Electronic Supplementary Material (ESI) for ChemComm.

Multiplex SNP Detection in Multiple Codons for Accurate Drug Therapy

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Supporting Information

1. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemicals, Korea. All oligonucleotides 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 Sung of 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 MDR-TB 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 primer oligonucleotide sequences

Table 1. Probe and primer sequences

Probes	Description	Sequences	TM (ºC)
Probe1	**c531WIL**D	5'-GGGGGGGGG CTTTATCATAAGCGCCGATTGTCGGC -3'	51℃
Probe2	c531**M1**	5'-GGGGGGGGG CTTTATCAT AAGCGCCGATTGTGGGC -3'	51℃
Probe3	c531**M2**	5'-GGGGGGGGG CTTTATCATAAGCGCCGATTGTTGGC -3'	49℃
Probe4	**c526WILD**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCC ACA AG-3'	51℃
Probe5	c526**M1**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCT ACA AG-3'	49℃
Probe6	c526**M2**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCA ACA AG-3'	49℃
Probe7	c526**M3**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCG ACA AG-3'	51℃
Probe8	c526**M4**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCT GCA AG-3'	51℃
Probe9	c526**M5**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCC GCA AG-3'	53℃
Probe10	c526**M6**	5'-GGGGGGGGG CTTTATCAT CGG GGT TGT CCC TCA AG-3'	51℃
Probe11	**c522 WILD**	5'-GGGGGGGGG CTTTATCAT AAC AAC CCT CTG TCG GG-3'	49℃
Probe12	c522 **M1**	5'-GGGGGGGGG CTTTATCAT AAC AAC CCT CTG TTG GG-3'	47℃
Probe13	c522 **M2**	5'-GGGGGGGGG CTTTATCAT AAC AAC CCT CTG CAG GG-3'	49℃
Probe14	**c516 WILD**	5'-GGGGGGGGG CTTTATCAT GAG CCA ATT CTT GGA CC-3'	47℃
Probe15	c516 **M1**	5'-GGGGGGGGG CTTTATCAT GAG CCA ATT CTT GGT CC-3'	47℃
Probe16	c516 **M2**	5'-GGGGGGGGG CTTTATCAT GAG CCA ATT CTT GTA CC-3'	45℃
Probe17	c516 **M3**	5'-GGGGGGGGG CTTTATCAT GAG CCA ATT CTT TTA CC-3'	45℃
Probe18	c516 **M4**	5'-GGGGGGGGG CTTTATCAT GAG CCA ATT CTT GGG CC-3'	49℃
Probe19	**c511 WILD**	5'-GGGGGGGGG CTTTATCAT CGG TAC CAG TCA GCT GA-3'	49℃
Probe20	c511 **M1**	5'-GGGGGGGGG CTTTATCAT CGG TAC CAG TCA GCC GA-3'	51℃
Probe21	PC	5'-GGG GGG GGG CTT TAT CAT GGC GGT CTG TCA CGT GA-3'	51℃
Probe22	PCR	5'-GGG GGG GGG CTT TAT CAT GCG CTG GGG CCC GGC-3'	53℃
Probe23	Cy5-Reverse Primer (Cy5-RP)	5'-Cy5-TCA CGT GAC AGA CCG CCG GG-3'	
Probe24	Forward Primer (FP)	5'-GTC GCC GCG ATC AAG GAG TTC-3'	

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 TAT CAT – vertical spacer group; c511, c516, c522, c526, and c531 – Codon containing mutation; M1-M6 – corresponds to the mutations in the corresponding codons.

Table 2: Controller DNA (COD) sequences

Controller DNA's corresponding to codons	Description	Sequence
c531	**531-C**	GCGCCGACTG
c526	**526-C**	GGGGTTGACC
c522	**522-C**	AGAACAACCC
c516	**516-C**	AGCCAATTCAT
c511	**511-C**	CGGCACCAGC
PC	**PC-C**	CCGGCGGTCTGT

4. Methods and experimental procedures:

4.1 DNA extraction from Human sputum of TB samples:

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°C. 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 MDR-TB 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°C for 5 min and then DNA polymerization for 60 cycles at 94°C for 10 s, 60°C for 10 s, and 72°C 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.

