Electronic Supplementary Material (ESI) for ChemComm.

Detection of Multiple Mutations in the Single Codon of Genomic DNA

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Supporting Information

1. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemicals, Korea. All probes were purchased from Bioneer, Korea. The PCR pre-mix and DNA extraction kits were obtained from the Bioneer, Korea. All washing solvents for the substrates are of HPLC grade from SK Chemicals, Korea. Ultrapure water (18 M Ω/cm) was obtained from a Milli-Q purification system (Millipore). The samples of drug resistant Mycobacterium Tuberculosis (DR-TB) strains were obtained from Dr. Nakmoon Sungof National Institute of Tuberculosis, Masan, South Korea.DNA's were extracted from the clinical samples and stored at -20℃ by following the reported method[1]. All DNA chips used in this study were obtained by following the previously reported method.[2]

2. Composition of different solutions used for hybridization and washing on 9G DNAChips

1. Immobilization solution (pH = 7.4): 15% glycerol, 50mM butyl amine, 600mM NH4Cl
2. Blocking buffer solution (pH = 7.4): 0.5% milk casein in 4xSSC
3. Hybridization buffer (pH = 7.4): 25% Formamide, 0.1% Triton X-100, 6xSSC
4. Washing buffer solution A (pH = 7.4): 0.1% SDS in 4xSSC
5. Washing buffer solution B (pH = 7.4): 4xSSC

3. Probe, Target, and primernucleotide sequences

Table S1. Probe and primer sequences

Probes	Description	Sequences	Tm (^{o}C)
Probe1	Wild	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCCACAAG-3'	51℃
Probe2	M1	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCTACAAG-3'	49℃
Probe3	M2	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCAACAAG-3'	49℃
Probe4	M3	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCGACAAG-3'	51℃
Probe5	M4	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCTGCAAG-3'	51℃
Probe6	M5	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCCGCAAG-3'	53℃
Probe7	M6	5'-GGGGGGGGGTTTTTTTTTCGGGGTTGTCCCTCAAG-3'	51℃
Probe8	HC	5'-GGGGGGGGGCTTTATCATGGCGGTCTGTCACGTGA-3'	51℃
Probe9	PC	5'-GGGGGGGGGCTTTATCATGGCGGTCTGTCACGTGA-3'	51℃
Probe10	PCR	5'-GGGGGGGGGCTTTATCATGCGCTGGGGCCCGGC-3'	53℃
Probe11	Cy5-Reverse Primer(Cy5-RP)	5'-Cy5-TCACGTGACAGACCGCCGGG-3'	63℃
Probe12	Forward Primer(FP)	5'-GTCGCCGCGATCAAGGAGTTC-3'	63℃
Probe 13	Splitter DNA	5'-GCTGGGGCCTGGCGGTCTGT-3'	65℃

PC– probe for the Positive control; PCR– probe for the PCR control; GGGGGGGGG (9G's) for immobilization of the probes on the AMCA slides (Ref. 2a); CTT TATCAT – vertical spacer group; c511, c516, c522, c526, and c531 – Codon containing mutation; M1-M6 – corresponds to the mutations in the corresponding codons.

Table S2: Controller DNA (CD) sequences (10mer) with the binding positions of 1-10, 12

Controller DNA(CD)	Description	Sequence	Tm
CD1	1mer open	TGACCCACAA	25℃
CD2	2mer open	TTGACCCACA	25℃
CD3	3mer open	GTTGACCCAC	27℃
CD4	4mer open	GGTTGACCCA	27℃
CD5	5mer open	GGGTTGACCC	29℃
CD6	6mer open	GGGGTTGACC	29℃
CD7	7mer open	CGGGGTTGAC	29℃
CD8	8mer open	TCGGGGTTGA	27℃
CD9	9mer open	GTCGGGGTTG	29℃
CD10	10mer open	TGTCGGGGTT	27℃
CD11	12mer open	GCTGTCGGGG	31℃

Table S3: Controller DNA (CD) sequenceswith the binding position of 7 and different lengths

Controller	Description	Sequence	Tm
CD12	5mer	TGACC	11℃
CD13	6mer	TTGACC	13℃
CD14	8mer	GGTTGACC	21℃
CD15	9mer	GGGTTGACC	25℃
CD16	10mer	GGGGTTGACC	29℃
CD17	12mer	TCGGGGTTGACC	35℃
CD18	14mer	TGTCGGGGTTGACC	41℃
CD19	16mer	GCTGTCGGGGTTGACC	49℃

4. Methods and experimental procedures:

4.1 DNA extraction from Human sputum:

200µl of sputum sample was transferred to a 1.5 mL eppendorf tube and diluted to 500 µL with isopropyl alcohol. The solution was vortexed for 2 min to get homogeneous mixture. The clear solution was transferred to another eppendorf tube. 100 µL of Binding buffer, 20 µL of Proteinase K were added to this solution and mixed. The mixture was incubated in water bath for 20 min at 60℃. Then 400 µL isopropanol was added to the mixture and kept aside for 10 min. The lysate was then carefully transferred into the upper reservoir of the binding column tube (fit in a 2 mL tube) and centrifuged. The Binding column was transferred to a new 2 mL tube for filtration. After addition of the 500 µL of washing buffer the tube was centrifuged (13,000rpm, 3min) to remove washing buffer (W). Then the Binding column tube was transferred to a new 1.5 mL tube for elution. Finally, the DNA was isolated by addition of the 100µl of elution buffer to binding column tube.

4.2 Amplification of genomic DNA by PCR:

4.2.1 Amplification of genomic DNA by PCR to obtain PCR product with 620 base pairs:

The MTB and genotype samples were amplified by PCR to generate amplicons of approximately 620bp. The extracted DNA was amplified with the single primer set of reverse primer with a sequence 5'-ACGGGTGCACGTCGCGGACCTCCA-3', and forward primer with a sequence 5'-GCTGTTGGACATCTACCGCAAGC-3'. 10 µL of the extracted DNA sample, 10 µL of Cy5-RP (4 pmol/µL), and 10 µL (2 pmol/µL) of FP were added to the PCR premix. Amplification was performed with the following steps: transcriptase denaturation at 95℃ for 5 min and then DNA polymerization for 60 cycles at 94℃ for 10 s, 60℃ for 10 s, and 72℃ for 10 s. The concentration of the PCR product was measured by the nanodrop as shown in the **Figure S1**. Then the dilutions were made as shown in the **Table S3** and the copy number in each solution was calculated. The 5 µL of each diluted PCR product was subjected to agarose gel electrophoresis, using a 2% agarose standard run in 1X Tris borate EDTA. 10µLof PCR products after dilution (1copy ~ 10^6 copies) were used were used to produce the 130 base pair Cy5 labeled PCR products.

