

Ultrasensitive Detection of Multidrug-Resistant *Mycobacterium tuberculosis* Using SuperSelective Primer-Based Real-Time PCR Assays

Anshika Narang ¹, Salvatore A. E. Marras ^{1,*}, Natalia Kurepina ², Varsha Chauhan ³, Elena Shashkina ², Barry Kreiswirth ², Mandira Varma-Basil ³, Christopher Vinnard ⁴ and Selvakumar Subbian ^{1,*}

¹ Public Health Research Institute, New Jersey Medical School, Rutgers University, Newark, NJ 07103, USA

² Center for Discovery and Innovation, Nutley, NJ 07110, USA

³ Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi 110021, India

⁴ New Jersey Medical School, Rutgers University, Newark, NJ 07103, USA

* Correspondence: marrassa@njms.rutgers.edu (S.A.E.M.); subbiase@njms.rutgers.edu (S.S.)

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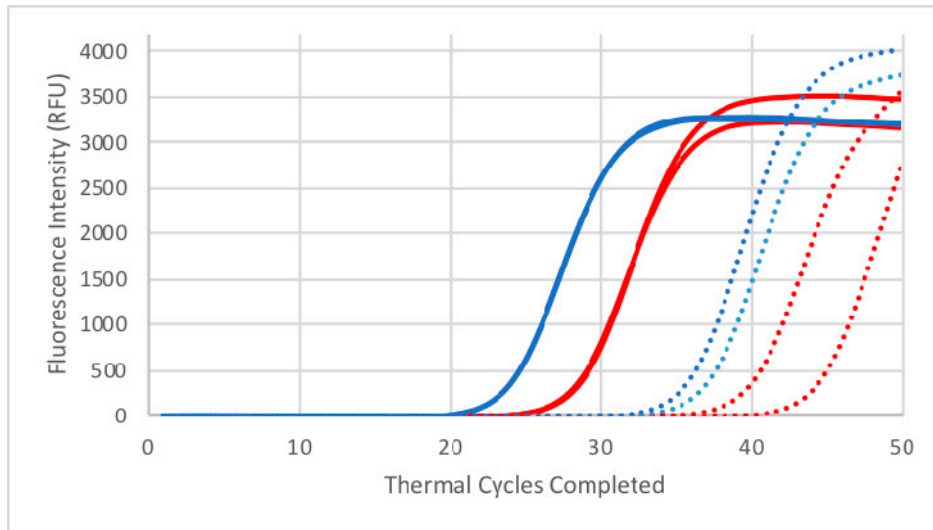
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a)



b)