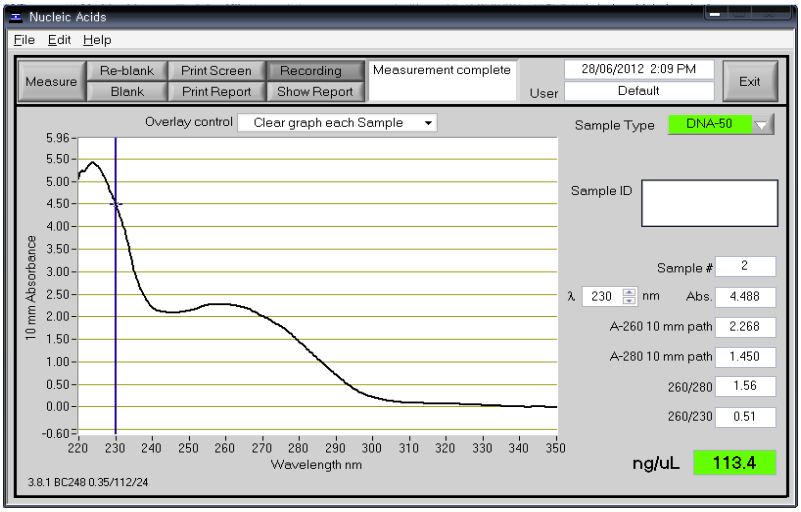


Figure S1: Determination of concentration in the final PCR product by using nanodrop

Table S3: DNA Concentration and Copy number for the PCR product of the 620 base pairs

Sr. no.	Initial concentration	Dilution	Final Concentration	Copy number
1	113ng/µL	1µL diluted to 113µL	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 MDR-TB 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°C for 5 min and then DNA polymerization for 60 cycles at 94°C for 10 s, 60°C for 10 s, and 72°C 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).

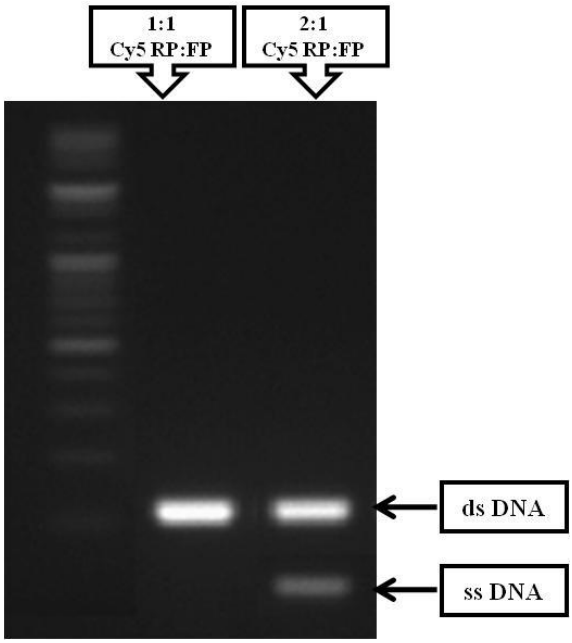
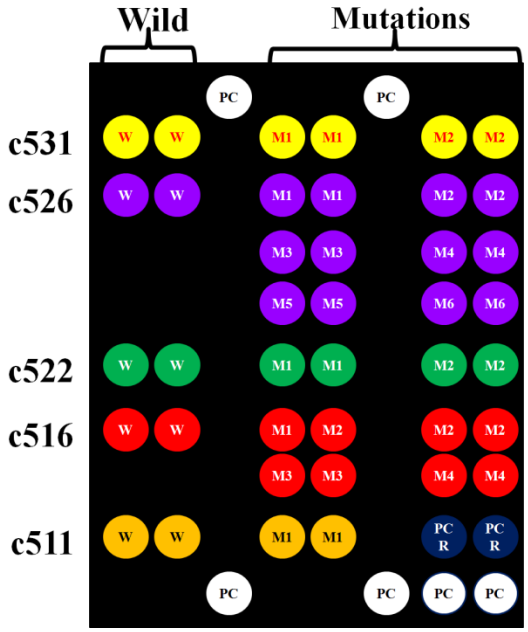


