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# Real Time PCR Using Molecular Beacons

*A New Tool to Identify Point Mutations and to Analyze Gene Expression in* Mycobacterium tuberculosis

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## 1. Introduction

### *1.1. Molecular Beacons*

Molecular beacons are a novel family of hybridization probes, which emit fluorescence upon interaction with their target. They are hairpin-shaped oligonucleotides with a central part complementary to the target, flanked by two 5–6 base pair (bp) inverted repeats, which can form a stable stem. A fluorescent moiety is covalently linked to the 5' end of the molecule, whereas the quenching moiety, 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), is covalently linked to the 3' end. The stem keeps the two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. When molecular beacons bind to their target, they undergo a conformational change that results in the restoration of fluorescence of the internally quenched fluorophore ***(1)*** (**Fig. 1**). Molecular beacons are extremely specific, and can clearly discriminate between targets differing only by a single nucleotide ***(2,3)***. When present in a PCR reaction where their target is the amplification product, molecular beacons can form a stable hybrid with the amplicon during the annealing step. The intensity of fluorescence at the annealing step in each amplification cycle is a direct measure of amplicon concentration ***(2,4)*** (**Fig. 2**). Another interesting feature of molecular beacons is that they can be coupled to a variety of differently colored fluorophores. This allows multiplex PCR reactions where different DNA fragments can be amplified and detected simultaneously in the same tube ***(2,3)***.

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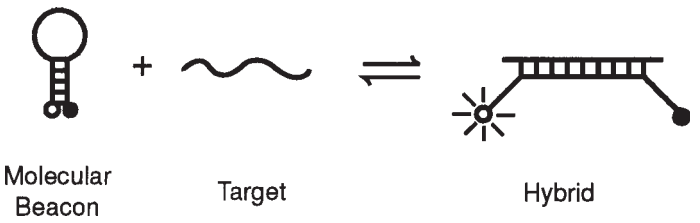


Fig. 1. Operation of molecular beacons. On their own, these molecules are nonfluorescent, because the stem hybrid keeps the fluorophore (○) close to the quencher (●). When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence ***(1)***.

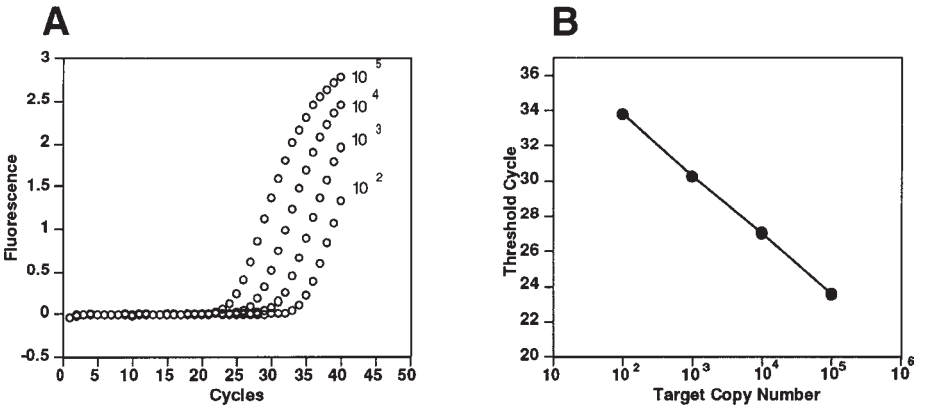


Fig. 2. Real time measurement of amplicon synthesis during PCR using molecular beacons. **(A)** Four PCR reactions were initiated with a different number of template molecules (indicated). The concentration of amplicons present after each cycle of amplification was determined by measuring fluorescence during the last few seconds of the annealing step. **(B)** Inverted relationship between the threshold cycle (the cycle at which the fluorescent signal becomes detectable above the background) and the logarithm of the initial number of template molecules. In this example, the target is *M. tuberculosis* H37Rv chromosomal DNA. The primers-molecular beacon set used in the reaction was specific for *sigA* (reprinted from **ref. *4***).