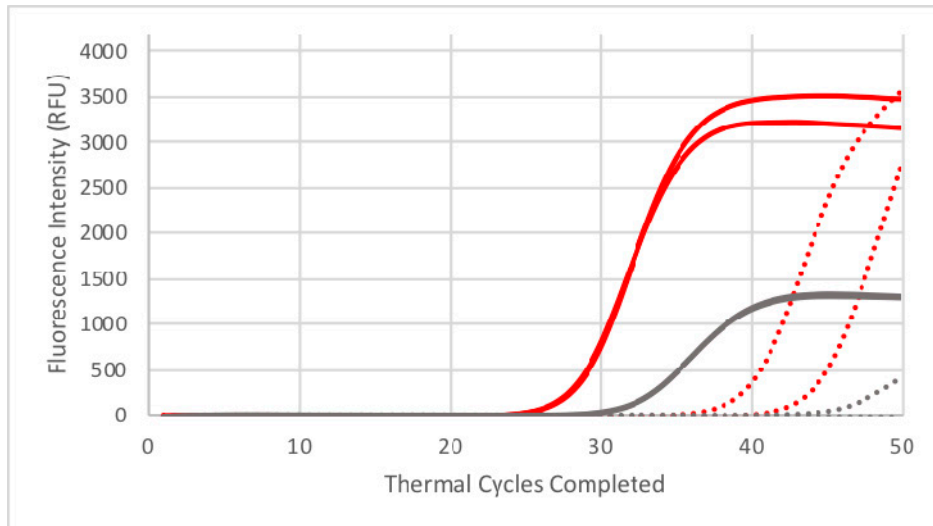
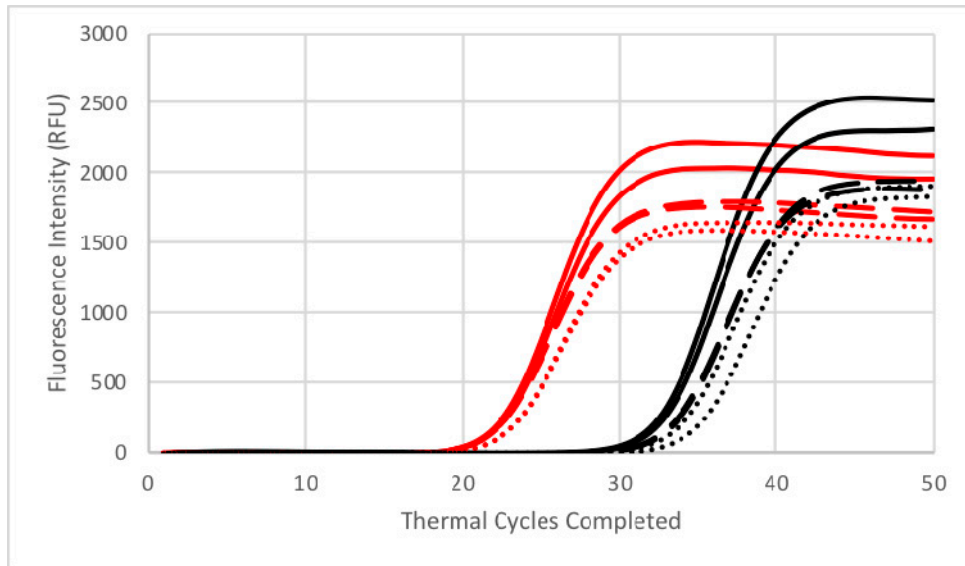


Figure S1. Effect of increase in length of the foot and intervening sequence of a SuperSelective primer on its performance. The plot shows the results of PCR assays for the detection of *inhA* promoter -15 mutation using 10^5 copies of mutant pDNA (solid lines) and 10^5 copies of WT pDNA (dotted lines). a. Comparison of threshold cycles with SuperSelective primer inh15_SSP3 (18-14/14-6:1:0) (red) and inh15_SSP4 (18-14/13-7:1:0) (blue) with foot length 7bp and 8bp respectively. b. Comparison of threshold cycles with SuperSelective primer inh15_SSP3 (18-14/14-6:1:0) (red) and inh15_SSP2 (18-14/21-6:1:0) (grey) with intervening sequence length 14bp and 21bp respectively.

a)



b)