Figure S2. Gel electrophoresis images of the PCR products

4.3 Typical method for the preparation of the MDR-TB 9G DNAChip:

The MDR-TB 9G DNAChips were prepared by spotting the immobilization solution containing oligonucleotide 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 (25^0C, 50% humidity) for 4h to immobilize the oligonucleotides. The slides were then suspended in the blocking buffer solution at 25^0C for 30 min, in order to remove the excess oligonucleotides 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 MDR-TB 9G DNAChips. Before hybridization, the MDR-TB 9G DNAChips were covered with Secure-Seal™ hybridization chambers.

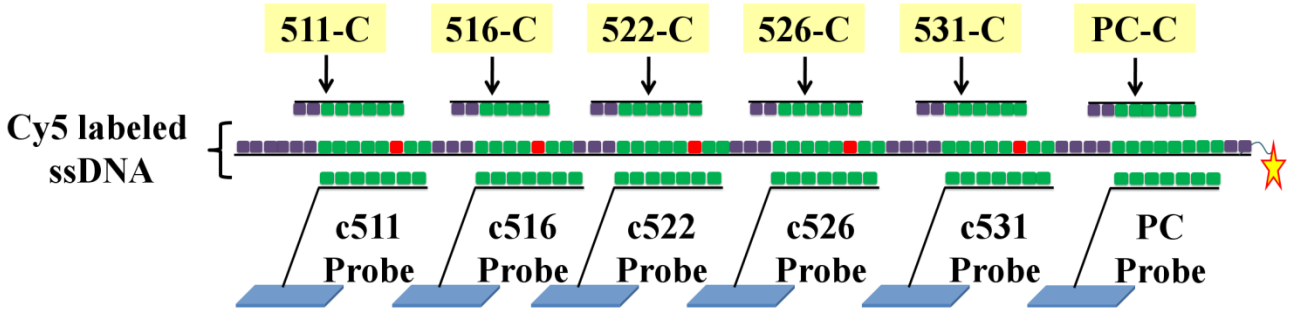


Codon	Wild	M1	M2	M3	M4	M5	M6
c531	TCG	TGG	TTG				
c526	CAC	TAC	AAC	GAC	TGC	CGC	CTC
c522	TCG	TTG	CAG				
c516	GAC	GTC	TAC	TTC	GGC		
c511	CTG	CCG					

PC = Positive Control
PCR = PCR Control

Scheme S1: Scheme for immobilization probes to produce MDR-TB 9G DNAChips

5. Positions of mutations, respective probes, and controller DNA's



Scheme S2: Positions of mutations on the Cy5 ssDNA and binding positions of the corresponding immobilized probes and controller DNA's (mutations are depicted in the red color).