### *1.2. Detection of Point Mutations*

The emergence of multidrug-resistance (MDR) *Mycobacterium tuberculosis* represents a major problem in tuberculosis treatment. Conventional testing for antibiotic susceptibility takes from 2–8 wk, during which time the patient's

health can deteriorate dramatically ***(5)***. Since almost all MDR *M. tuberculosis* strains are resistant to rifampin, resistance to this antibiotic has been proposed as a marker for MDR tuberculosis ***(6)***. Rifampin resistance is usually associated with mutations in an 81-bp region of the *rpoB* gene ***(6)***. Analysis of this region of *rpoB* has been used to predict rifampin susceptibility of clinical strains using sequence analysis ***(7,8)***, single-strand conformational polymorphism analysis ***(6)***, heteroduplex analysis ***(9)***, and RNA/RNA mismatch assays ***(10)***. All of these techniques are significantly faster than the conventional culture-based techniques for drug sensitivity, but are still time consuming and require sophisticated technical expertise. Piatek et al. ***(11)*** recently developed a very easy and effective method for the sequence analysis of the 81-bp region of the *rpoB* gene using a set of five overlapping molecular beacons, each capable of distinguishing various single point mutations. The 81-bp fragment of *rpoB* was amplified in five parallel PCR reactions, each of which contained a molecular beacon specific for about 20 bp of the amplicon. Each molecular beacon was designed to hybridize only to the wild-type sequence. The absence of fluorescence in one or more reactions indicated the presence of one or more mutations. Each of the five reactions also contained primers and a molecular beacon specific for the amplification and the detection of a fragment of the mycobacterial 16S rRNA as a positive control. The molecular beacon for 16S rRNA was coupled with tetramethylrhodamine (TET) and the molecular beacon for *rpoB* was coupled with fluorescein (FAM) so that the two reactions could be distinguished. A sixth reaction was carried out with primers and molecular beacon specific for the insertion element IS*6110* to confirm that the DNA of the sample was from *M. tuberculosis*.

This technique can be easily adapted for the detection of other mutations known to result in drug resistance in *M. tuberculosis* (for example, mutations in *katG* and *inhA* conferring resistance to isoniazid) and for the identification of mycobacteria in clinical samples.

In this chapter, we describe how to design and characterize a molecular beacon capable of discriminating between two targets differing only in a single nucleotide and we also describe a protocol to perform the assay.

### *1.3. Gene Expression in* M. tuberculosis

*M. tuberculosis* must adapt to a changing and challenging environment during infection; moreover, it has been proposed to be able, under certain circumstances, to enter a "persistent" state ***(12)***. This suggests that the regulation of gene expression plays a major role in its pathogenicity as it is expected that different genes would be induced or repressed during different stages of the infection process.

Several methods have been used to monitor gene epression in *M. tuberculosis*: fusion with reporter genes such as *lacZ* or *gfp*, RNAse protection assays, differential display PCR, Northern blot analysis, customized amplification libraries, and reverse transcriptase-PCR (RT-PCR) ***(13,14)***. The limited number of mycobacteria that can be recovered from samples such as infected tissue or macrophages makes PCR-based techniques the methods of choice for quantitative measurements of gene expression. We recently developed a quantitative RT-PCR assay coupled with molecular beacons that enabled us to study the differential expression of 10 genes in cultures of *M. tuberculosis* exposed to various stresses ***(4)***. The method relies upon the use of a constitutively expressed gene (*sigA*) as an internal control to correct for variability in the efficiency of the reverse transcription reaction as well as for differences in RNA concentrations from sample to sample. A standard reverse transcription reaction is followed by a quantitative PCR with molecular beacons. The use of molecular beacons to detect and measure the reaction product enhances both the sensitivity and the specificity of the PCR reaction. Moreover, by following the PCR reaction in real time, the investigator can easily recognize the onset of the linear phase of the reaction, which permits accurate quantitation.

In this chapter, we describe this RT-PCR technique as well as a method for the preparation of mycobacterial DNA and RNA from infected human macrophages. The method is designed to provide seven identical sets of seven samples collected at various times during an infection of 72 h. These seven samples consist of two samples of extracellular bacteria and five samples of intracellular bacteria ($T_1$, $T_6$, $T_{24}$, $T_{48}$, and $T_{72}$). The RNA can be used to study the differential gene expression during growth in macrophages, whereas the DNA can be used to measure the growth of the intracellular bacteria.

## 2. Materials

### 2.1. Synthesis of Molecular Beacons

Molecular beacons are synthesized from oligonucleotides containing terminal aminosulfhydryl functional groups by coupling them to activated dyes. The detailed procedures are available on the worldwide web at http://www.molecular-beacons.org. They can also be obtained from a number of oligonucleotide synthesis companies such as Research Genetics (Muntsville, AL), Midland Certified Reagents (Midland, TX), Life Technologies (Gaithersburg, MD), Biosearch Technologies (Novato, CA), Stratagene (La Jolla, CA), and TriLink BioTechnologies (San Diego, CA) in the United States and Eurogentec (Herstal, Belgium), Oswel (Southampton, UK), and TIB Molbiol (Berlin, Germany) in Europe.