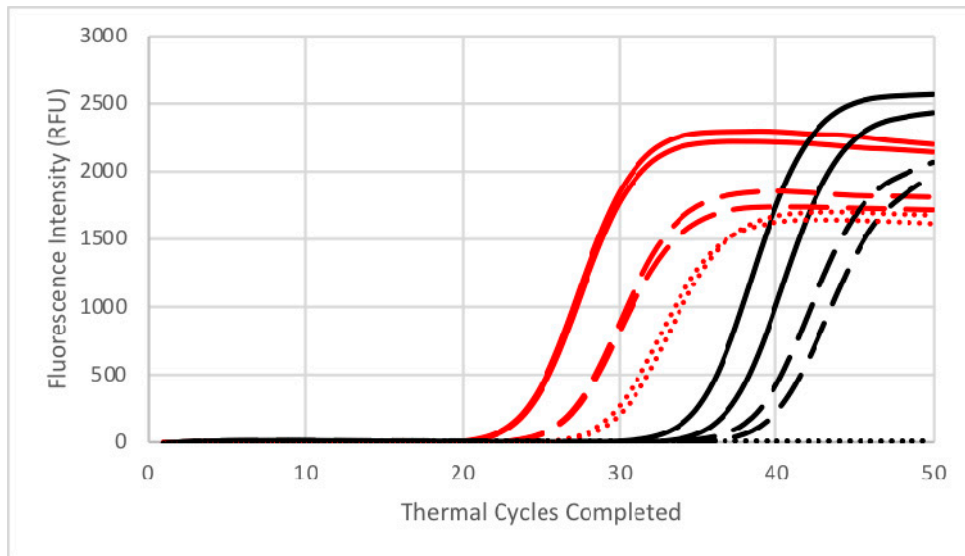


Figure S2. Efficiency of SuperSelective primer designs with symmetric and asymmetric bubble under different annealing temperatures. a. inh15_SSP8 (18-8/8-7:1:0) – symmetric bubble, b. inh15_SSP7 (18-20/8-7:1:0) – asymmetric bubble. The plots show the results of PCR assays carried out for the detection of *inhA* promoter -15 mutation using 10^5 copies of mutant pDNA (red) and 10^5 copies of WT pDNA (black) at an annealing temperature of 60°C (Solid lines), 64°C (Broken lines), and 66°C (Dotted lines).

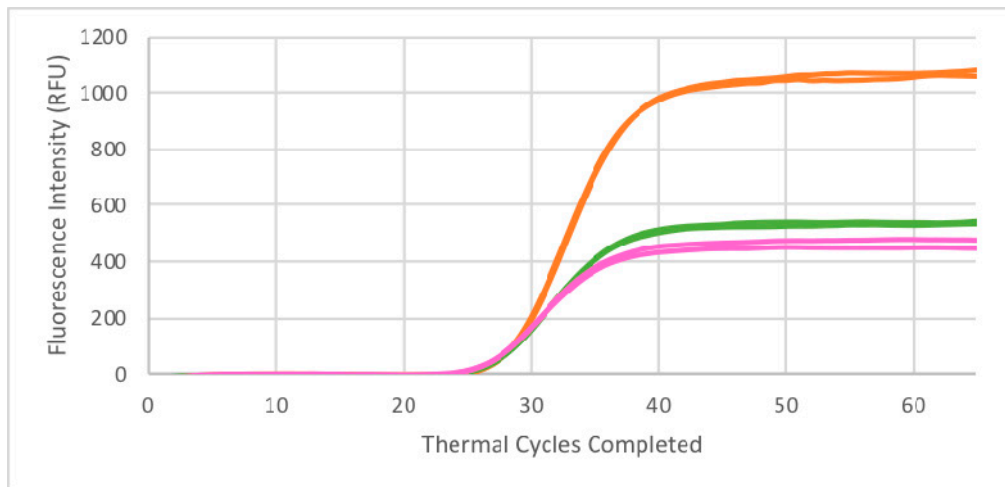


Figure S3. Fluorescence signal intensity of *inhA* probe at different molecular beacon concentrations. The plot shows amplification of 10^5 copies of mutant target DNA template using SuperSelective primer inh8_SSP1 (18-14/14-6:1:0) and a conventional reverse primer for the detection of *inhA* promoter mutation -8T-->A at molecular beacon probe concentrations 0.25 μM (orange), 0.125 μM (green) and 0.1 μM (pink). There was no significant difference between the signal intensity with 0.1 μM and 0.125 μM probe; however, 0.25 μM probe resulted in signals with the highest fluorescence intensity.