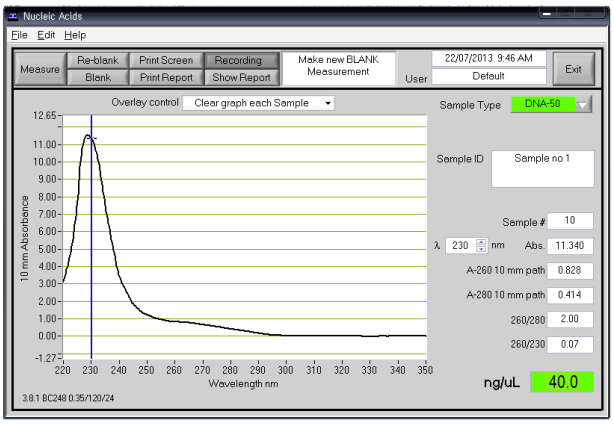


Figure S1: Determination of concentration in the final PCR product by using nanodrop

Table S4: DNA Concentration and Copy number for the PCR product of the 620 base pairs

Sr. no.	Initial concentration	Dilution	Final Concentration	Copy number
1	40ng/µL	1µL diluted to 40µL	1ng/µL	10^9 copies
2	1ng/µL	10µL diluted to 100µL	100pg/µL	10^8 copies
3	100pg/µL	10µL diluted to 100µL	10pg/µL	10^7 copies
4	10pg/µL	10µL diluted to 100µL	1pg/µL	10^6 copies
5	1pg/µL	10µL diluted to 100µL	100fg/µL	10^5 copies
6	100fg/µL	10µL diluted to 100µL	10fg/µL	10^4 copies
7	10fg/µL	10µL diluted to 100µL	1fg/µL	10^3 copies
8	1fg/µL	10µL diluted to 100µL	100ag/µL	10^2 copies
9	100ag/µL	10µL diluted to 100µL	10ag/µL	10^1 copies
10	10ag/µL	10µL diluted to 100µL	1ag/µL	1 copy

Note: Solutions of 1 copy (10ag/µL) to 10^6 copies (10pg/µL) were used for further experiments

4.2.2 Symmetric (1:1 Cy5-RP:FP) and Asymmetric(2:1 Cy5-RP:FP) PCR amplification of genomic DNA to obtain Cy5 labeled PCR product with 130 base pairs:

The MTB and genotype samples were amplified by PCR to generate amplicons of approximately 130bp. The extracted DNA was amplified with the single primer set of primers (**Table S1**). Similar method was followed for the symmetric as well as asymmetric PCR except for the concentrations of the Cy5-RP and FP. For symmetric PCR, 10 µL of the extracted DNA sample, 10 µL of Cy5-RP (10pmol/µL), and 10 µL (10pmol/µL) of FP were added to the PCR premix.For asymmetric PCR, 10 µL of the extracted DNA sample, 10 µL of Cy5-RP (20pmol/µL), and 10 µL (10pmol/µL) of FP were added to the PCR premix. Amplification was performed with the following steps: transcriptase denaturation at 95℃ for 5 min and then DNA polymerization for 60 cycles at 94℃ for 10 s, 60℃ for 10 s, and 72℃ for 10 s. Then 5 µL of

PCR products were subjected to agarose gel electrophoresis, using a 2% agarose standard run in 1X Tris borate EDTA (**Figure S2**). The PCR was run to increase the concentrations by 10 times of the initial genomic DNA (620 base pairs).

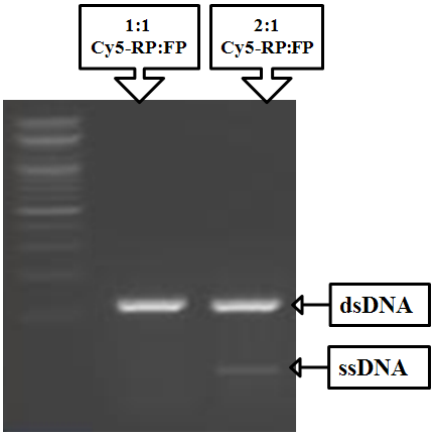
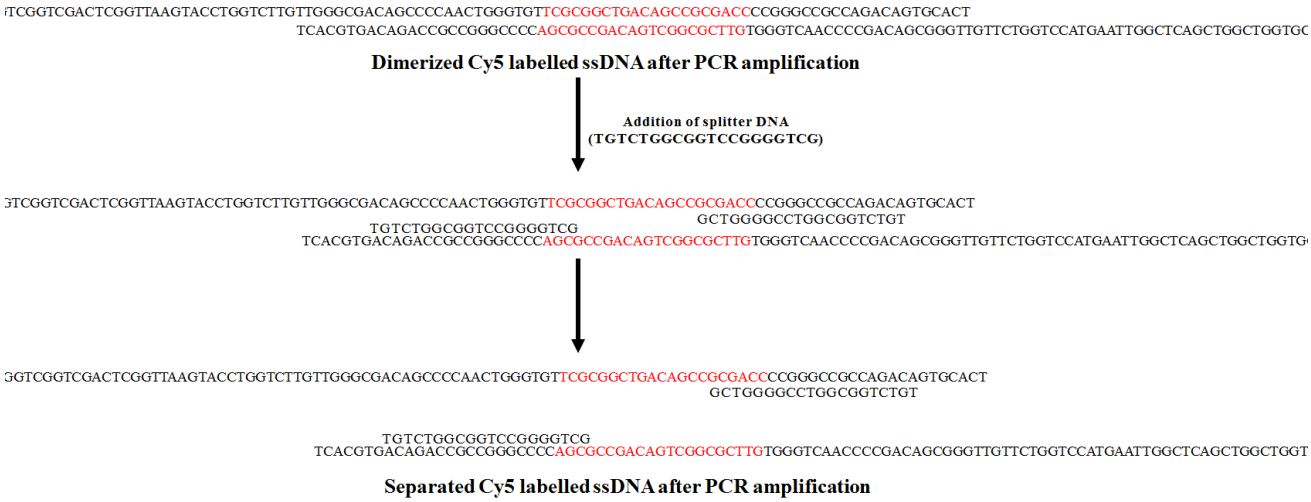