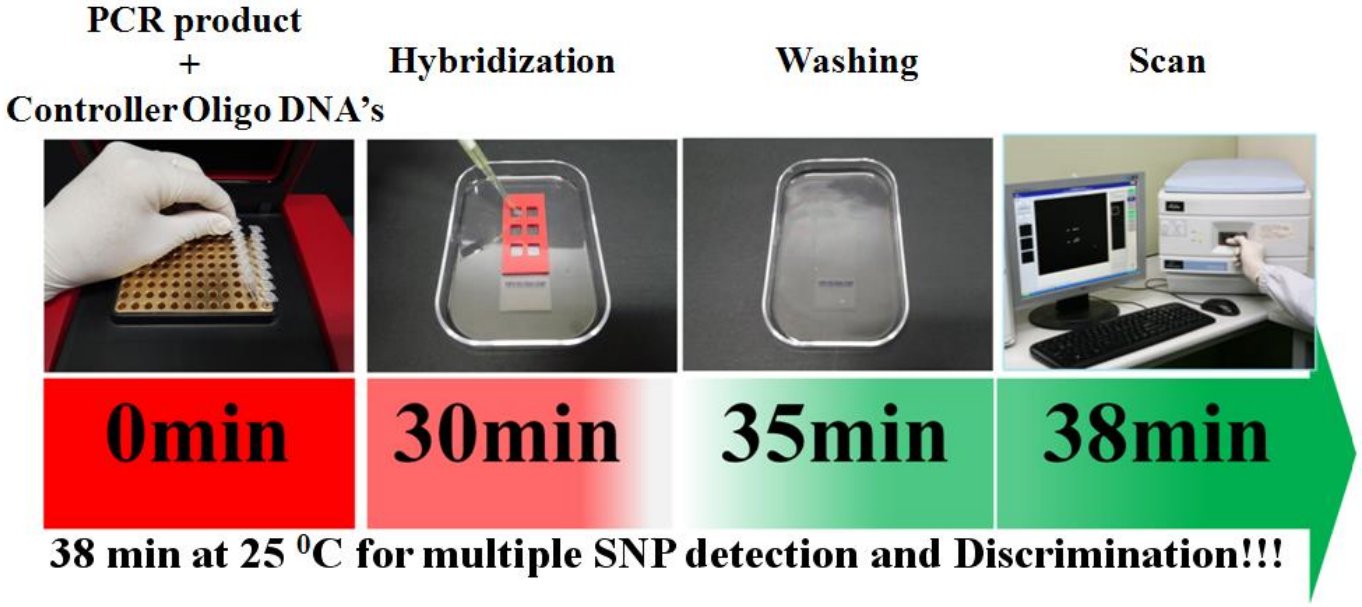
6. Hybridization and washing method

6.1 Hybridization with the Cy5 Labeled PCR product of TB (in absence of controller DNA's)

Hybridizations were done by using the 100fmol of the Cy5 labeled PCR products of the TB at 25^0C for 30min in the commercial incubator. Then MDR-TB 9G DNAChips was rinsed with washing buffer solutions A and B successively for 2 min each at 35^0C, in order to remove the excess target DNA, and dried with commercial centrifuge (1000 rpm). The fluorescence signal of the microarray was measured on ScanArrayLite, and the images were analyzed by Quant Array software (Packard Bioscience).

6.2 Hybridization of the Cy5 Labeled PCR products of TB, MDR-TB strains (in presence of controller DNA's)

The 100fmol of the Cy5 labeled PCR products of the MTB-DR strains were first mixed with 100 pmol of each of the controller DNA's in the hybridization buffer. Then this mixture was loaded on the MDR-TB 9G DNAChips and allowed to hybridize at 25^0C for 30min in the commercial incubator. Then the MDR-TB 9G DNAChips were rinsed with washing buffer solutions A and B successively for 2 min each at 35^0C, 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.

6.3 Effect of the single stranded Cy5 Labeled PCR product (Cy5 labeled ssDNA) and the double stranded Cy5 Labeled PCR product (Cy5 labeled dsDNA) on the hybridization yield.

For the discrimination of multiple SNP's, the probes immobilized on the MDR-TB 9G DNAChips were allowed to hybridize at 25 ^{0}C for 30 min with the 100 fmol of the Cy5 ssDNA of the wild TB strain, the results are depicted in the **Figure S3**. The **Figure S3** demonstrate that, the PCR product obtained by the 1:1 ratio of reverse primer (RP): forward primer (FP) did not show good hybridization ability with the immobilized probes. The probes corresponding to the positive control (PC), codon 511 (c511), and codon 516 (c516) showed target specific hybridizations but with a very low signal intensities (15000 - 36000). However, the probes corresponding to the codon 522 (c522) codon 526 (c526), and codon 531 (c531) did not hybridize with the PCR product at all.

