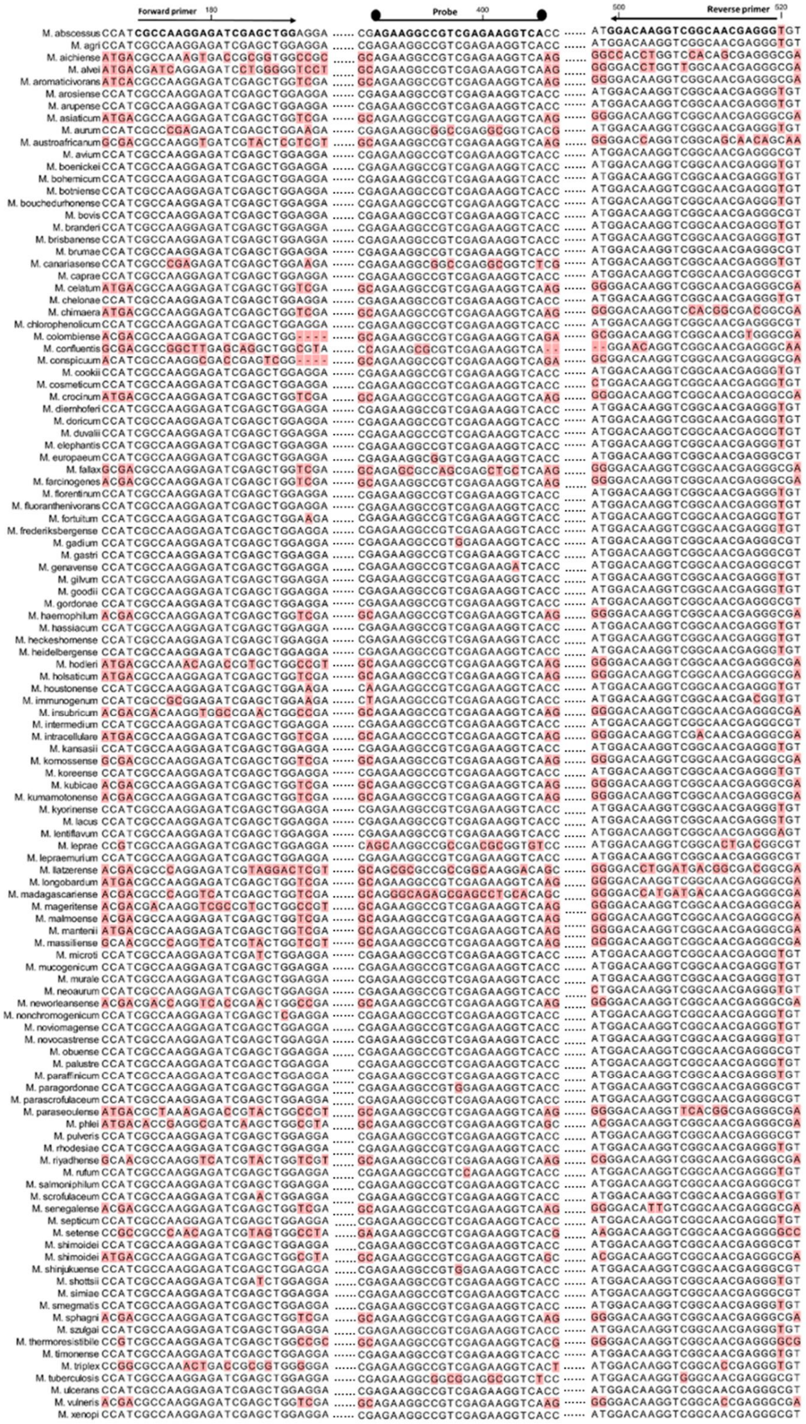
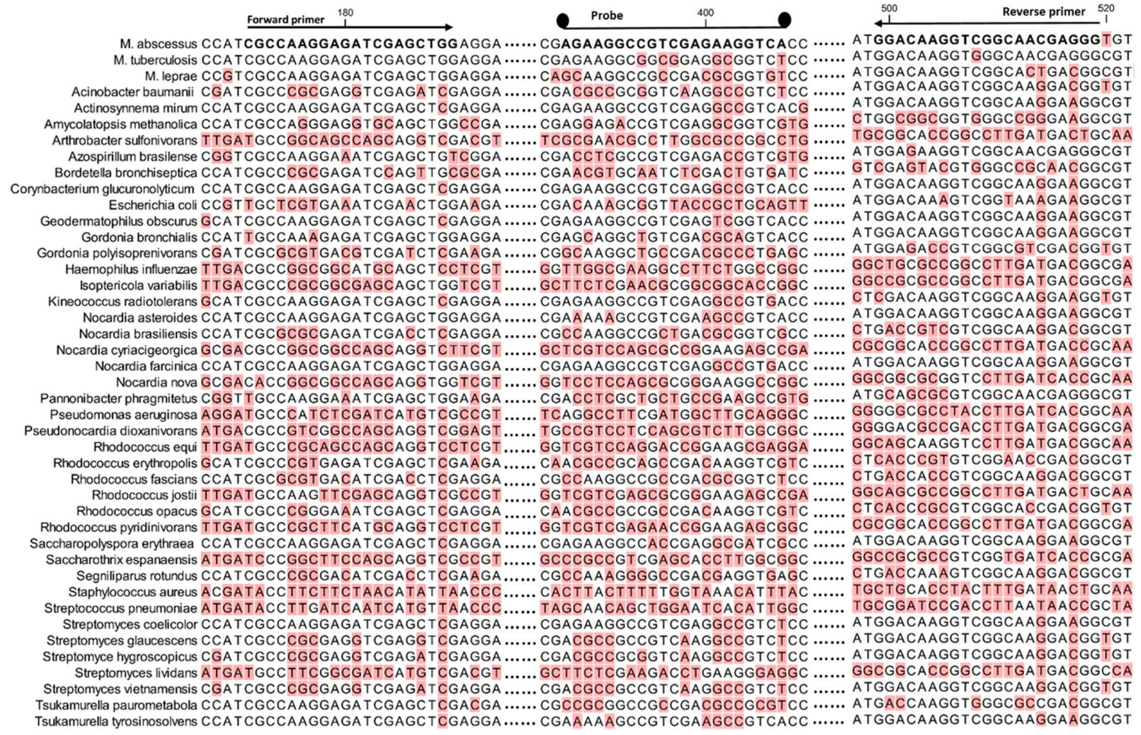
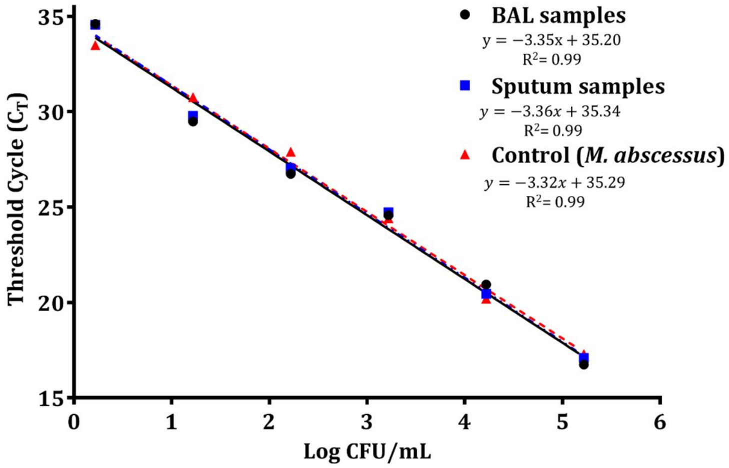