Figure S2: Gel electrophoresis images of the PCR products

4.2.3 Addition of a splitter DNA for the separation of dimerized ssDNA in the PCR product

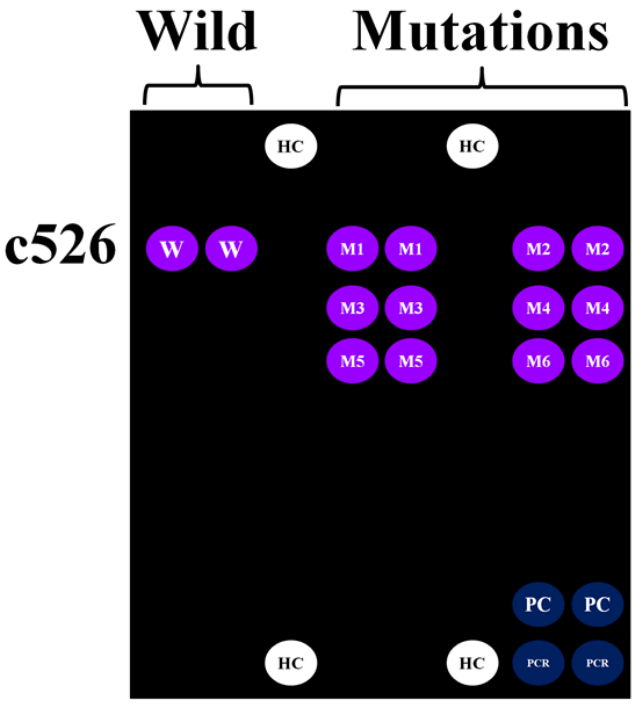


Scheme S1: Addition of a splitter DNA for the separation of dimerized ssDNA in the PCR product

4.3 Typical method for the preparation of the 9G DNAChip:

The 9G DNAChips were prepared by spotting the immobilization solution containingnucleotide probes Probe1-Probe25 on the 9G slides, with the microarrayer as reported earlier.[2] The spots were arranged as demonstrated in the **Scheme S1**. The microarrayed 9G slides were then kept in the incubator

(25^{0}C, 50% humidity) for 4h to immobilize the probes. The slides were then suspended in the blocking buffer solution at 25^{0}C for 30 min, in order to remove the excess probes and to deactivate the non-spotted areas. Then the slides were rinsed with washing buffer solutions A and B for 5min each, and then dried with commercial centrifuge to obtain the 9G DNAChips. Before hybridization, the 9G DNAChipswere covered with Secure-Seal™ hybridization chambers.



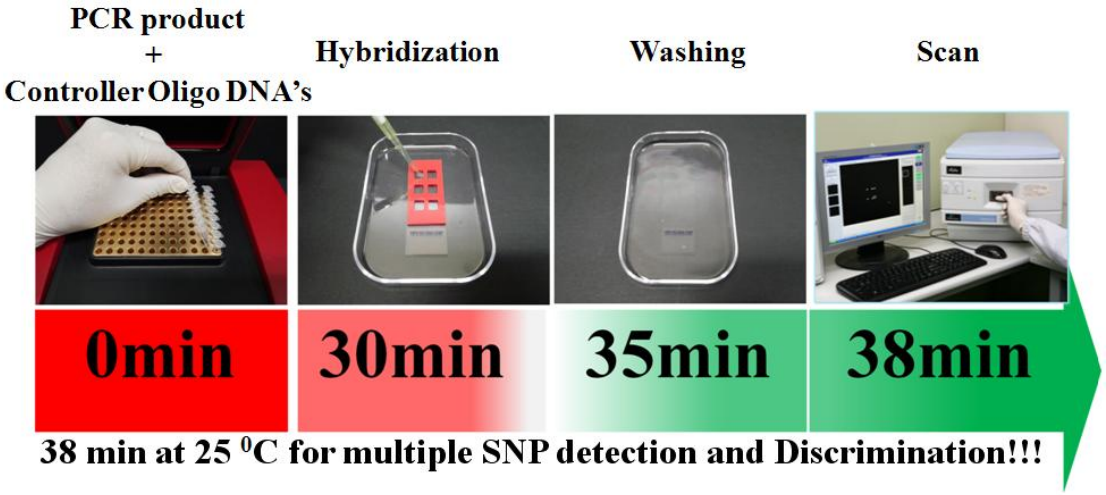
Codon	Wild	M1	M2	M3	M4	M5	M6
C526	CAC	TAC	AAC	GAC	TGC	CGC	CTC

Scheme S2: Scheme for immobilization probes to produce 9G DNAChips

4.4 Hybridization and washing method

The 100copies of the Cy5 labeled PCR products of the MTB-DR strains were first mixed with 100pmol of splitter DNA (5'-GCTGGGGCCTGGCGGTCTGT-3') and 100pmol of controller DNA in the hybridization buffer. Then this mixture was loaded on the 9G DNAChips and allowed to hybridize at 25^{0}C for 30min in the commercial incubator. Then the 9G DNAChips were rinsed with washing buffer solutions A and B successively for 2 min each at 35^{0}C, in order to remove the excess target DNA, and dried with commercial centrifuge (1000 rpm). The fluorescence signals were measured on ScanArrayLite, and the

images were analyzed by Quant Array software (Packard Bioscience). The hybridization, washing, and scanning processes are depicted in the **Scheme S2**.



Scheme S3: Experimental steps for the multiple SNP detection and discrimination.