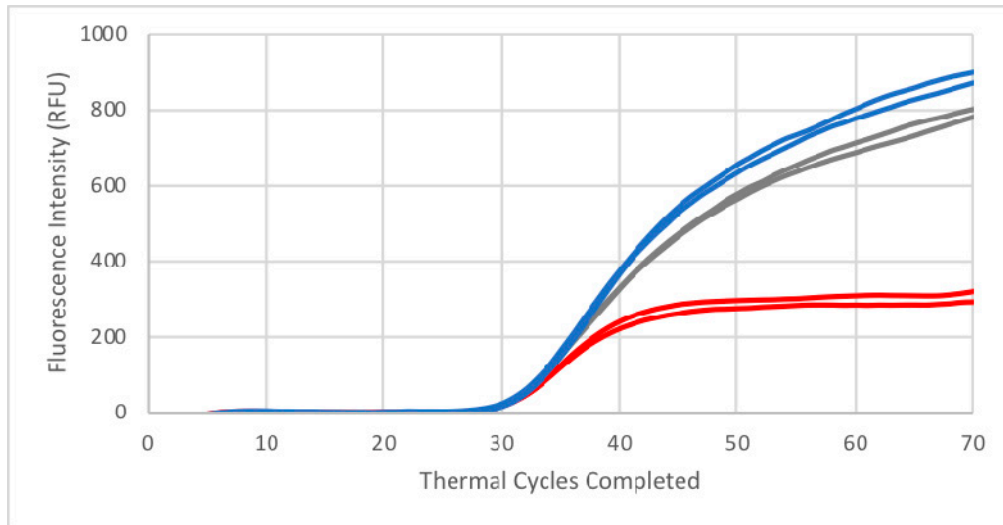


Figure S4. Fluorescence signal intensity of *rpoB* probe in asymmetric PCR. The plot shows the amplification of 10^5 copies of *rpoB* D516V GAC-->GTC mutant template with SuperSelective primer *rpoB*516_SSP4 (18-14/13-7:1:0) and a conventional reverse primer in the ratio 1:1, 1:5 and 1:10. Asymmetric PCR with forward primer 0.1 μ M, reverse primer 0.5 μ M (grey) and 1 μ M (blue) resulted in higher fluorescence intensity as compared to symmetric PCR with forward and reverse primer 0.1 μ M (red). However, there was no significant difference in fluorescence intensity with reverse primer 0.5 μ M and 1 μ M.

Table S1: RT-PCR assay conditions tested for the optimization of SuperSelective primers for the detection of *inhA* promoter mutation -15C→T.

Forward Primer	Reverse Primer	Annealing Temperature (°C)	TMAC (mM)*
inh15_SSP2 (18-14/21-6:1:0)	inhA_rev1	60	25
inh15_SSP3 (18-14/14-6:1:0)	inhA_rev1	60, 62	25
inh15_SSP3 (18-14/14-6:1:0)	inhA_rev2	60	25
inh15_SSP4 (18-14/13-7:1:0)	inhA_rev1	60, 62, 66	25
inh15_SSP4 (18-14/13-7:1:0)	inhA_rev3, inhA_rev4	60	25
inh15_SSP5 (18-15/13-7:1:0)	inhA_rev1	60, 62	25
inh15_SSP6 (18-17/13-7:1:0)			
inh15_SSP7 (18-20/8-7:1:0)	inhA_rev1	60, 64, 66	25
inh15_SSP8 (18-8/8-7:1:0)			
inh15_SSP9 (18-8/20-7:1:0)			
inhA_for2	SSP10 (18-14/14-6:1:0)	60	25
inh15_SSP4 (18-14/13-7:1:0)			
inh15_SSP4 (18-14/13-7:1:0)	SSP10 (18-14/14-6:1:0)	60, 64	0, 25
inh15_SSP7 (18-20/8-7:1:0)			
inhA_for2	SSP11 (18-14/13-7:1:0)	60	0, 25
inhA_for4			
inh15_SSP4 (18-14/13-7:1:0)			
inh15_SSP7 (18-20/8-7:1:0)			
inhA_for2	SSP12 (18-14/8-6:1:0)	60	0
inhA_for4	SSP13 (18-14/8-7:1:0)		
inh15_SSP4 (18-14/13-7:1:0)			
inh15_SSP7 (18-20/8-7:1:0)			
inh15_SSP4 (18-14/13-7:1:0)	SSP10 (18-14/14-6:1:0)	64, 66	0
inh15_SSP7 (18-20/8-7:1:0)	SSP11 (18-14/13-7:1:0)		
	SSP12 (18-14/8-6:1:0)		
	SSP13 (18-14/8-7:1:0)		
inh15_SSP4 (18-14/13-7:1:0)	SSP17,18	60	25
inh15_SSP22	SSP10 (18-14/14-6:1:0)	60	0
inh15_SSP14-16, 19-21	inhA_rev1	60	25
inh15_SSP23-25	SSP10 (18-14/14-6:1:0)	60	25
inh15_SSP3 (18-14/14-6:1:0)	inhA_rev1	60	0
inh15_SSP4 (18-14/13-7:1:0)			
inh15_SSP7 (18-20/8-7:1:0)			

*Addition of TMAC was always accompanied by Tween 20 (0.25%)

Table S2: List of primers used for *katG*, *inhA* and *rpoB* mutations.