### 2.2. Media for M. tuberculosis

1. Middlebrook 7H9 liquid medium (Difco, Detroit, MI) supplemented with 10% bovine serum albumin, dextrose, and sodium chloride (ADC), 0.2% glycerol, and 0.05% Tween-80.
2. Middlebrook 7H10 solid medium (Difco) supplemented with 10% ADC, 0.2% glycerol, and 0.05% Tween-80.

### 2.3. Cell Cultures

1. RPMI 1640 medium (Life Technologies) with 2 m*M* L-glutamine adjusted to 1.5 g/L sodium bicarbonate (Life Technologies), 4.5 g/L glucose (Life Technologies), 10 m*M* HEPES (Life Technologies), and 1.0 m*M* sodium pyruvate (Life Technologies), supplemented with $5 \times 10^{-5}$ *M* 2-mercaptoethanol (Life Technologies) and fetal bovine serum, 10% (HyClone, Logan, UT).
2. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) (*see* **Note 1**).
3. Phosphate-buffered saline, pH 7.2 (PBS) (Life Technologies).
4. Tissue culture flasks (Becton Dickinson, Lincon Park, NJ).
5. 24-well plates (Becton Dickinson).
6. Trypan Blue solution (0.4%) (Sigma-Aldrich).
7. Inverted microscope.
8. $CO_2$ incubator.

### 2.4. Extraction of Nucleic Acids

1. TRI Reagent (Molecular Research Center, Cincinnati, OH).
2. 1-Bromo-3-chloropropane (BCP) (Molecular Research Center).
3. Polyacryl Carrier (Molecular Research Center).
4. TRI reagent/carrier solution: TRI reagent + 1/100 vol polyacryl carrier.
5. 0.1-mm sterile zirconia/silica beads (Biospec Products, Bartlesville, OK).
6. 2-mL screw cap microcentrifuge tubes with O-rings (Fisher Scientific, Pittsburgh, PA).
7. 0.5% w/v sodium dodecyl sulfate (SDS)
8. BeadBeater (Biospec Products).
9. Isopropanol.
10. 75% ethanol.
11. 0.1 *M* sodium citrate in 10% ethanol.
12. 10 m*M* Tris-HCl, pH 7.5.
13. Diethylpyrocarbonate (DEPC)-treated $H_2O$.

### 2.5. Reverse Transcription

1. Gene-specific reverse primers.
2. AMV reverse transcriptase (USB, Cleveland, OH) (*see* **Note 2**).
3. 5X AMV RT buffer (provided with the enzyme).
4. dNTP solution (1.0 m*M* each) (Promega, Madison, WI).

### 2.6. PCR with Molecular Beacons

1. Applied Biosystem 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer, Norwalk, CT) (*see* **Note 3**).
2. Ampli*Taq* Gold DNA Polymerase (Perkin-Elmer) (*see* **Note 4**).
3. TaqMan Buffer A without $MgCl_2$ (provided with the enzyme) (*see* **Note 5**).
4. 25 m*M* $MgCl_2$ (provided with the enzyme).
5. Optical micro tubes (Perkin-Elmer).
6. dNTP solution (2.5 m*M* each) (Promega).
7. Primers:
   *sigA*: 5'-GAGATCGGCCAGGTCTACGGCGTG-3'
   5'-CTGACATGGGGGCCCGCTACGTTG-3' ***(4)***
   IS*6110*: 5'-CTAACCGGCTGTGGGTAG-3'
   5'-GTCTTTCAGGTCGAGTAC-3' ***(11)***
   16S rRNA: 5'-GAGATACTCGAGTGGCGAAC-3'
   5'-GGCCGGCTACCCGTCGTC-3' ***(11)***
8. Molecular beacons (arm sequences are underlined):
   *sigA*: FAM-5'-<u>GCGAG</u>AGTTGCGCCATCCGA<u>CTCGC</u>-3'-DABCYL ***(4)***
   IS*6110*: FAM-5'-<u>GCACC</u>GAGGTGGCCATCGTGGAAGCG<u>GGTGC</u>-3'-DABCYL ***(11)***
   16S rRNA: TET–5'-<u>CGAGC</u>ATAGGACCACGGGATGCA<u>GCTCG</u>-3'-DABCYL

## 3. Methods

### 3.1. How to Design a Molecular Beacon

In order to detect synthesis of products during PCR, molecular beacons should be designed so that they are able to hybridize to their targets at annealing temperatures used for PCR, whereas in the absence of the target, the molecular beacons remain closed and nonfluorescent. This can be ensured by choosing the length of the probe and arm sequences appropriately. In order to discriminate amplicons that differ from each other only by a nucleotide, the length of the probe sequence should be such that it would dissociate from its perfectly complementary target at temperatures 7–10°C higher than the annealing temperature of the PCR. The melting temperature of the probe target-hybrid can be predicted with the percent GC rule (*see* **Note 6**) using the probe sequence without the stem. In practice, lengths of the probe sequences usually range from 15–30 nucleotides.