5. Hybridization of Cy5 labeled PCR product of mutant strain

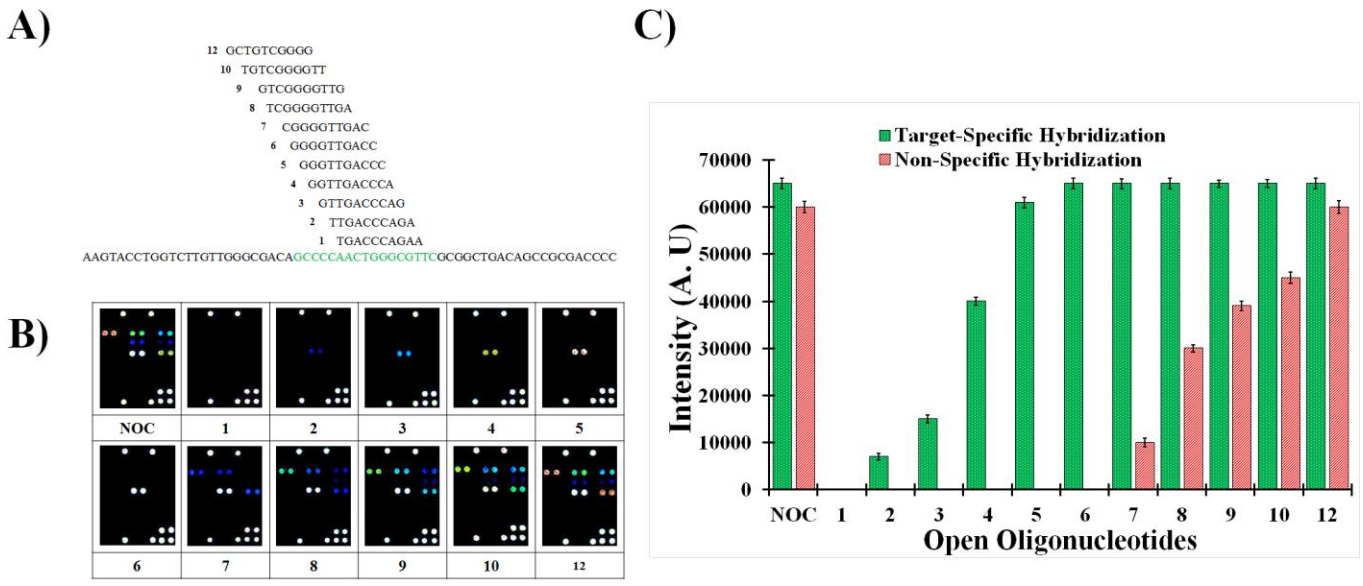


Figure S3: Fig. 1 A) Controller DNA's (with a starting position of 2-11 and 13 leaving 1-10and 12 nucleotides open, respectively) and the partial sequence of Cy5 labelled ssDNA, B) Fluorescence images after hybridization of the Cy5 labelled ssDNA of mutant TB strain (c526 CAC -> CGC) with the immobilized probes in absence (NOC) and presence of controller DNA's (with a starting position of 2-11, 13), and C) Corresponding graph, PMT= 53.

6. Sensitivity of Controller DNA Technology

6.1 Sensitivity of CDT in the multiple SNP detection of wild and mutant TB strains in the individual sample

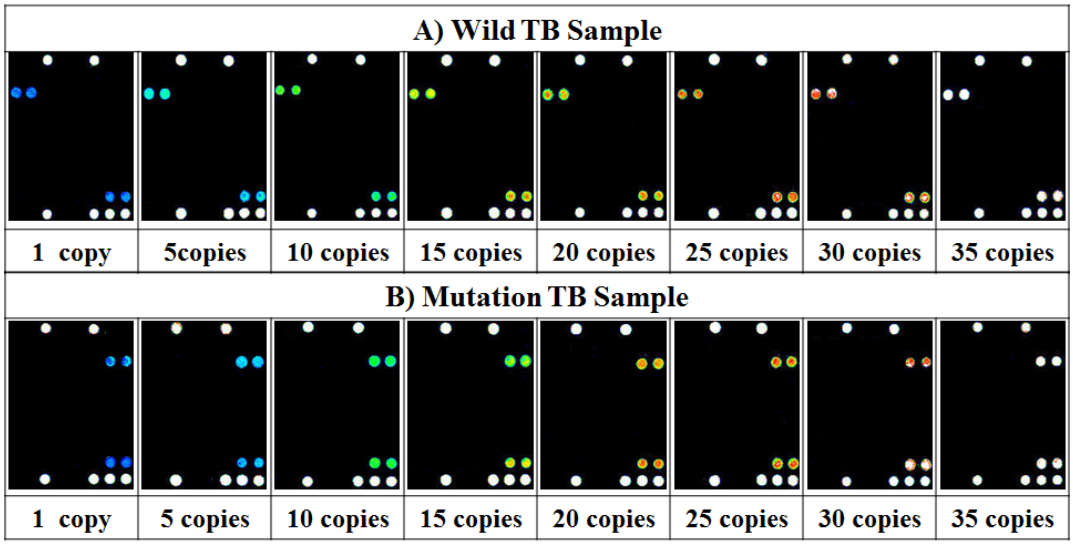


Figure S4: A) Determination of sensitivity of CDT by using 1 – 35 copies of genomic DNA of the wild TB strain, B) Determination of sensitivity of CDT by using 1 – 35 copies of genomic DNA of the mutant TB strain

6.2 Sensitivity of CDT in the multiple SNP detection of wild and mutant TB strains in the mixed sample

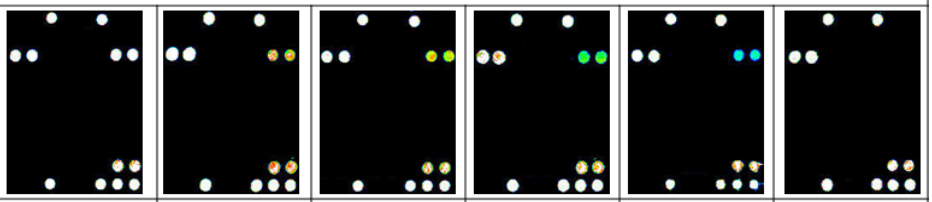
Sensitivity of CDT in the detection and discrimination of Wild and Mutant TB strains in a mixed sample						
						
Copies of genomic DNA's of wild TB strain	**100**	**100**	**100**	**100**	**100**	**100**
Copies of genomic DNA's of Mutant TB strain	**100**	**30**	**20**	**10**	**5**	**0**

Figure S5: A) Determination of sensitivity of CDT in the multiple SNP detection of wild and mutant TB strains in the mixed sample (Sample contained 100 copies of genomic DNA of wild TB strain in presence of 0, 5, 10, 20, 30 and 100 copies of the genomic DNA of mutant TB strain)

7. Detection of the multiple mutations at c526 of genomic DNA of TB in the clinical samples
(The fluorescenceimages of results of hybridization are embedded in the spectra of sequencing analysis for each sample)

Sample 1: Wild (CAC)