Gene	Mutation	Primer ID	Strand d +/-	Design	Sequence
<i>katG</i>	S315T AGC-->ACA	katG315T2_SSP1	+	20-14/14-5:2:0	<u>GCTCGTATGGCACCGGAACCAC</u> <u>ACTACCGCGACCCACCACA</u>
<i>katG</i>	S315T AGC-->ACC	katG315T1_SSP1	+	20-14/14-5:1:0	<u>GCTCGTATGGCACCGGAACCAC</u> <u>ACTACCGCGACCCACCAC</u>
<i>katG</i>	S315T AGC-->ACC	katG315T1_SSP2	+	20-14/13-6:1:0	<u>GCTCGTATGGCACCGGAACCTC</u> <u>ACTACAGAAACCTCACCAC</u>
<i>katG</i>		katG_rev1	-		ATTTCGTCGGGGTGTTTCGTC
<i>katG</i>		katG_rev2	-		TCGTAGCCGTACAGGATCTCG
<i>inhA</i>	-8T-->A	inh8_SSP1	-	18-14/14-6:1:0	<u>GTTTGGCCCCCTTCAGTGGAAATA</u> <u>TTGTTGATAACCGACAT</u>
<i>inhA</i>	-8T-->A	inh8_SSP2	-	20-14/14-6:1:0	<u>GGGTTTGGCCCCCTTCAGTGGAA</u> <u>TATTGTTGATAACCGACAT</u>
<i>inhA</i>	-15C-->T	inh15_SSP2	-	18-14/21-6:1:0	<u>GTTTGGCCCCCTTCAGTGGAAATA</u> <u>TTGTCATGTTCCCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP3	-	18-14/14-6:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> <u>TTGTCATGTTCCCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP4	-	18-14/13-7:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> <u>TTGTCATGTTACCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP5	-	18-15/13-7:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> <u>TTAGTCATGTTACCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP6	-	18-17/13-7:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> <u>TTATCGTCATGTTACCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP7	-	18-20/8-7:1:0	<u>CAGTGGCTGTGGCAGTCATCTA</u> <u>GTACACTCGTCATGTTACCTATC</u> <u>A</u>
<i>inhA</i>	-15C-->T	inh15_SSP8	-	18-8/8-7:1:0	<u>CAGTGGCTGTGGCAGTCAGTCA</u> <u>TGTTACCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP9	-	18-8/20-7:1:0	<u>GTTTGGCCCCCTTCAGTGGATCAT</u> <u>GTTACCTATCA</u>
<i>inhA</i>	-15C-->T	inh15_SSP10	+	18-14/14-6:1:0	<u>CTCGTGGACATACCGATTGTTG</u> <u>TTCATCAGTAGCGAGAT</u>
<i>inhA</i>	-15C-->T	inh15_SSP11	+	18-14/13-7:1:0	<u>CTCGTGGACATACCGATTGTTG</u> <u>TTCATCAGTGGCGAGAT</u>
<i>inhA</i>	-15C-->T	inh15_SSP12	+	18-14/8-6:1:0	<u>CTCGTGGACATACCGATTGTTG</u> <u>TTCAGCGAGAT</u>
<i>inhA</i>	-15C-->T	inh15_SSP13	+	18-14/8-7:1:0	<u>CTCGTGGACATACCGATTGTTG</u>