After selecting a probe sequence, two complementary arm sequences should be added on either side of the probe sequence. In order to ensure that the molecular beacons remain closed in the absence of the target, the length, sequence, and GC content of the stem should be chosen such that the melting temperature of the stem is 7–10°C higher than the detection temperature (annealing temperature). The melting temperature of the stem can not be predicted by the

percent GC rule since the stem forms by an intramolecular hybridization event. Instead, a DNA folding program (such as Zuker folding program available on the Internet, at http://www.ibc.wustl.edu/~zuker/) should be utilized to estimate the free energy of formation of the stem hybrid, from which its melting temperature can be predicted. Usually the stems are 5–7 nucleotides long. In general, GC-rich stems of 5, 6, and 7 bp will melt at between 55°C and 60°C, 60°C and 65°C, and 65°C and 70°C, respectively.

It is important that the conformation of free molecular beacons is the intended hairpin, rather than other structures that do not place the fluorophore in the immediate vicinity of the quencher or form extremely long stems. The former will cause high background signals and the latter will slow the molecular beacon denaturation and reduce its ability to bind its target. A folding of the selected sequence by the Zuker DNA folding program will reveal such problems. If the alternative structures are a result of the choice of the stem sequence, the stem sequence can be altered, whereas if the alternative structures arise from the probe sequence itself, the frame of the probe can be moved along the target to obtain probe sequences that are not self-complementary.

As with PCR primers in general, the sequence of the molecular beacon should be checked against those of the primers to make sure there are no areas of complementarity that may cause the molecular beacon to bind to primers and increase background. The primers used in PCR experiments with molecular beacons should be designed to produce a relatively short amplicon, in general less than 150 bp. The rationale of choosing a short amplicon is that the molecular beacon is an internal probe which must compete with the opposite strand of the amplicon for binding to its complementary target. Having a shorter amplicon allows the molecular beacon to compete more efficiently for binding to its target and, therefore, produces better results during molecular beacon PCR experiments.

### 3.2. Characterization of Molecular Beacons

Following the design and synthesis, the melting characteriztics of the molecular beacon in PCR buffer conditions should be verified experimentally to ensure that the molecular beacon is functioning as expected. This also allows determination of the appropriate annealing temperature for subsequent PCR experiments.

1. Prepare three tubes containing 200 n*M* molecular beacon, 4 m*M* $MgCl_2$, and 10 m*M* Tris-HCl, pH 8.0, in a volume of 50 μL.
2. Add an oligonucleotide perfectly complementary to the molecular beacon (without the self-complementary arms) to one of the tubes and an oligonucleotide differing from the first one in only one position to another tube, at a final concentration of 400 n*M*.

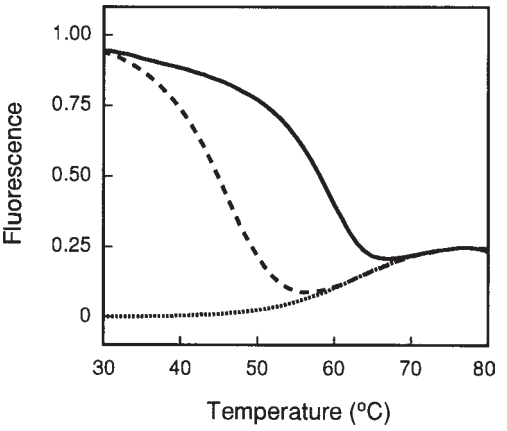


Fig. 3. Thermal denaturation profiles of a molecular beacon (dotted line), of its hybrid with a perfectly complementary target (continuous line) and of its hybrid with a mismatched target (dashed line).

3. Determine the fluorescence of each solution as a function of temperature using the spectrofluorometric thermal cycler. Decrease the temperature of the tubes from 80°C to 30°C in 1°C steps, with each step lasting 1 min, while monitoring the fluorescence during each step.