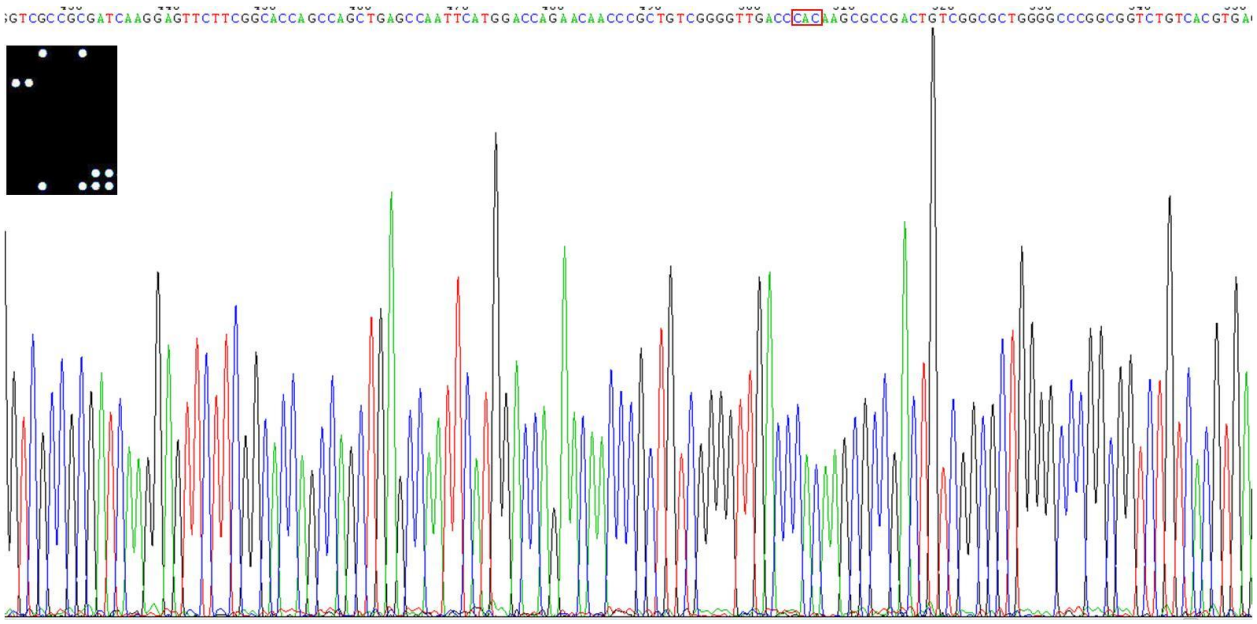


Figure S6: Spectra of sequencing analysis embedded with the fluorescence image for Sample 1.

Sample 2: Wild (CAC)

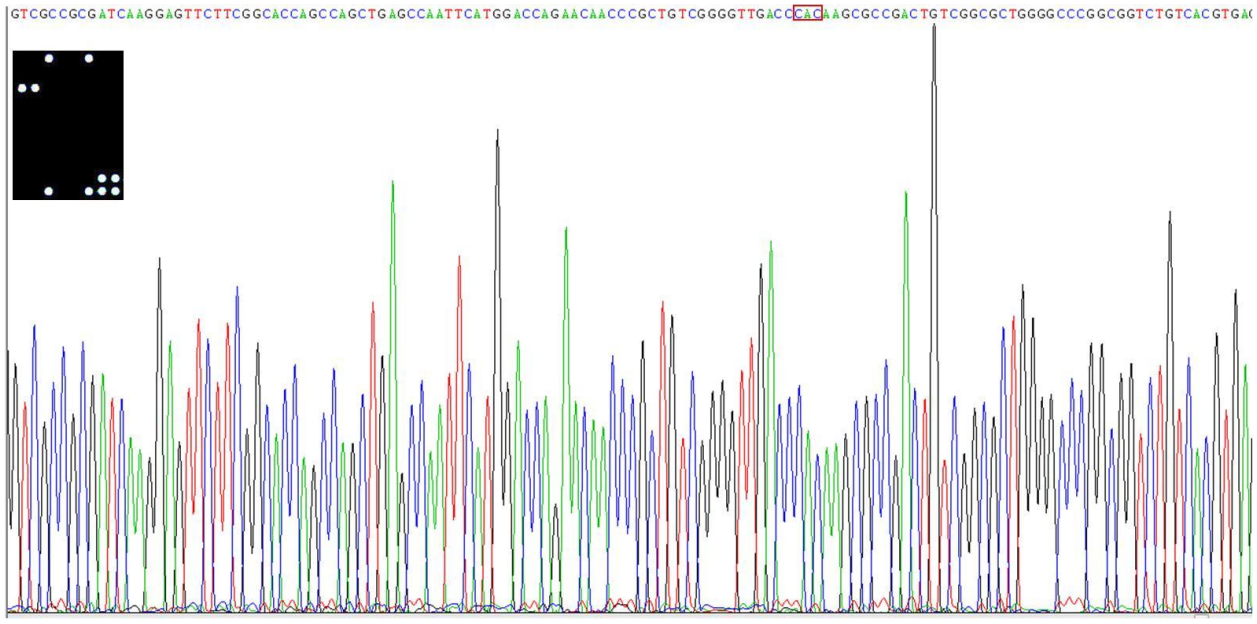


Figure S7: Spectra of sequencing analysis embedded with the fluorescence image for Sample 2.
Sample 3: Wild (CAC)

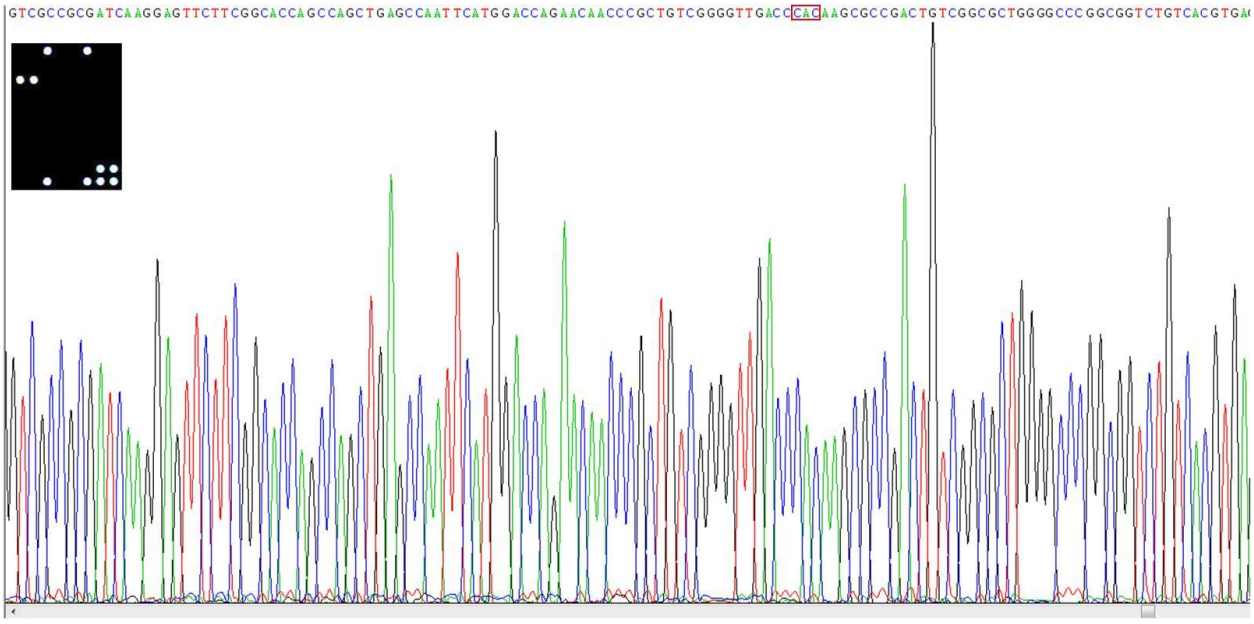


Figure S8: Spectra of sequencing analysis embedded with the fluorescence image for Sample 3.