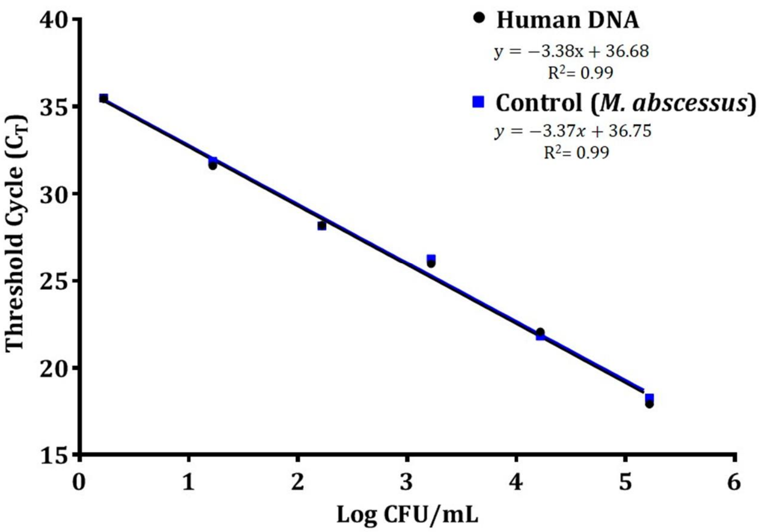
Supplementary Figure 1: Sequence alignment of the forward primer, reverse primer and probe binding regions for 107 representative mycobacterial species. Red shading indicates nucleotide mismatches.