**Figure 3** illustrates a typical molecular beacon melting profile. The dotted line represents the fluorescence obtained with the molecular beacon alone, the continuous line represents the fluorescence of its hybrid with a perfectly complementary target, and the dashed line represents the fluorescence of its hybrid with a target that is mismatched by a single nucleotide. An annealing temperature should be chosen at which the molecular beacon will bind efficiently to its perfectly complementary target but not to the mismatched target, and at which the molecular beacon will adopt a stem-loop conformation if the target is not present. Therefore, in the example illustrated in **Fig. 3** the annealing temperature should be 55°C (the center of the window of discrimination).

### 3.3. Detection of Point Mutations

1. Design a FAM-coupled molecular beacon homologous to the region that is subjected to search for point mutations. It must be able to discriminate between the wild-type sequence and one with a single mismatch (*see* **Subheading 3.2.** and **Note 7**).
2. Subject the sample to a multiplex PCR reaction containing the primers-molecular beacon set specific for 16S rRNA (whose molecular beacon is coupled with TET),

and the primers-molecular beacon set specific for the target sequence subjected to search for point mutations (whose molecular beacon is coupled with FAM). Also perform a PCR reaction with the primers-molecular beacon set for the insertion sequence IS*6110*.

3. Set up each PCR reaction in final volume of 25 μL to contain 1X TaqMan Buffer A, 4 m*M* $MgCl_2$, 0.25 m*M* each dNTP, 1.75 U Ampli*Taq* Gold polymerase, 0.5 μ*M* of each primer, 0.3 μ*M* of the appropriate molecular beacon, and 1.75 μL of template (1–5 ng of chromosomal DNA).
4. Perform the reactions in the Applied Biosystem 7700 Prism spectrofluorometric thermal cycler (which is able to differentiate between fluorescence emitted from the two different fluorophores). After 10 min at 94°C to activate the enzyme, perform 40 cycles with the following conditions: 94°C denaturation for 30 s; annealing for 1 min at a suitable temperature for the particular primers-beacon sets (*see* **Subheading 3.2.**); 72°C extension for 30 s. Measure fluorescence during the annealing steps.
5. If the wild-type sequence is present, fluorescence from both FAM (molecular beacon specific for the wild-type sequence) and TET (molecular beacon for 16S rRNA) should be detected. If a mutation is present in the region of interest, only fluorescence from TET will be detected. If no fluorescence from TET is detected, the analysis will be considered to be indeterminate. Samples in which no fluorescence will be detected in the reaction with the primers-molecular beacon set for the insertion sequence IS*6110* will be considered as negative for the presence of *M. tuberculosis* DNA.

### 3.4. Infection of Macrophages

1. Resuspend $6 \times 10^7$ THP-1 cells in 80 mL of warm RPMI with 50 n*M* PMA—final cell concentration $7.5 \times 10^5$/mL (*see* **Note 8**).
2. Seed three 24-well tissue culture plates with 1 mL/well.
3. Incubate at 37°C for 24 h.
4. Dilute a sample of *M. tuberculosis* H37Rv growing exponentially in warm RPMI and another sample in warm 7H9 up to a concentration of $3.7 \times 10^5$ cfu/mL.
5. Replace the media of 55 wells with the RPMI inoculated with mycobacteria (infected macrophages). Replace the media of the other wells with uninoculated RPMI (uninfected control macrophages).
6. Simultaneously seed 7 wells of a 24-well tissue culture plate not containing macrophages with 1 mL of inoculated RPMI, and 7 wells with 1 mL of inoculated 7H9.
7. Incubate the plates at 37°C for 1h.
8. Remove the media from all wells containing macrophages, wash them twice with warm PBS, and add fresh media.
9. At the same time transfer the inoculated media from the plate not containing macrophages (**step 6**) to 1.5 mL microcentrifuge tubes, chill on ice for 3 min and centrifuge at 5000*g* for 5 min at 4°C. Remove the supernatant, resuspend each pellet in 300 μL of TRI Reagent/+ 1/100 vol of polyacryl carrier, transfer to a

2-mL screw cap micro centrifuge tube with O-rings and freeze on dry ice. Store at –70°C (*see* **Note 9**).

10. At each time point (1; 6; 24, 48,72 h):
    a. Remove media from two wells containing infected macrophages, lyse the cells by adding 1 mL 0.05% SDS and plate the bacteria at appropriate dilutions on 7H10 plates for cfu determination.
    b. Remove media from two wells, one containing infected, the other uninfected macrophages and stain with 20 μL of Trypan-Blue for analysis of macrophage viability (*see* **Note 10**).
    c. Treating one well at a time, remove the media from 7 wells containing infected macrophages, lyse the cells of each well by adding 300 μL of cold TRI Reagent/carrier, transfer the lysate immediately to a 2-mL screw cap microcentrifuge tube with O-rings and freeze on dry ice (*see* **Note 11**).
    d. Store at –70°C.

