising 7 decimal dilutions with the highest concentration as 10ng/μl to 10^{-5}ng/μl
6. Total RNA present in the sample prepared from step 2 will be derived from the standard curve constructed from step 5 (more information on data analysis can be found in section 3.5)
7. Plot the CFU data against the corresponding amount of total RNA.

3.5 qPCR data interpretation and bacterial load quantification

3.5.1 Principle

The principle of the MBL assay is absolute quantification based on a standard curve consisting of a set of RNA templates with known concentration. The standard curve is used to calculate the *M. marinum* concentration of an unknown sample.

IC standard curve is used to justify the efficiency of the extraction. If the amount of IC detected from unknown sample is no less than 10% of the spiked in IC, the extraction will be treated as a successful one, otherwise it will be treated as a failed extraction that should be repeated. Extraction efficiency could be achieved by divide the amount of IC from the sample by the spiked in IC, which can be used for normalization of the *M. marinum* MBL data. Standard curves must be constructed for each real-time PCR instrument (see **Note 8**).

3.5.2 Standard curves construction

1. *M. marinum* RNA extracted from culture with concentrations of 10^8 CFU/mL or higher and IC RNA at 50 ng/μl.
2. Dilute the extracted RNA decimally to create a series of standards. Add 10 μL of extracted RNA into 90 μL of RNase-free water, mix by vortexing for 5 s.
3. Set up the RT-PCR master mixes as outlined above in **Table 2**.
4. The standards are amplified in duplicates (along with the samples or on their own).

5. In RotorGene Q software, label the standards in sample sheet and assign them corresponding concentration and units, e.g. 10^8 for first 1 in 10 dilution (if the RNA is extracted from culture with 10^9 CFU/mL).

3.5.3 Standard curve data analysis

The standard curve can be prepared in a separate run for the use with RotorGene Q and it can be further incorporated for data analysis of samples with unknown bacterial load.

1. Analyze the amplification curves in appropriate fluorescence channel, i.e. green channel for Mtb (FAM labelled probe), yellow channel for IC (VIC or HEX labelled probe).
2. Set the fluorescence threshold to 0.02 and examine the curves in exponential view and then in logarithmic mode.
3. Go to "Analysis" option and select the channel and sample sheet you are going to analyze.
4. Click on "Slope correct" in order to minimize the fluorescence fluctuations.
5. When standards and their respective concentrations are assigned in the sample sheet, the analysis software will automatically populate a standard curve.
6. Examine the parameters of the standard curve. The parameters are:
    a. Slope (M), informs on assay efficiency
    b. Correlation coefficient (R^2), informs on assay linearity and the dynamic range (or limits of quantification)
    c. Intercept, shift in C_T value on the y axis

7. The PCR efficiency can be evaluated by the parameters of standard curve. The equation for an ideal standard curve and a 100% amplification efficiency (E=1) is:

   C_T = slope×Log(concentration) – intercept

   or

   C_T = -3.32×Log(concentration) – intercept

   Aim for the efficiency of 90%-100%, i.e. E=0.9 to 1.0. The efficiency can be calculated from the slope of the standard curve using the equation:

   $E=10^{-1/-3.32}-1$

8. Very high or too low RNA concentrations in the RT-PCR reaction can cause fluctuations in reverse transcription and PCR efficiency. These result in outlying C_T values. Outlier C_T values can be also caused by errors in pipetting, dilutions' preparation, and insufficient homogeneity of a PCR mastermix, evaporation during reaction and improperly placed rotor.
9. Consider careful removal of the outliers.
10. Interpretation of the data is illustrated in table 3.

Table 1. Taq-Man assay for M.marinum 16s rRNA list of sequences for primers and probes

Name	Sequence	Channel	Target
M.marinum 16s rRNA Forward	5'-GAA CTC AAT AGT GTG TTT GGT GGT-3'		Mycobacterium Marinum 16s rRNA
M.marinum 16s rRNA reverse	5'-CCC ATC CAA AGA CAG GTG AA-3'		
M.marinum 16s rRNA probe	FAM-TTG TCC GCC TCT TTT TCC CGT TT-BHQ1	Fam	
IC forward	5'-GTG TGA TAC TGT TGT TGA-3'		Internal control
IC Reverse	5'-CCG ATA TAG GGC TCT AAA-3'		
IC probe	Hex-TAC TCT CAG CCA CTA CCT CTC CAT-BHQ1	Hex	
Thermal cycles			
Step 1	50°C	20 mins	1 cycle
Step2	94°C	45 secs	40 cycles
	60°C	45 sec	