					TTCAGGCGAGAT
<i>inhA</i>	-15C-->T	inh15_SSP14	-	18-14/14-6:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTCTCTCA
<i>inhA</i>	-15C-->T	inh15_SSP15	-	18-14/13-7:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTACCTCTCA
<i>inhA</i>	-15C-->T	inh15_SSP16	-	18-20/8-7:1:0	<u>CAGTGGCTGTGGCAGTCATCTA</u> <u>GTACACTCGTCATGTTACCTCTC</u> A
<i>inhA</i>	-15C-->T	inh15_SSP17	+	20-14/14-6:1:0	<u>CGCTCGTGGACATACCGATTGT</u> TGTTTCATCAGTAGCGAGAT
<i>inhA</i>	-15C-->T	inh15_SSP18	+	20-14/13-7:1:0	<u>CGCTCGTGGACATACCGATTGT</u> TGTTTCATCAGTGGCGAGAT
<i>inhA</i>	-15C-->T	inh15_SSP19	-	18-14/14-6:1:2	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTCTATCATC
<i>inhA</i>	-15C-->T	inh15_SSP20	-	18-14/13-7:1:2	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTACCTATCATC
<i>inhA</i>	-15C-->T	inh15_SSP21	-	18-20/8-7:1:2	<u>CAGTGGCTGTGGCAGTCATCTA</u> <u>GTACACTCGTCATGTTACCTATC</u> ATC
<i>inhA</i>	-15C-->T	inh15_SSP22	-	18-14/14-3:1:2	<u>TTCAGTGGCTGTGGCAGTATTG</u> TTACATTATAATCATC
<i>inhA</i>	-15C-->T	inh15_SSP23	-	18-14/14-3:2:1	<u>TTCAGTGGCTGTGGCAGTATTG</u> TTACATTATAATCACC
<i>inhA</i>	-15C-->T	inh15_SSP24	-	18-14/14-6:2:1	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTCTATCACC
<i>inhA</i>	-15C-->T	inh15_SSP25	-	18-14/14-6:2:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTCTATCAC
<i>inhA</i>	-15C-->T	inh15_SSP26	-	18-10/14-6:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTCTATCA
<i>inhA</i>	-15C-->T	inh15_SSP27	-	20-14/13-7:1:0	<u>GCCCCCTTCAGTGGCTGTGGCTA</u> TATTGTCATGTTACCTATCA
<i>inhA</i>	-15C-->T	inh15_SSP28	-	18-14/12-8:1:0	<u>CCCTTCAGTGGCTGTGGCTATA</u> TTGTCATGTTAACCTATCA
<i>inhA</i>	-17G-->T	inh17_SSP1	-	18-14/15-6:1:0	<u>CCTTCAGTGGCTGTGGCATATA</u> TTGTCATGTTTATCGTA
<i>inhA</i>	-17G-->T	inh17_SSP2	-	18-14/14-6:1:0	<u>CTTCAGTGGCTGTGGCAGTATA</u> TTGTCATGTTTATCGTA
<i>inhA</i>	-17G-->T	inh17_SSP3	-	18-14/13-7:1:0	<u>CTTCAGTGGCTGTGGCAGTATA</u> TTGTCATGTTCTATCGTA
<i>inhA</i>	-17G-->T	inh17_SSP4	-	20-14/14-6:1:0	<u>CCCTTCAGTGGCTGTGGCAGTA</u> TATTGTCATGTTTATCGTA