### 3.5. RNA Extraction

1. Add 150 μL of zirconia/silica beads to the frozen lysate (from **Subheading 3.4.**).
2. Place the tubes with the frozen samples in the BeadBeater and subject to 2 × 1 min pulses with a 2 min rest on ice.
3. Transfer the sample to a clean tube.
4. Add 100 μL of TRI reagent/carrier to the original tube to wash the beads and add it to the previously transferred sample.
5. Incubate the sample at room temperature for 10 min.
6. Spin for 10 min at 12,000*g* at 4° C.
7. Transfer the supernatant to a clean tube (save the pellet for DNA extraction).
8. Add 30 μL of BCP and shake vigorously for 15 s (do not vortex).
9. Incubate 10 min at room temperature and then spin at 12,000*g* for 15 min at 4°C.
10. Transfer the upper phase to a new tube (save the lower phase for DNA extraction) (*see* **Note 12**).
11. Add 150 μL of isopropanol.
12. Incubate 5 min at room temperature and then spin at 12,000*g* for 8 min at 4°C.
13. Remove supernatant and add 300 μL of 75% ethanol (*see* **Note 13**).
14. Mix and then spin at 7500*g* for 5 min at 4°C.
15. Remove the supernatant and air dry the pellet 3–5 min.
16. Dissolve in 30 μL DEPC-treated $H_2O$.
17. Add 300 μL of TRI reagent/carrier and repeat from **steps 8**–**15**.
18. Dissolve in 12 μL DEPC-treated $H_2O$.
19. Store at –70°C.

### 3.6. DNA Extraction

1. Resuspend the pellet from **step 7** of **Subheading 3.5.** with the lower phase collected during **step 10** of **Subheading 3.5.**
2. Add 200 μL 95% ethanol, mix by inverting and incubate for 5 min at room temperature to allow the DNA to precipitate.

3. Spin at 2000*g* for 5 min at 4°C.
4. Remove supernatant and wash the pellet twice with 300 mL of 0.1 *M* sodium citrate in 10% ethanol. For each wash, incubate the pellet with the solution for 5 min at room temperature and spin at 3,000*g* for 5 min at 4°C (*see* **Note 14**).
5. Wash the DNA pellet with 500 μL of 75% ethanol, and spin at 2000*g* for 5 min at 4°C.
6. Remove the supernatant and air dry the pellet 5 min at room temperature.
7. Dissolve the pellet in 100 μL of 10 m*M* Tris (pH 7.5) (*see* **Note 15**).
8. Remove cell debris by centrifuging at 12,000*g* for 10 min.

### 3.7. Reverse Transcription (see Note 16)

1. In a PCR tube add 2.5 pmol of each antisense primer (up to four different primers plus the primer for *sigA*), 2 μL of 5X AMV RT buffer and 2 μL of RNA sample, in a final volume of 10 μL (*see* **Note 17**).
2. Denature in a thermocycler at 95°C for 1 min 30 s. Carry out the annealing between the RNA and the antisense primers for 3 min at 65°C followed by 3 min at 57°C (*see* **Note 18**). Place the tube on ice.
3. Add 3.5 μL of annealing mixture to 1.1 μL of dNTP solution (1 m*M* each), 0.4 mL of 5X AMV RT buffer and 8 U of AMV in a final volume of 5.5 μL (*see* **Note 19**).
4. Incubate 30 min at 47°C (*see* **Note 20**), inactivate the enzyme for 1 min at 95°C and place the tube on ice.
5. Dilute with 70 μL of $H_2O$ (*see* **Note 21**).
6. Prepare identical samples not treated with AMV as a control to monitor the amount of DNA carryover during RNA purification (mock reverse transcription).