Table 2. PCR reaction components

	RT+ reaction V per reaction	RT- reaction V per reaction
Quantitect Mastermix	10µL	10µL
M. Marinum 16S F+ R primer mix	0.4µL	0.4µL
M.Marinum 16S-FAM probe	0.2µL	0.2µL
IC F+R primer mix	0.4µL	0.4µL
EC probe	0.2µL	0.2µL
RT enzyme	0.2µL	-------
Molecular grade water	4.6µL	4.8µL
Sample	**4µL**	**4**µL
Total	**20 µL**	**20 µL**

Table 3. Validation of assay

Target (Marinum)	IC	Result
+	+	+
+	-	+*
-	+	-
-	-	**Invalid**

+ = Positive shown by Cycle threshold (Ct) from the RT-qPCR

- = Negative shown by no Ct from the RT-PCR

*** = The Mtb presence result is positive, but the result cannot be used for quantitative analysis or data normalization.**

4. Notes

1. The *M. marinum* can be grown in Middle brook 7h9 broth supplemented with OADC and incubated at 30°C.
2. The method for RNA extraction DNA digestion and PCR master mix presented in this chapter is optimized using the kits noted but alternatives can be used.
3. The assay presented in this chapter is optimized for the Rotor-Gene (Qiagen) but other machines with similar characteristics can be used and we have adapted similar assays to a wide range of machines.
4. After this procedure the material can be stored at -80^{o}C
5. After defrosting of the Multiplex quantitect master mix, aliquot them in 500uL and store them at -20°C. Avoid multiple freeze and thaw of the master

mix which can reduce its efficiency. If the mix is not finished at a single use, it can be stored at 4°C for up to a week for further use.

6. Incubation time can be up to 1 hour.
7. Optimization has been carried out to ensure there is no cross reaction between these two reactions.
8. Extraction efficiency can be achieved by dividing the IC in the post extraction sample by the spiked in amount. Actual 16s rRNA present in the pre-extraction sample can therefore be retrieved based on the extraction efficiency of each individual sample. Efficiency of *M. marinum* 16s rRNA (Figure 1A) and IC (Figure 1B) Taq-man assay was tested by running a standard curve composing pure RNA with six decimal dilutions, with the highest concentration of 10ng/ul while the lowest concentration of 10^{-5}ng/ul. Based on three replicate experiments, efficiencies of these two assays are above 95%. To reflect the CFU of *M. marinum* from each sample, correlation between CFU and the amount of total RNA detected by 16s rRNA assay was established. However, such correlation is used only as an indicator of the approximate amount of CFU present in the sample but not to conclude the actual amount of CFU. Such recommendation is based on the observation that relationship between CFU and 16s rRNA varies amongst different growth phase and when under different stress conditions.

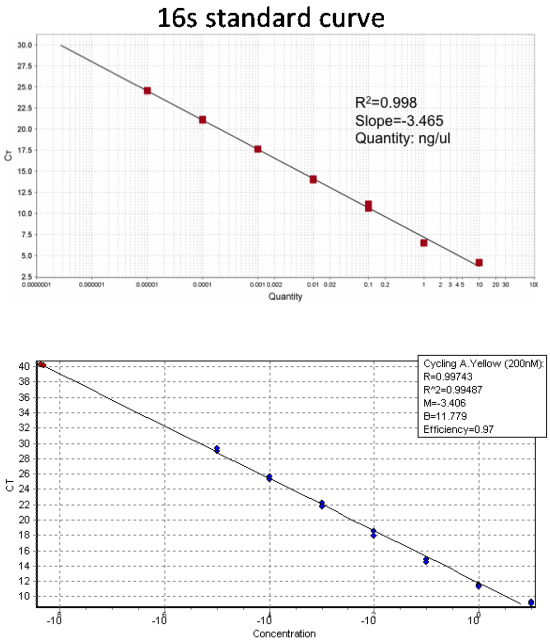

Figure 1A, standard curve of M. marinum 16s rRNA, Figure 1B, standard curve of IC

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