Supplementary Figure 2: Sequence alignment of the forward primer, reverse primer and probe to the *hsp65* gene region for 40 non-mycobacterial species, *M. abscessus*, *M. tuberculosis* and *M. leprae*. Red shading indicates nucleotide mismatches.



Supplementary Figure 3: Relationship between threshold C_T values and log CFU/mL in reactions spiked with DNA extracts from culture-negative BAL or sputum. C_T values are based on three independent replicates.



Supplementary Figure 4. Relationship between threshold C_T values and log CFU/mL in reactions spiked with purified human DNA. C_T values are based on three independent replicates.

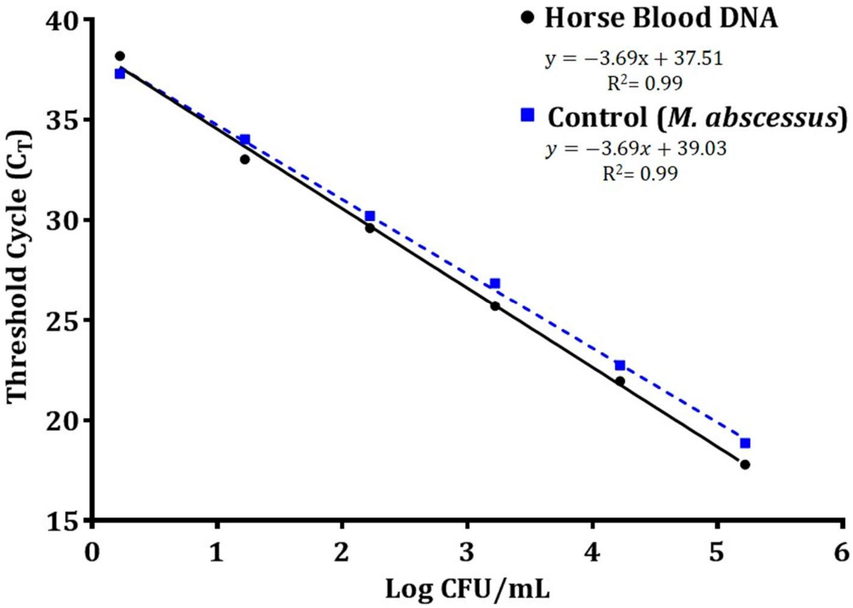


Figure 5: Relationship between threshold C_T values and log CFU/mL in reactions spiked with horse blood. C_T values are based on three independent replicates.

Supplementary Table 1: The 75 mycobacterial species with complete sequence homology at primer and probe binding sites

***Mycobacterium* species**	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. abscessus	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. agri	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. aromaticivorans	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. arosiense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. arupense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. asiaticum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. avium	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. boenickei	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. bohemicum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. botniense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. bouchedurhonense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. bovis	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. branderi	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. brisbanense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. brumae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. caprae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60

Supplementary Table 1, continued

Mycobacterium Species	Forward sequence 5'-3'	Annealing temp (°C)	Probe sequence 5'-3'	Annealing temp (°C)	Reverse sequence 5'-3'	Annealing temp (°C)
M. celatum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. chelonae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. chlorophenolicum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. colombiense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. cookii	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. cosmeticum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. crocinum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. diernhoferi	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. doricum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. duvalii	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. elephantis	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. farcinogenes	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. florentinum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. fluoranthenivorans	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. fortuitum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. frederiksbergense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. gastri	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60