It was understood that the PCR product obtained by the 1:1 ratio of Cy5-RP:FP mainly contain the dsDNA, which do not show good hybridization ability on the microarray **Figure S3 A**. Therefore to generate ample amount of ssDNA, an asymmetric PCR was performed by using 2:1 ratio of Cy5-RP:FP.[3] The PCR product obtained by the asymmetric PCR (2:1 ratio of Cy5-RP:FP) demonstrated improved hybridization ability with the immobilized probes as shown in the **Figure S3 B**. The probes corresponding to the PC, c511, and c516 showed increased intensities (46000 - 65000). Moreover, the probes corresponding to the c522, c526, and c531 also showed hybridization but with a very low signal intensities (4000 - 4800). It is very clear from the **Figure S3 B** that, the probes corresponding to the PC, c511, and c516 bind more strongly than the probes corresponding to the c522, c526, and c531 to the PCR product.

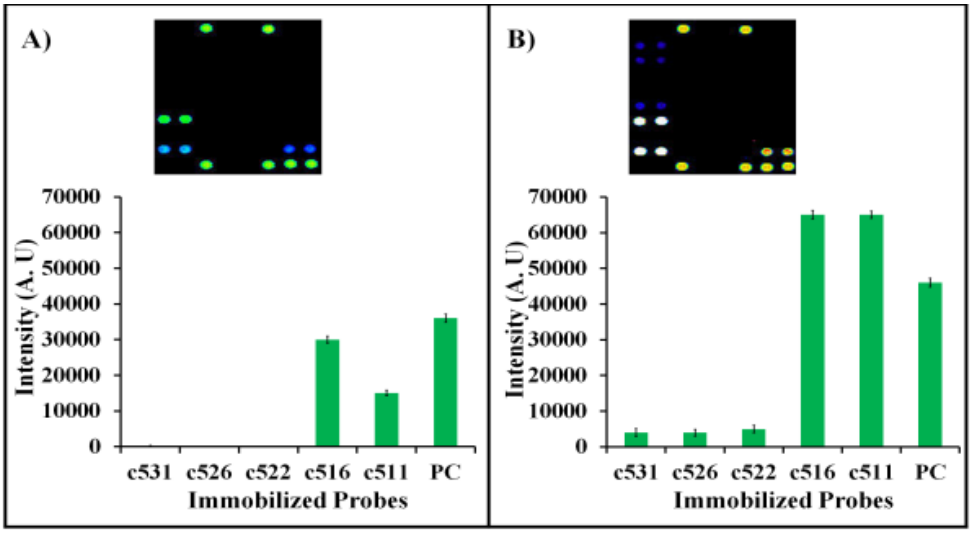


Fig. S3 Fluorescence image and respective graph after hybridization of immobilized probes with PCR product of wild TB strain obtained by Cy5-RP: FP ratio of A) 1:1 and B) 2:1, PMT gain = 53.

7. Sensitivity of Controller DNA Technology

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

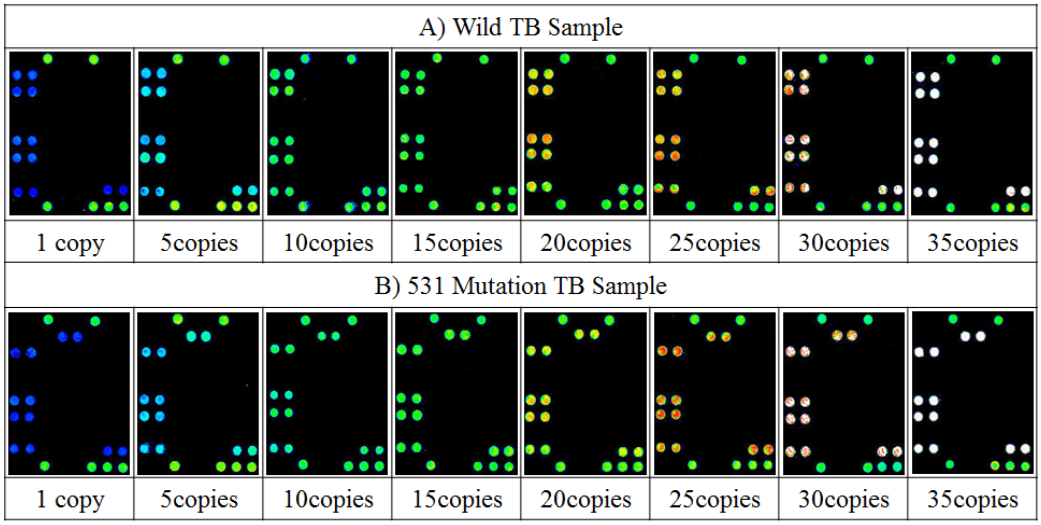


Figure S4: A) Determination of sensitivity of CDT by using 1 – 35 copies of genomic DNA of the wild TB strain, B) Determination of sensitivity of CDT by using 1 – 35 copies of genomic DNA of the mutant TB strain (mutation at c531).

