

Standard Operating Procedure for Identification of Bovine Tuberculosis Pathogen by Real-Time Fluorescent Quantitative PCR

1 References

Real-time Fluorescent PCR Detection Method for Tuberculosis Pathogens
*GB/T27639*2011

2 Equipment and Materials

2.1 Tissue grinding consumables, PBS, ddH₂ O, nucleic acid extraction kit, fluorescent nucleic acid amplification kit, primers and probes (see Table 1).

2.2 Water bath, tissue homogenizer, real-time fluorescent quantitative PCR instrument, pipettors, centrifuge, biosafety cabinet.

Table 1 Primers and Probes for Real-Time Fluorescent Quantitative PCR

Category	Primer Name	Sequence (5'-3')
Mycobacterium tuberculosis complex	IS6110-F	GCA GGG TTC GCC TAC GTG
	IS6110-R	CGG GTC CAG ATG GCT TGC
	IS6110-P	FAM-TGT CAC CGA CGC CTA CGC TCG C-BHQ1
Mycobacterium tuberculosis complex	IS1081-F	CCA GGA ACG CCT CAA CC
	IS1081-R	CGA CGA GGC GGA TGA TC
	IS1081-P	FAM-AGA GGT ACG ACG CCG AAC CGA C-BHQ1

Mycobacterium tuberculosis	MTB-F	CGG TGT AAT CAG TTT TGA AGC
	MTB-R	CGA TTG GAA CGG CGA AGC
	MTB-P	FAM-TAG GTA GTC CAG TAG AGC CCC ATA GCC A-BHQ1
Mycobacterium bovis	MB-F	ACG CCT TCC TAA CCA GAA TTG
	MB-R	GGC TAT TGA CCA GCT AAG ATA TCC
	MB-P	FAM-AAT TCA TAC AAG CCG TAG TCG TGC AGA A-BHQ1

3 Operating Procedures

3.1 Sample Treatment

3.1.1 Tissue Samples

Take a tissue block of approximately 1 cm³, cut it into small pieces using sterilized scissors and forceps, transfer to a 1.5 mL tissue grinding tube containing 1 mL PBS. Homogenize for 5 min, perform brief centrifugation. Transfer the supernatant and inactivate in a water-bath at 80 °C for 1 h, then proceed to nucleic acid extraction.

3.1.2 Milk Samples

Transfer 200 µL milk sample into a 1.5 mL centrifuge tube containing 1 mL PBS. Inactivate in an 80 °C water-bath for 1 h. Centrifuge at 12 000 g for 5 min. Discard supernatant and lipid layer, then proceed to nucleic acid extraction.

3.1.3 Bacterial Culture

Scrape a small amount (1-2 loopfuls) of mycobacteria from solid medium and transfer into a 1.5 mL screw-cap centrifuge tube with 100 µL PBS. Mix well, inactivate in an 80 °C water-bath for 1 h, then proceed to nucleic acid extraction.

Note: Culture handling shall be performed in a BSL-3 laboratory.

3.2 Nucleic Acid Extraction from Samples

3.2.1 Prepare n sterilized 1.5 mL centrifuge tubes, where n equals the total number of test samples, positive controls and negative controls. Label each tube clearly.

3.2.2 Commercial Kit Extraction Method

Extract sample DNA using a commercial nucleic acid extraction kit. Finally elute DNA with 100 μ L sterile double-distilled water to obtain DNA template. Templates may be stored at 4 °C for short-term use; for long-term preservation store at -80 °C.

3.3 Real-Time Fluorescent Quantitative PCR Reaction System

Table 2 RT-PCR Reaction System

Reagent	Volume (μ L)
2×Taq PCR Master Mix	10.0
Forward Primer 10 μ mol/L	0.8
Reverse Primer 10 μ mol/L	0.8
Probe 10 μ mol/L	0.4
ddH ₂ O	6.0
DNA Template	2.0
Total Volume	20.0

Note: n = total count of test samples, positive controls and negative controls. Prepare $n+1$ reaction mixes. Mix thoroughly and perform brief centrifugation to collect all reaction components at the bottom of tubes.

3.4 Real-Time Fluorescent Quantitative PCR Controls

Two controls shall be set up:

Positive control: DNA of reference standard strain.

Negative control: DNA-free ddH₂O.

3.5 Thermal Cycling Programme

After sample loading, place the reaction plate in the real-time fluorescent quantitative PCR instrument. Reaction conditions are as follows:

95 °C for 15 min; followed by 45 amplification cycles (95 °C for 15 s, 60 °C for 1 min). Fluorescence signal is collected at the annealing-extension step of each cycle.

3.6 Analysis of Real-Time Fluorescent Quantitative PCR Results

Analyse amplification results with the software bundled with the real-time PCR instrument. Synthesize all output parameters. Use instrument default value as reference for baseline setting. Threshold shall be set just above the highest point of amplification curve of valid negative controls, and adjusted according to instrument background noise. Analyse signals from the fluorescence channel corresponding to the labelled reporter dye.

4 Result Interpretation

4.1 Test Validity

A test is valid only if: no Ct value and no amplification curve (or no obvious logarithmic growth phase) for negative control; positive control yields $Ct \leq 35$ with typical amplification curve. Otherwise the test is invalid.

4.2 Under valid test conditions: if the test sample shows $Ct \leq 37$ with typical amplification curve, the real-time PCR result is positive.

4.3 For samples with $37 < Ct < 40$, repeat the test with newly prepared sample. If repeated test gives $Ct < 40$ and obvious logarithmic growth phase on amplification curve, interpret as positive; otherwise interpret as negative.

4.4 If no Ct value and no amplification curve are observed for the test sample, the real-time PCR result is negative.