Supplementary Table 1, continued

Mycobacterium Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. genavense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. gilvum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. goodii	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. gordonae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. haemophilum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. hassiacum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. heckeshornense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. heidelbergense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. holsaticum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. houstonense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. interjectum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. intermedium	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. kansasii	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. komossense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. koreense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. kubicae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. kumamotonense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60

Supplementary Table 1, continued

Mycobacterium Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. kyorinense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. lacus	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. lentiflavum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. lepraemurium	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. longobardum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. malmoense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. mantenii	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. mucogenicum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. murale	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. neoaurum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. noviomagense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. novocastrense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. obuense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. palustre	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. paraffinicum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. parascrofulaceum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60

Supplementary Table 1, continued

Mycobacterium Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. pulveris	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. salmoniphilum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. septicum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. shimoidei	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. simiae	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. smegmatis	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. sphagni	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. szulgai	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. thermoresistibile	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. timonense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. ulcerans	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60

Supplementary Table 2: In silico evaluation of 14 mycobacterial species that have < 100% primer sequence homology, but which are amplifiable at annealing temperatures Ó55.5°C. Mismatched bases, as compared to the *M. abscessus* reference sequence, are highlighted in red.

Mycobacterium Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. vulneris	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGTTCCAGCCGTGGCTCCC	59.9
M. intracellulare	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGTTCCAGCTGTTGCTCCC	59.2
M. riyadhense	CGCCAAGGTCATCGTACTGG	58.8	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. rhodesiae	CGCCAAGGAGATCGAGCTGG	58	TCTTCCGGCAGCTCTTCTAGT	58.0	GGACAAGGTCGGCAACGAGGG	60
M. nonchromogenicum	CGCCAAGGAGATCGAGCTCG	57.4	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. conspicuum	CGCCAAGGAGATCGAACTGG	57.2	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. shottsii	CGCCAAGGAGATCGAACTGG	57.2	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. microti	CGCCAAGGAGATCGAACTGG	57.2	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. rufum	CGCCAAGGAGATCGAGCTGG	58	TCTTCCGGCAGGTCTTCCAGT	57.0	GGACAAGGTCGGCAACGAGGG	60
M. scrofulaceum	CGCCAAGGAGATCGAACTGG	58.8	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. senegalense	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGTAACAGCCGTTGCTCCC	56.5
M. paragordonae	CGCCAAGGAGATCGAGCTGG	58	TCTTCCGGCACCTCTTCCAGT	55.5	GGACAAGGTCGGCAACGAGGG	60
M. gadium	CGCCAAGGAGATCGAGCTGG	58	TCTTCCGGCACCTCTTCCAGT	55.5	CCTGTTCCACCCGTTGCTCCC	59.2
M. shinjukuense	CGCCAAGGAGATCGAGCTGG	58	TCTTCCGGCACCTCTTCCAGT	55.5	CCTGTTCCACCCGTTGCTCCC	59.2

Supplementary Table 3: Mycobacterial and non-mycobacterial species that require an annealing temperature Ò55.3°C for amplification.

Bacterial Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
*Geodermatophilus obscurus*_CP001867.1	GCGGTTCCTCTAGCTCGAGC	57.4	TCTTCCGGCAGCTCAGCCAGT	55.3	GGACAAGGTCGGCAAGGAAGG	58.7
M. immunogenum	GCGGGCCCTCTAGCTCGACC	54.9	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGTTCCAGCCGTTGCTGCC	60
M. hodleri	CGCCAAACAGACCGTGCTGG	53.9	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. europaeum	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCGGTCGAGAAGGTCA	53.2	GGACAAGGTCGGCAACGAGGG	60
M. tuberculosis H37Rv complete genome	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCGGCGGAGGCGGTCT	49.3	CCTGTTCCACCCGTTGCTCCC	59.2
*Actinosynnema mirum*_CP001630.1	GCGGTTCCTCTAGCTCGAGC	57.4	TCTTCCGGCAGCTCCGGCAGT	48.5	GGACAAGGTCGGCAAGGAAGG	50.8
Streptomyces coelicolor 24427855	GCGGTTCCTCTAGCTCGAGC	57.4	TCTTCCGGCAGCTCCGGCAGA	47.4	GGACAAGGTCGGCAAGGAAGG	50.8
M. triplex	CGCCAAACTGACCGCGGTGG	47.2	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCACCGAGGG	56.6
M. setense	CGCCCAACAGATCGTAGTGG	47.1	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. paraseoulense	CGCTAAAGAGACCGTACTGG	44.2	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTTCACGGCGAGGG	44.5
*Nocardia farcinica*_IFM_10152	CGCGCGTGACATCGACCTCG	48.1	GGTTCCGGCGGCTGCGCCAGA	42.1	GACCACCGTCGGCAAGGACGG	50.6
M. aichiense	CGCCAAAGTGACCGCGGTGG	47.2	AGAAGGCCGTCGAGAAGGTCA	62.9	CCACCTGGTCCACAGCGAGGG	41.9
M. alvei	CGATCAGGAGATCCTGGGGG	41.6	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGGACCAACCGTTGCTCCC	50.5

Supplementary Table 3, continued

Bacterial Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. fallax	CGCCAAGGAGATCGAGCTGG	58	AGAGCGCCAGCGAGCTGCTCA	41.0	GGACAAGGTCGGCAACGAGGG	60
*Nocardia farcinica*_AF352577.1	CGCGCGTGACATCGACCTCG	48.1	TCTTCCGGCAGCTCCGGCACT	40.5	GGACAAGGTCGGCAAGGAAGG	50.8
*Kineococcus radiotolerans*_CP000750.2	GCGGTTCCTCTAGCTCGAGC	57.4	TCTTCCGGCAGCTCCGGCACT	40.5	CGACAAGGTCGGCAAGGAAGG	49.7
M. canariasense	GCGGGCTCTCTAGCTCGACC	48.1	TCTTCCGCCGGCTCCGCCAGA	39.9	GGACAAGGTCGGCAACGAGGG	60
M. mageritense	CGACAAGGTCGCCGTGCTGG	39.3	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. insubricum	CGACAAGGTGGCCGAACTGG	39.3	AGAAGGCCGTCGAGAAGGTCA	60	GGACAAGGTCGGCAACGAGGG	60
*Rhodococcus opacus*_CP008947.1	GCGGGCCCTTTAGCTCGAGC	41.0	TCTTCCGGCAGCTCCGGCAGT	48.5	GTGGGCGCAGCCGTGGCTGCC	38.1
*Rhodococcus opacus*_B4 226237899	GCGGGCCCTTTAGCTCGAGC	41.0	TCTTCCGGCAGCTCCGGCAGT	48.5	GTGGGCGCAGCCGTGGCTGCC	38.1
M. leprae	CGCCAAGGAGATCGAGCTGG	58	CGTTCCGGCGGCTGCGCCACA	37.9	CCTGTTCCAGCCGTGACTGCC	50.6
*Rhodococcus pyridinivorans*_CP006996.1	ACGGGCGAAGTACGTCCAGG	37.5	AGCAGCTCTTGGCCTTCTCGC	46.4	GCCGTGGCCGGAACTACTGCC	47.8
*Nocardia asteroides*_AF352019.1	CGCCAAGGAGATCGAGCTGG	58	TTTTTCGGCAGCTTCGGCAGT	36.4	GGACAAGGTCGGCAAGGAAGG	50.8
M. chimaera	CGCCAAGGAGATCGAGCTGG	58	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGTTCCAGGTGCCGCTGCC	35.7