<i>inhA</i>	-17G-->T	inh17_SSP5	-	20-14/13-7:1:0	<u>CCCTTCAGTGGCTGTGGCAGTA</u> <u>TATTGTCATGTTCTATCGTA</u>
<i>inhA</i>		inhA_rev1	+		GGAAATCGCAGCCACGTTAC
<i>inhA</i>		inhA_rev2	+		TTACGCTCGTGGACATACCG
<i>inhA</i>		inhA_rev6	+		CGTAACCCCACTGCGAAAGT
<i>inhA</i>		inhA_for2	-		CTCGTGGACATACCGATT
<i>inhA</i>		inhA_for4	-		CACGTTACGCTCGTGGACAT
<i>rpoB</i>	D516V (GAC-->GTC)	rpoB516_SSP1	-	20-14/14-6:1:0	<u>TCGGCGCTTGTGGGTCAACCTG</u> <u>CTTGTATACACATTCTGGA</u>
<i>rpoB</i>	D516V (GAC-->GTC)	rpoB516_SSP2	-	18-14/14-6:1:0	<u>GGCGCTTGTGGGTCAACCTGCT</u> <u>TGTATACACATTCTGGA</u>
<i>rpoB</i>	D516V (GAC-->GTC)	rpoB516_SSP3	-	20-14/13-7:1:0	<u>TCGGCGCTTGTGGGTCAACCTG</u> <u>CTTGTATACACAGTTCTGGA</u>
<i>rpoB</i>	D516V (GAC-->GTC)	rpoB516_SSP4	-	18-14/13-7:1:0	<u>GGCGCTTGTGGGTCAACCTGCT</u> <u>TGTATACACAGTTCTGGA</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP1	-	20-14/14-6:1:0	<u>GACAGACCGCCGGGCCCCAGT</u> <u>ATTACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP2	-	16-14/22-6:1:0	<u>ACGTGACAGACCGCCGTATTAC</u> <u>AGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP3	-	16-14/21-7:1:0	<u>ACGTGACAGACCGCCGTATTAC</u> <u>AGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP4	-	18-14/14-6:1:0	<u>CAGACCGCCGGGCCCCAGTATT</u> <u>ACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP5	-	18-14/22-6:1:0	<u>TCACGTGACAGACCGCCGTATT</u> <u>ACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP6	-	20-14/13-7:1:0	<u>GACAGACCGCCGGGCCCCAGT</u> <u>ATTACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP7	-	18-14/13-7:1:0	<u>CAGACCGCCGGGCCCCAGTATT</u> <u>ACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526D (CAC-->GAC)	rpoB526D_SSP8	-	18-14/21-7:1:0	<u>TCACGTGACAGACCGCCGTATT</u> <u>ACAGTAAATTGCTTGTC</u>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP1	-	20-14/14-6:1:0	<u>GACAGACCGCCGGGCCCCAGT</u> <u>ATTACAGTAAATTGCTTGTA</u>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP2	-	16-14/22-6:1:0	<u>ACGTGACAGACCGCCGTATTAC</u> <u>AGTAAATTGCTTGTA</u>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP3	-	16-14/21-7:1:0	<u>ACGTGACAGACCGCCGTATTAC</u> <u>AGTAAATTGCTTGTA</u>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP4	-	18-14/14-6:1:0	<u>CAGACCGCCGGGCCCCAGTATT</u> <u>ACAGTAAATTGCTTGTA</u>
<i>rpoB</i>	H526Y	rpoB526Y_SSP5	-	18-14/22-6:1:0	<u>TCACGTGACAGACCGCCGTATT</u>

	(CAC-->TAC)				<i>ACAGTAAATTGCTTGTA</i>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP6	-	20-14/13-7:1:0	<u>GACAGACCGCCGGGCCCCAGT</u> <i>ATTACAGTAAATTCGCTTGTA</i>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP7	-	18-14/13-7:1:0	<u>CAGACCGCCGGGCCCCAGTATT</u> <i>ACAGTAAATTCGCTTGTA</i>
<i>rpoB</i>	H526Y (CAC-->TAC)	rpoB526Y_SSP8	-	18-14/21-7:1:0	<u>TCACGTGACAGACCGCCGTATT</u> <i>ACAGTAAATTCGCTTGTA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP1	-	20-14/14-6:1:0	<u>CCCGGCACGCTCACGTGACATG</u> <i>TTATACTAAATTAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP2	-	20-14/12-6:1:0	<u>CGGCACGCTCACGTGACAGAT</u> <i>GTTATACTAAATTAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP3	-	18-14/14-6:1:0	<u>CGGCACGCTCACGTGACATGTT</u> <i>ATACTAAATTAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP4	-	20-14/13-7:1:0	<u>CCCGGCACGCTCACGTGACATG</u> <i>TTATACTAAATTCAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP5	-	20-14/11-7:1:0	<u>CGGCACGCTCACGTGACAGAT</u> <i>GTTATACTAAATTCAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB531_SSP6	-	18-14/13-7:1:0	<u>CGGCACGCTCACGTGACATGTT</u> <i>ATACTAAATTCAGCGCCA</i>
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB_rev1	+		GAGGCGATCACACCGCAGAC
<i>rpoB</i>	S531L (TCG-->TTG)	rpoB_rev2	+		GCCGCGATCAAGGAGTTCTTC