Sample 4: Wild (CAC)

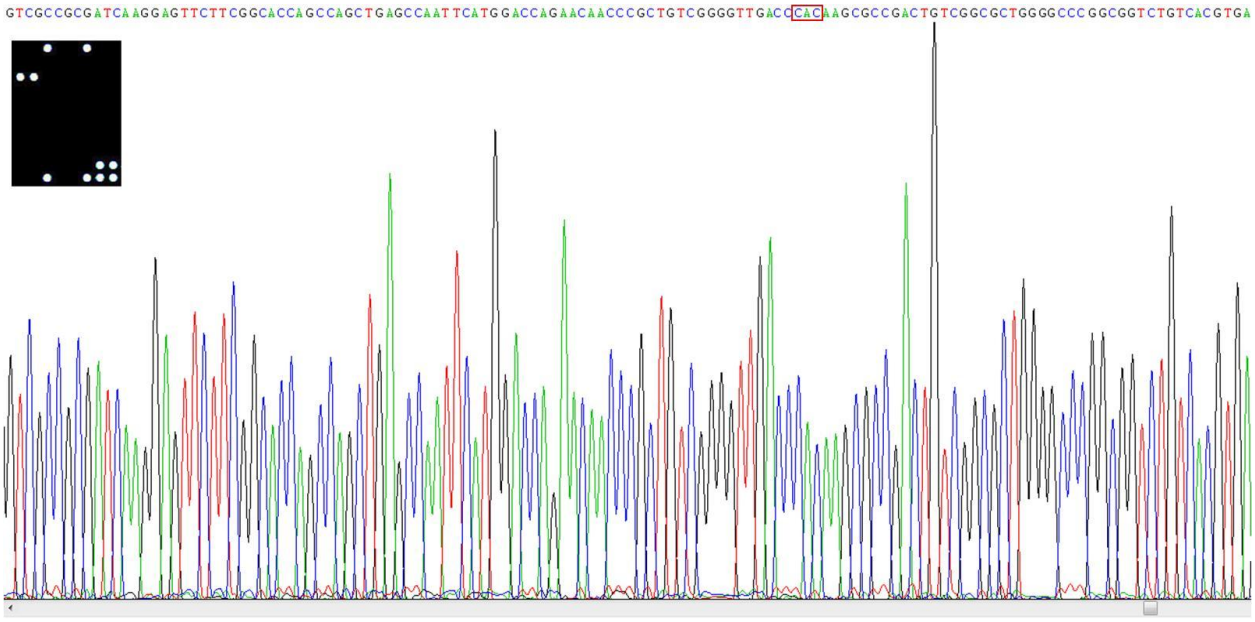


Figure S9: Spectra of sequencing analysis embedded with the fluorescence image for Sample 4.

Sample 5: M1 (CAC->TAC)

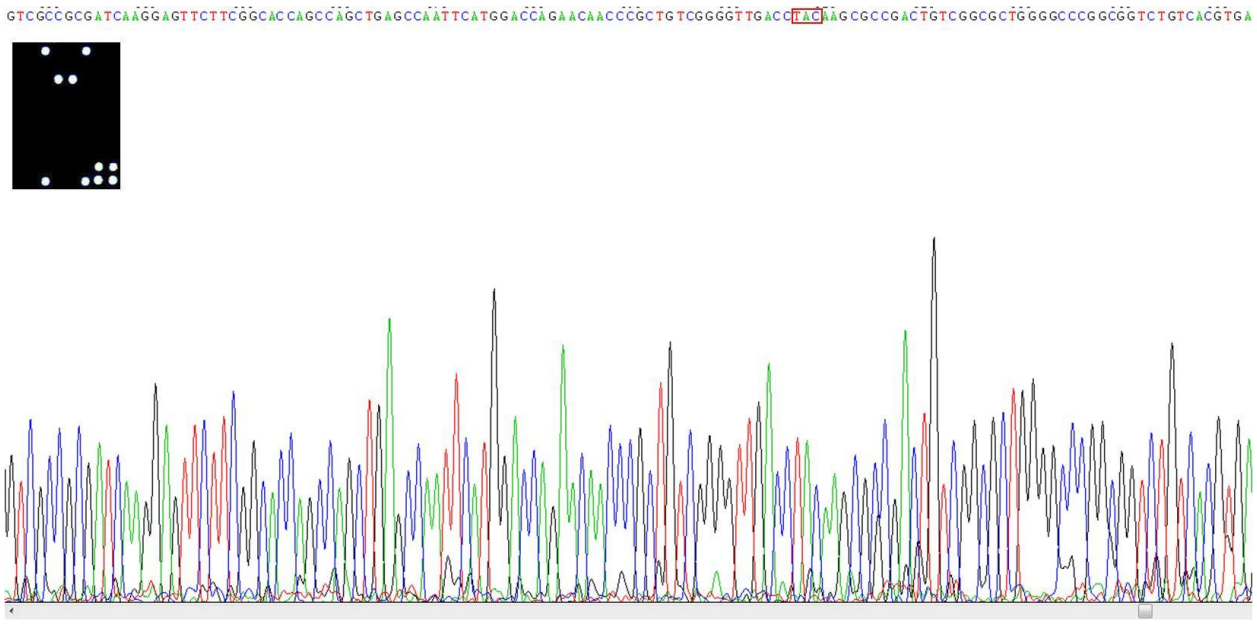


Figure S10: Spectra of sequencing analysis embedded with the fluorescence image for Sample 5.