Supplementary Table 3, continued

Bacterial Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
M. aurum	GCGGGCTCTCTAGCTCGACC	48.1	TCTTCCGCCGGCTCCGCCAGT	35.5	GGACAAGGTCGGCAACGAGGG	60
M. massiliense	GCGGGTCCAGTAGCATGACC	35.0	AGAAGGCCGTCGAGAAGGTCA	62.9	GGACAAGGTCGGCAACGAGGG	60
M. madagascariense	CGCCCAGGTCATCGAGCTGG	50.7	AGGGCAGAGCGAGCCTGCACA	34.7	CCTGGTACTACTGTTGCTCCC	37.3
M. austroafricanum	GCGGTTCCACTAGCATGAGC	38.4	AGAAGGCCGTCGAGAAGGTCA	62.9	CCTGGTCCAGCCGTCGTTGTC	34.5
M. llatzerense	CGCCCAGGAGATCGTAGGAC	46.8	AGCGCGCCGCCGGCAAGGACA	45.0	GGACCTGGATGACGGCGACGG	34.3
M. neworleansense	CGACCAGGTCACCGAACTGG	31.5	AGAAGGCCGTCGAGAAGGTCA	60	GGACAAGGTCGGCAACGAGGG	60
Nocardia cyriacigeorgica 374843763	CGCCGGCGGCCAGCAGGTCT	46.2	AGCAGGTCGCGGCCTTCTCGG	45.0	GCCGTGGCCGGAACTACTGGC	28.0
Tsukamurella tyrosinosolvens	GCGGTTCCTCTAGCTCGAGC	57.4	TTTTTCGGCAGCTTCGGCAGT	27.7	GGACAAGGTCGGCAAGGAAGG	50.8
*Pseudonocardia dioxanivorans*_CP002593.1	GCGGCAGCCGGTCGTCCAGC	40.1	GGCAGGAGGTCGCAGAACCGC	26.9	CCTGCGGCTGGAACTACTGCC	27.5
*Azospirillum brasilense*_HE577327	GCGGTTCCTTTAGCTCGACA	58.4	TGGAGCGGCAGCTCTGGCAGCA	26.8	GGAGAAGGTCGGCAACGAGGG	59.4
*Saccharothrix espanaensis*_DSM_44229	AGGGCCGAAGGTCGTCCAGC	27.8	TCTTCCGGTGGCTCCGCTAGC	31.3	GGCGCGGCAGCCACTAGTGGC	25.9
*Gordonia bronchialis*_CP001802.1	AGCCAATGAGATCGAGCTGG	55.3	TCGTCCGACAGCTGCGTCAGT	25.0	GGACAAGGTCGGCAAGGAAGG	50.8
*Tsukamurella paurometabola*_CP001966.1	CGCCAAGGAGATCGAGCTGG	57.4	GGCGCCGGCGGCTGCGGCGCA	24.2	GACCAAGGTGGGCGCCGACGG	47.2
*Pseudomonas aeruginosa*_CP000438.1	TGCCCATCTCGATCATGTCGC	48.2	TCCGGAAGCTACCGAACGTCC	20.6	CCCGCGGATGGAACTAGTGCC	58.6

Supplementary Table 3, continued

Bacterial Species	**Forward sequence 5'-3'**	**Annealing temp (°C)**	**Probe sequence 5'-3'**	**Annealing temp (°C)**	**Reverse sequence 5'-3'**	**Annealing temp (°C)**
Saccharopolyspora erythraea	GCGGTTCCTCTAGCTCGAGC	57.4	GGCGGCAGCTCGTGGAACCGC	20	GGACAAGGTCGGCAAGGAAGG	50.8
*Pannonibacter phragmitetus*_CP013068.1	ACGGTTCCTTTAGCTCGACC	55.4	TGGAGCGACGACGGCTTCGGC	18.6	CGTCGCGCAGCCGTTGCTCCC	44.0
*Escherichia coli*_MC4100	ACGAGCACTTTAGCTTGACC	18.2	TGTTTCGCCAATGGCGACGTC	20.2	GGACAAAGTCGGTAAAGAAGG	37.0
*Nocardia brasiliensis*_ATCC_700358	GCGCGCGCTGTAGCTGGAGC	21.5	GGTTCCGGCGACTGCGCCAGC	26.8	CTGGCAGCAGCCGTTCCTGCC	16.1
*Streptomyces lividans*_TK24_CP009124.1	ACGGAAGCCGCTAGTACAGC	27.6	AAGAGCTTCTGGACTTCCCTC	15.6	GCCGTGGCCGGAACTACTGCC	47.8
Streptococcus pneumoniae 749204398	ATGGAACTAGTTAGTACAAT	27.2	CGTTGTCGACCTTAGTGTAAC	14.6	GCCTAGGCTGGAATTATTGGC	27.8
*Rhodococcus erythropolis*_CP003761.1	GCGGGCACTCTAGCTCGAGC	40.9	TGCGGCGTCGGCTGTTCCAGC	13.9	CACCCGTGTCGGAACCGACGG	15.7
*Segniliparus rotundus*_CP001958.1	GCGGGCGCTGTAGCTGGAGC	28.4	GGTTTCCCCGGCTGCTCCACT	13.4	TGGTTTCAGCCGTTCCTGCC	40.5
*Staphylococcus aureus*_NCTC_8325	ATGGAAGAAGATTGTATAAT	24.9	GAATGAAAAACCATTTGTAAA	20.8	GACGTGGATGAAACTATTGAC	12.7
*Streptomyces hygroscopicus*_CP003720.1	CGCCCGCGAGGTCGAGATCG	43.4	TGCGGCGCCAGTTCCGGCAGA	11.7	CCTGTTCCAGCCGTTCCTGCC	51.7
Streptomyces hygroscopicus.CP003275.1	CGCCCGCGAGGTCGAGATCG	42.5	TGCGGCGCCAGTTCCGGCAGA	11.7	CCTGTTCCAGCCGTTCCTGCC	51.7
*Isoptericola variabilis*_225_CP002810.1	GCGGGCGCCGCTCGTCGACC	12.7	AAGAGCTTGCGCCGCCGTGGC	26.4	GGCGCGGCCGGAACTACTGCC	11.0
*Streptomyces glaucescens*_CP009438.1	CGCCCGCGAGGTCGAGATCG	43.4	TGCGGCGGCAGTTCCGGCAGA	10.7	CCTGTTCCAGCCGTTCCTGCC	51.7