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

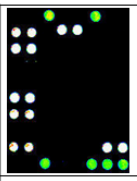
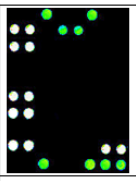
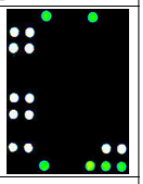
Sensitivity of CDT in the detection and discrimination of Wild and Mutant TB strains in mixed sample						
						
Copies of genomic DNA's of wild TB st rains	100	100	100	100	100	100
Copies of genomic DNA's of mutant TB strains (mutation at c531)	100	30	20	10	5	0

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 (mutation at c531))

8. Detection of the MDR-TB strains in the 24 clinical samples

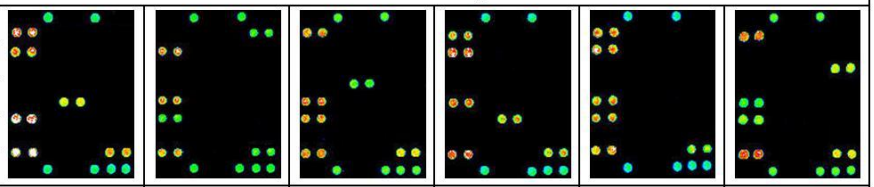
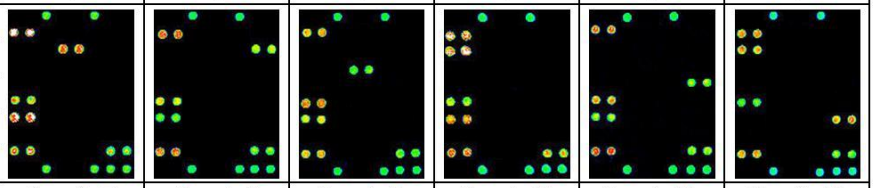
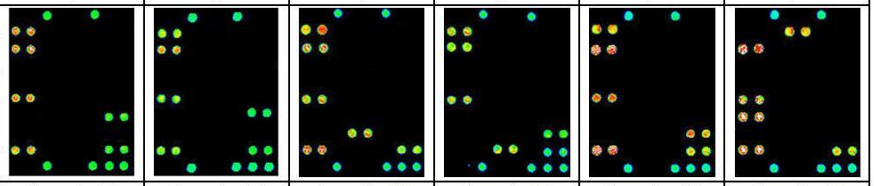
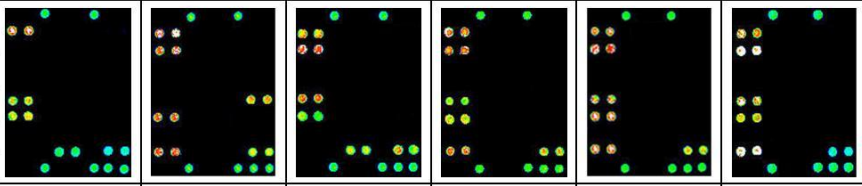
Sample	Mutation codon	Clinical Sample					
Sample-1	522 (TCG→TTG)						
Sample-2	531(TCG→TTG)						
Sample-3	526(CAC→CGC)						
Sample-4	516(GAC→GTC)						
Sample-5	Susceptible						
Sample-6	526(CAC→TGC)	Sample-1	Sample-2	Sample-3	Sample-4	Sample-5	Sample-6
Sample-7	526 (CAC→TAC)						
Sample-8	526(CAC→AAC)						
Sample-9	526(CAC→GAC)						
Sample-10	Susceptible						
Sample-11	526(CAC→CTC)						
Sample-12	516(CAC→TAC)	Sample-7	Sample-8	Sample-9	Sample-10	Sample-11	Sample-12
Sample-13	516 (GAC→TAC)						
Sample-14	516(GAC→TAC)						
Sample-15	516(GAC→TTC)						
Sample-16	511(CTG→CCG) 516(GAC→GGC)						
Sample-17	516(GAC→GGC)						
Sample-18	531(TCG→TGG)	Sample-13	Sample-14	Sample-15	Sample-16	Sample-17	Sample-18
Sample-19	511(CTG→CCG) 526(CAC→CAA)						
Sample-20	522 (TCG→CAG)						
Sample-21	511(CTG→CCG)						
Sample-22	Susceptible						
Sample-23	Susceptible						
Sample-24	Susceptible	Sample-19	Sample-20	Sample-21	Sample-22	Sample-23	Sample-24