Sample 6: M2 (CAC->AAC)

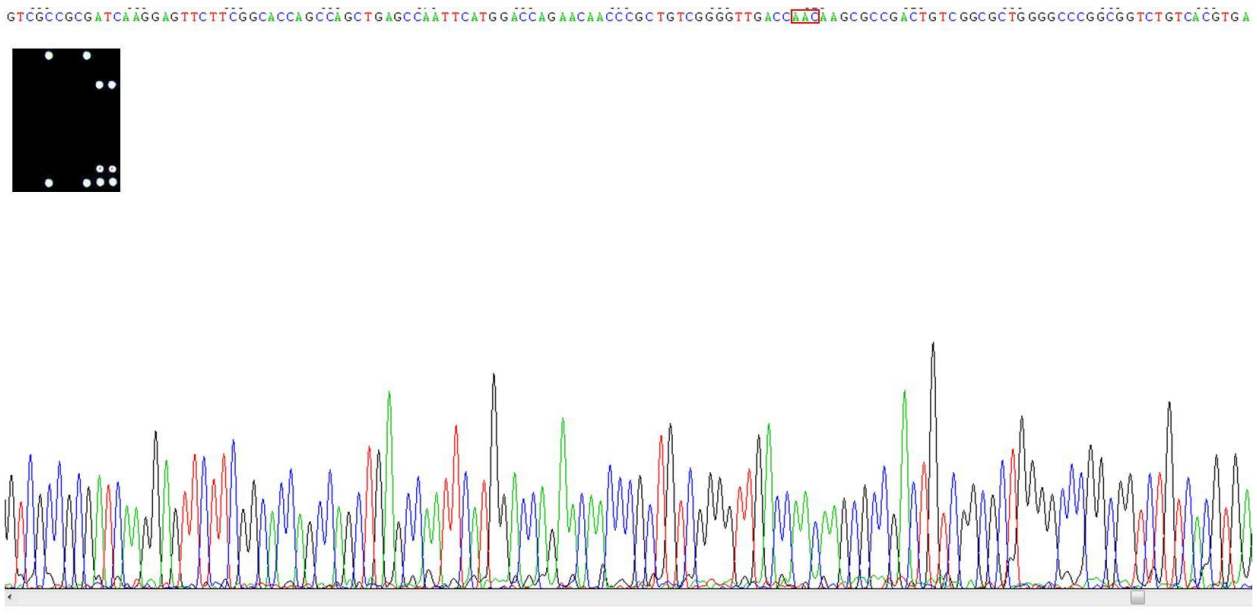


Figure S11: Spectra of sequencing analysis embedded with the fluorescence image for Sample 6.

Sample 7: M3 (CAC->GAC)

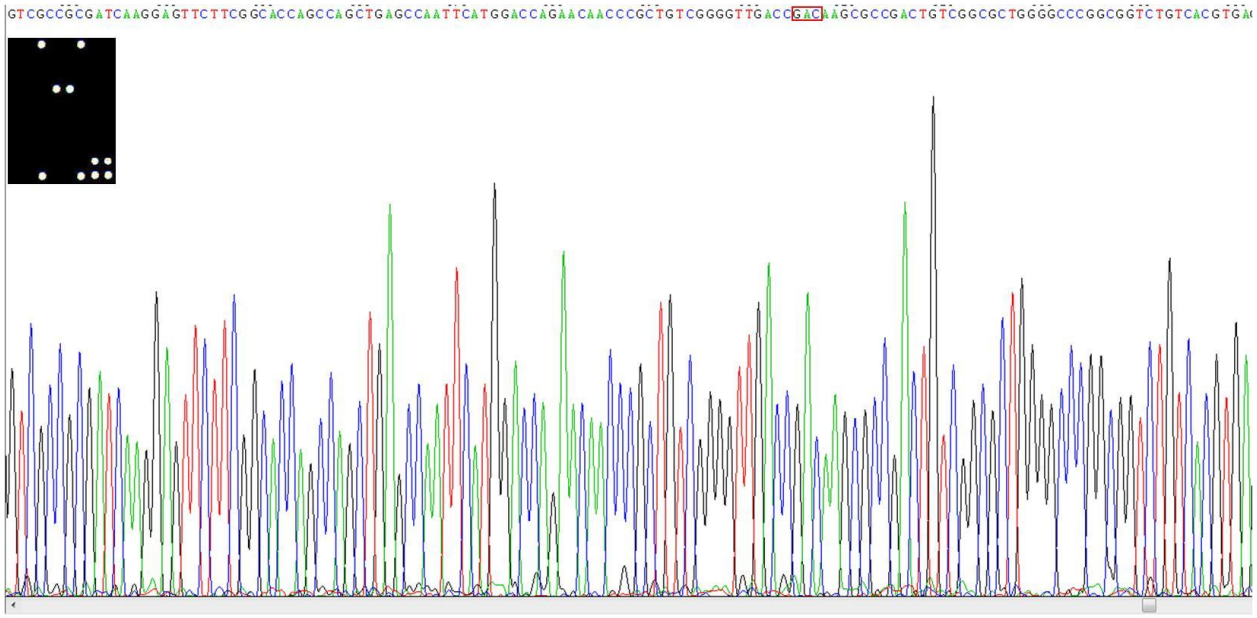


Figure S12: Spectra of sequencing analysis embedded with the fluorescence image for Sample 7.

Sample 8: M4 (CAC->TGC)

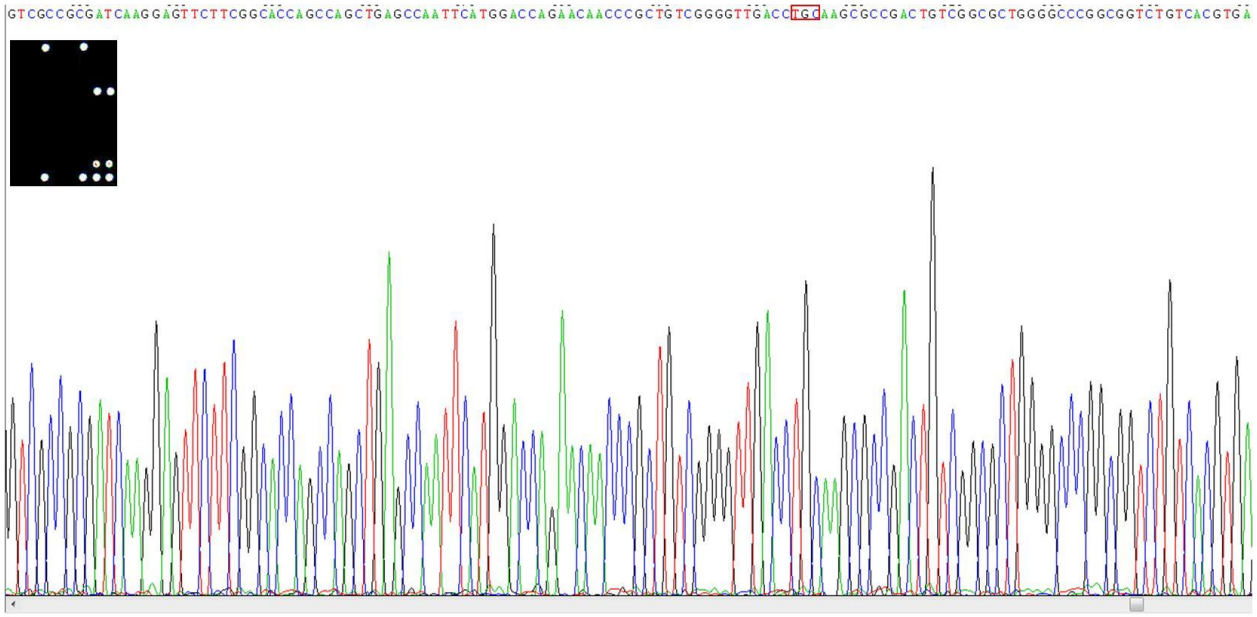


Figure S13: Spectra of sequencing analysis embedded with the fluorescence image for Sample 8.