Supplementary Table 3, continued

Mycobacterium Species	Forward sequence 5'-3'	Annealing temp (°C)	Probe sequence 5'-3'	Annealing temp (°C)	Reverse sequence 5'-3'	Annealing temp (°C)
*Streptomyces vietnamensis*_CP010407.1	CGCCCGCGAGGTCGAGATCG	43.4	TGCGGCGGCAGTTCCGGCAGA	10.7	CCTGTTCCAGCCGTTCCTGCC	51.7
*Rhodococcus equi*_103S_ATCC33701	ACGGGCGTCGGTCGTCCAGG	24.3	AGCAGGTCCTGGCCTTCGCTC	17.6	GTCGCGGCCGGAACTACTGCC	8.8
*Gordonia polyisoprenivorans*_CP003119.1	GCGCGCACTGCAGCTAGAGC	8.3	CGTTCCGACGGCTGCGGGACT	26.2	CCTCTGGCAGCCGCAGCTGCC	30.0
Gordonia sp.KTR9_CP002907.1	GCGGGCGCTCTAGGTCAACG	29.3	AACGTGCAATCTCGACTGTGA	17.8	CCTCATGCACCCGGCGTTGCC	7.8
Rhodococcus jostii.RHA1_CP000431.1	ACGGTTCAAGCTCGTCCAGC	16.1	TCGTCGAGCGCGGGAAGAGCC	7.7	GTCGCGGCCGGAACTACTGAC	15.5
*Amycolatopsis methanolica*_CP009110.1	GCGGTCCCTCCACGTCGACC	37.7	TCCTCTGGCAGCTCCGCCAGC	40.4	CGCCGCCACCCGGCCCTTCC	7.3
Arthrobacter sp 674646427	GCGGCCGCCGTACGTCGAGG	5.3	AACCGCTTCCGGAAGACCGGC	10.2	GACGCGGCCGGAACTACTGCC	11.1
*Nocardia nova*_SH22a_CP006850.1	GTGGCCGCCGGTCGTCCACC	2.1	AGGAGGTCGCGCCCTTCCGGC	6.1	GCCGCGCCAGGAACTAGTGGC	46.7
*Arthrobacter sulfonivorans*_CP013747.1	ACGGCCGTCGGTCGTCCAGC	26.1	CGCTTGCGGAACCGCGGCCGG	29.2	CGGCACCGGCCTTGATGACTG	2.05

Supplementary Table 4. Details of clinical samples used for assay validation.