### 3.8. Quantitative PCR with Molecular Beacons (see Note 22)

1. Each reaction (25 μL) consists of 1X TaqMan Buffer A, 4 m*M* $MgCl_2$, 0.25 m*M* each dNTP, 1.75 U Ampli*Taq* Gold polymerase, 0.5 μ*M* of each primer, 0.3 μ*M* of the appropriate molecular beacon, and 1.75 μL of template, which could be cDNA (when measuring gene expression) or DNA (when measuring the growth of the intracellular bacteria).
2. Perform the reactions in the Applied Biosystem 7700 Prism spectrofluorometric thermal cycler. After 10 min at 94°C to activate the enzyme, perform the following two sets of cycles. First set (15 cycles): touchdown with the annealing temperature decreasing 0.5°C per cycle starting from 65°C. 94°C denaturation; 72°C extension (all steps 30 s long). Second set (25 cycles): 94°C denaturation for 30 s; 57°C annealing for 1 min; 72°C extension for 30 s. Measure the fluorescence during the annealing steps of the second sets of cycles (*see* **Note 23**).
3. In order to obtain a standard curve for each primers-beacon set, run four different PCR reactions in parallel with the uncharacterized samples, using as template 10-fold dilutions of known amounts of H37Rv chromosomal DNA ($10^2$, $10^3$, $10^4$, and $10^5$ copies).

### 3.9. Data Analysis

1. Plot the threshold cycles obtained from amplification of the DNA standard as a function of the logarithm of the number of target molecules. This curve is described by the equation $n = a + b \log(x)$, where n is the number of target copies, $x$ is the threshold cycle obtained amplifying $n$ target copies, $a$ is the intercept and $b$ is the slope of the resulting curve. Solve the equation in order to calculate the constants $a$ and $b$ specific of each primers-beacon set (*see* **Note 24**).
2. Using the constants calculated from above, solve the equation for the number of target copies present in the uncharacterized samples.
3. When the uncharacterized sample consists of cDNA, subtract the values obtained amplifying the mock reverse transcriptions from those obtained amplifying the reverse transcription (*see* **Note 25**).
4. For each gene analyzed, calculate the ratio between the amount of cDNA in the samples from intracellular mycobacteriathat in the samples from extracellular mycobacteria, normalizing for the amount of *sigA* cDNA: (*geneX* $\text{cDNA}^{\text{intracellular}}$/*sigA* $\text{cDNA}^{\text{intracellular}}$)/(*geneX* $\text{cDNA}^{\text{extracellular}}$/*sigA* $\text{cDNA}^{\text{extracellular}}$). This value represents the ratio of the amount of mRNA of a given gene found in intracellular bacteria to that found in extracellular bacteria, normalized to *sigA* mRNA.

## 4. Notes

1. Resuspend PMA in $H_2O$ at a concentration of 1.5 m*M* and keep frozen at –70°C in small aliquots.
2. We have been using AMV reverse transcriptase, since it works well at 47°C, which is desirable when dealing with nucleic acids with a high GC content. However a number of companies have recently advertised new recombinant reverse transcriptases designed for quantitative RT-PCR which, according to the producer, are highly efficient with GC-rich templates (Omniscript RT, Quiagen, Hilden, Germany; ThermoScript, Life Technologies).
3. Two other instruments that can perform PCR and monitor fluorescence have become available: Icycler (Biorad, Hercules, CA) and Lightcycler (Roche Diagnostic, Nutley, NJ)
4. Ampli*Taq* Gold DNA polymerase is completely inactive until it is activated at 94°C. This is very useful because it minimizes false priming without requiring a hot start.
5. This buffer contains the dye 6-carboxy-X-rhodamine (6-rox). The fluorescence of this dye is used by the spectrophotometric thermal cycler as a normalization reference.
6. $T_m$ (in degrees Celsius) = 81.5 + 16.6 × log $[Na^+]$ + 0.41 × (%GC) – 675/length. For the calculation set the $Na^+$ concentration to 1 *M*.
7. When the sequence of the mutant allele is not known, or as in the case of *rpoB* in *M. tuberculosis*, many different mutations can result in drug resistance, the most reliable method consists of performing two different PCR reactions in the same tube. The first PCR product will be the DNA fragment being studied, whose

molecular beacon will be coupled to FAM and will be specific for the wild-type sequence. The second PCR product will be a fragment of the gene encoding 16S rRNA; the molecular beacon specific for it will be coupled to TET and it will represent the positive control for the amplification.

When the sequence of all the different alleles to discriminate is known, it is possible to perform a single PCR reaction amplifying the fragment containing the region which includes the differences among the various alleles. Molecular beacons specific for each allele, coupled with different fluorophores, will be included in the PCR reaction. The color of the fluorescence resulting from the hybridization of the molecular beacons to their targets will reveal which allele is present in the sample ***(15)***. This technique, using rRNA as target sequence, could be extremely valuable in tests for identification of pathogenic mycobacteria in clinical specimens.