Sample 8: M5 (CAC->CGC)

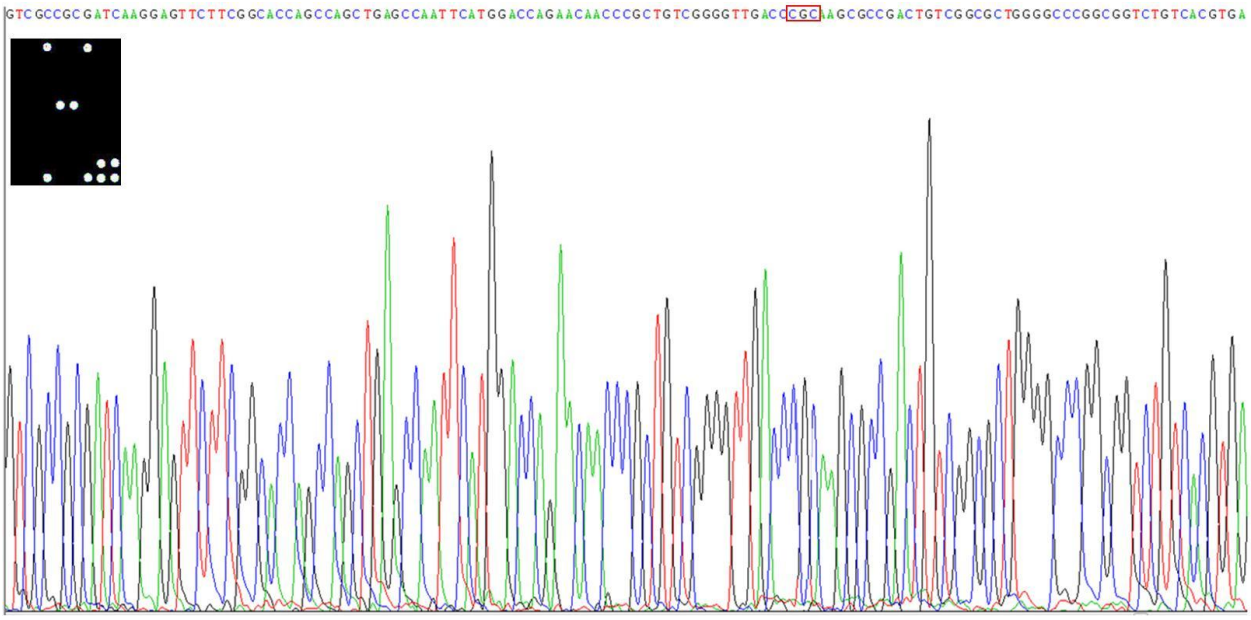


Figure S14: Spectra of sequencing analysis embedded with the fluorescence image for Sample 9.

Sample 9: M6 (CAC->CTC)

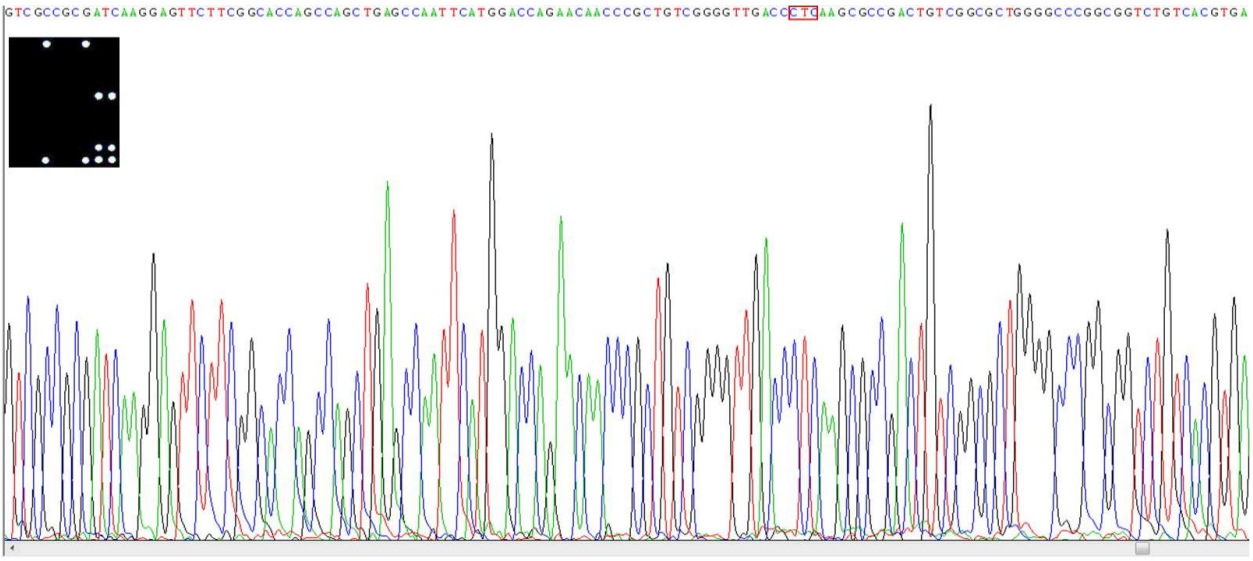


Figure S15: Spectra of sequencing analysis embedded with the fluorescence image for Sample 10.

8. References:

1. J. Wang, L. Lee, C. Chou, C. Huang, S. Wang, H. Lai, P. Hsueh, K. Luh, *J. Clin. Microbiol.*2004,4599 - 4603.

2. a)K. Song, S. B. Nimse, J. Kim, J. Kim, Van-Thuan Nguyen, V. Ta, T. Kim, *Chem. Commun.* 2011, **47**, 7101; b)Van-Thuan Nguyen, S. B. Nimse, K. Song, J. Kim, J. Kim, V. Ta, T. Kim, *Chem. Commun*. 2012, **48**, 4582-4584