Subject	**Gender**	**Age**	**Sample type**	**Diagnosis**
1	Female	64	BAL	Bronchiectasis
2	Female	75	BAL	Rheumatoid arthritis with immunosuppression
3	Female	67	BAL	Bronchiectasis
4	Female	81	BAL	Bronchiectasis and gastro oesophageal reflux disease
5	Male	87	BAL	Chronic obstructive pulmonary disease
6	Female	62	BAL	Asthma and lingular bronchiectasis
7	Female	72	BAL	Non-cystic fibrosis bronchiectasis
8	Female	71	BAL	Non-cystic fibrosis bronchiectasis
9	Female	59	BAL	No current diagnosis
10	Male	69	BAL	Stem cell transplant patient
11	Female	60	BAL	Thyroiditis with chronic cough
12	Male	96	BAL	Lung cancer
13	Female	64	BAL	Non-cystic fibrosis bronchiectasis
14	Female	52	BAL	Chronic cough
15	Female	40	BAL	Mild bronchiectasis
16	Female	73	BAL	Non-cystic fibrosis bronchiectasis
17	Female	75	BAL	Alpha-1 antitrypsin deficiency
18	Female	81	BAL	Non-cystic fibrosis bronchiectasis
19	Female	82	BAL	No current diagnosis
20	Female	69	BAL	Chronic cough
21	Female	74	BAL	Non-cystic fibrosis bronchiectasis
22	Female	14	Sputum	Cystic fibrosis
23	Male	10	Sputum	Cystic fibrosis
24	Female	4	Sputum	Cystic fibrosis
25	Female	8	Sputum	Cystic fibrosis
26	Male	10	Sputum	Cystic fibrosis
27	Male	17	Sputum	Cystic fibrosis
28	Male	10	BAL	Cystic fibrosis
29	Male	10	BAL	Cystic fibrosis
30	Male	10	Sputum	Cystic fibrosis
31	Female	15	Sputum	Cystic fibrosis
32	Male	10	BAL	Cystic fibrosis
33	Female	12	Sputum	Cystic fibrosis
34	Male	7	BAL	Non-cystic fibrosis bronchiectasis
35	Female	12	BAL	Cystic fibrosis
36	Male	4	BAL	Cystic fibrosis
37	Female	4	BAL	Cystic fibrosis
38	Female	9	BAL	Cystic fibrosis
39	Female	7	BAL	Non-cystic fibrosis bronchiectasis
40	Female	8	Sputum	Cystic fibrosis
41	Female	16	Sputum	Cystic fibrosis
42	Female	12	Sputum	Cystic fibrosis

Supplementary Table 5: Detection of NTM species from respiratory samples using culture-based and molecular approaches.

Patient	Sample type	C_T value	CFU/mL	qPCR/sequencing	Diagnostic microbiology
1	BAL	-			
2	BAL	-			
3	BAL	30.5	1.36×10^5	*M. avium*	*M. avium*
4	BAL	-			
5	BAL	-			
6	BAL	30.9	1.02×10^5	*M. avium*	*M. avium*
7	BAL	32.1	4.58×10^4	*M. intracellulare*	*M. intracellulare*
8	BAL	35.4	1.13×10^4	*M. avium*	*M. avium*
9	BAL	-			
10	BAL	-			
11	BAL	34	8.70×10^4	*M. avium*	-
12	BAL	-			
13	BAL	32.7	3.02×10^4	*M. avium*	*M. avium*
14	BAL	-			
15	BAL	-			
16	BAL	-			
17	BAL	-			
18	BAL	-			
19	BAL	-			
20	BAL	-			
21	BAL	-			
22	Sputum	31.1	3.22×10^4	*M. massiliense*	*M. massiliense*
23	Sputum	31.7	2.08×10^4	*M. flavescens*	-
24	Sputum	-			
25	Sputum	-			
26	Sputum	31.5	2.44×10^4	*M. abscessus*	*M. abscessus*
27	Sputum	33.4	6.43×10^3	*M. avium*	-
28	BAL	-			
29	BAL	-			
30	Sputum	-			
31	Sputum	35.5	1.49×10^3	*M. avium*	*M. avium*
32	BAL	-			
33	Sputum	-			
34	BAL	-			
35	BAL	-			
36	BAL	-			
37	BAL	-			
38	BAL	-			
39	BAL	-			
40	Sputum	-			
41	Sputum	-			
42	Sputum	-			