8. THP-1 is a human monocytic cell line. THP-1 cells grow in a nonadherent fashion until induced with PMA; at this point they differentiate into macrophages, stop cell division and adhere to tissue culture flasks ***(16)***. It is very important that THP-1 cells not be passaged continuously. In our experience cell cultures passaged less than 45 d differentiate stably after 24 h induction with 50 n*M* PMA and can be maintained after infection with *M. tuberculosis* for at least another week. During this time, intracellular bacteria double almost once a day. It is important to avoid high concentrations of PMA: we noticed that cells differentiated for longer than 24 h or with concentrations of PMA higher than 50 n*M* die when infected with *M. tuberculosis*. If cells are subcultured for more than 45 d, higher concentrations of PMA or longer times of induction are needed in order to obtain stable differentiation and often cells become nonadherent after several days of incubation.
9. The RNA from bacteria incubated in 7H9 represents the standard for calculation of differential gene expression during growth in macrophages. The RNA from bacteria incubated in RPMI will be used to determine if exposure to RPMI prior to uptake by macrophages alters the expression of the studied genes.
10. Do not allow the macrophages to dry before adding the dye, and observe them under the microscope immediately. If the cells are allowed to dry, they will die quickly. Dead cells stain blue, live cells remain colorless. Usually about 95% of the macrophages remain viable until the end of the experiment and little, if any, difference can be noted between viability of infected and uninfected cells.
11. Treating one well at a time reduces the time required between collection and freezing of the sample. This is important since bacterial RNA has a very short half-life and could be degraded during the manipulation of the sample. TRI Reagent solubilizes most bacteria and eucaryotic tissues almost immediately and is designed to stabilize the RNA, but it does not lyse mycobacteria.
12. It is extremely important to avoid touching the interface with the pipet-tip, since this contains the DNA.
13. Usually the pellet is almost invisible after the isopropanol precipitation, since it is very transparent. However, after the wash in 75% ethanol, it will be more visible.

14. Be careful when removing the supernatant, since the pellet might be very loose.
15. It is very important to use Tris buffer to resuspend this pellet, since it is almost insoluble in $H_2O$.
16. Reverse transcription of all the studied genes and the control (*sigA*) is carried out in the same tube. This is necessary in order to correct for tube to tube variability in reverse transcription as well as for differences in the amounts of total RNA in the different samples.
17. Prepare one annealing reaction for each of the 7 RNA samples (5 intracellular time points and two extracellular controls). To minimize sample to sample variability prepare a master-mix with all the antisense primers and aliquot it into 7 tubes before adding the templates.
18. For a higher degree of reproducibility perform annealing in a thermal cycler. Optimal annealing temperature should be determined empirically for each primer, but the use of many primers at the same time makes this impractical. We designed all our primers so that 57°C was a suitable annealing temperature.
19. Prepare a master-mix with all the reagents, aliquot it into 7 tubes, and then add the templates. Be very careful to mix the master-mix well after adding the enzyme, since the presence of glycerol makes the solution particularly difficult to disperse evenly.
20. Since slight variations in the temperature could affect the efficiency of the reverse transcriptase it is advisable to perform the incubation in a thermal cycler.
21. It is necessary to dilute the reverse transcriptase reaction, since it can inhibit the PCR reaction.
22. Due to the extreme sensitivity of PCR it is very important to avoid any kind of external DNA contamination which can invalidate the results. The use of molecular beacons decreases the risk of template contamination since there is no need to run the PCR product on an agarose gel (the most frequent source of contamination). Nonetheless, it is preferable to perform all the manipulations in a controlled environment (preferably a laminar flow hood) which can be UV-irradiated. It is also important to have a set of pipetors dedicated solely to PCR and to use aerosol barrier tips when pipeting samples containing nucleic acids in order to avoid contamination of the pipettors.
23. The use of a touchdown reduces the probability for nonspecific PCR products, which could compete with the specific amplicon and thus modify the kinetics of the reaction. Although an optimal annealing temperature should be determined for each primers-beacon set, the use of many different sets at the same time makes this impractical. We designed all our primers and beacons so that 57°C would be a suitable annealing temperature.
24. The values of the constants can be easily calculated using most graphic software packages such as DeltaGraph (DeltaPoint, Monterey, Ca) or KaleidaGraph (Abelbeck Software, Reading, PA).
25. Usually, the amount of DNA that copurifies with the RNA is not significant, thus making the subtraction superfluous. Nonetheless, it is advisable to perform the control to check the performance of the reverse transcriptase and because in the

case of genes expressed at very low level, levels of DNA contamination may become significant.

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