Figure S6: Detection of the multiple mutations in the MDR-TB strains in the clinical samples.

9. Sequencing analysis of sample -1 -24

Add the sequencing data with the embedded chip result for the same sample. (24 samples 24 sequencing spectra)

Sample 1: 522 (TCG→TTG)

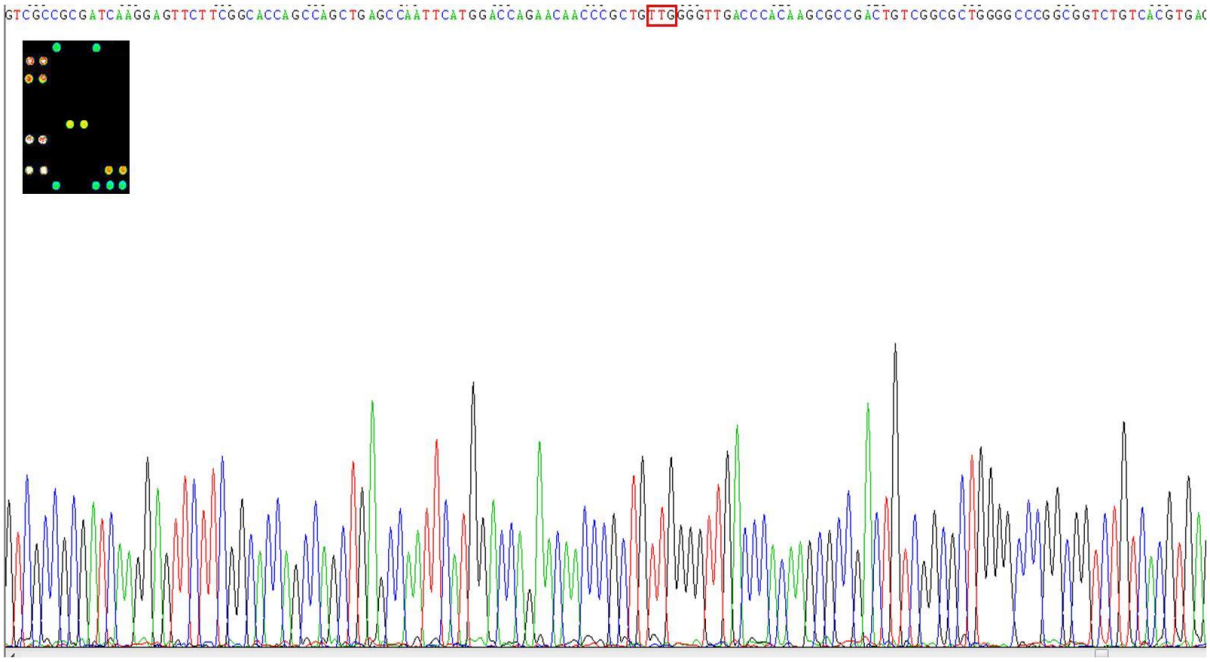


Figure S7: Spectra of sequencing analysis embedded with the fluorescence image for Sample1

Sample 2: 531(TCG→TTG)

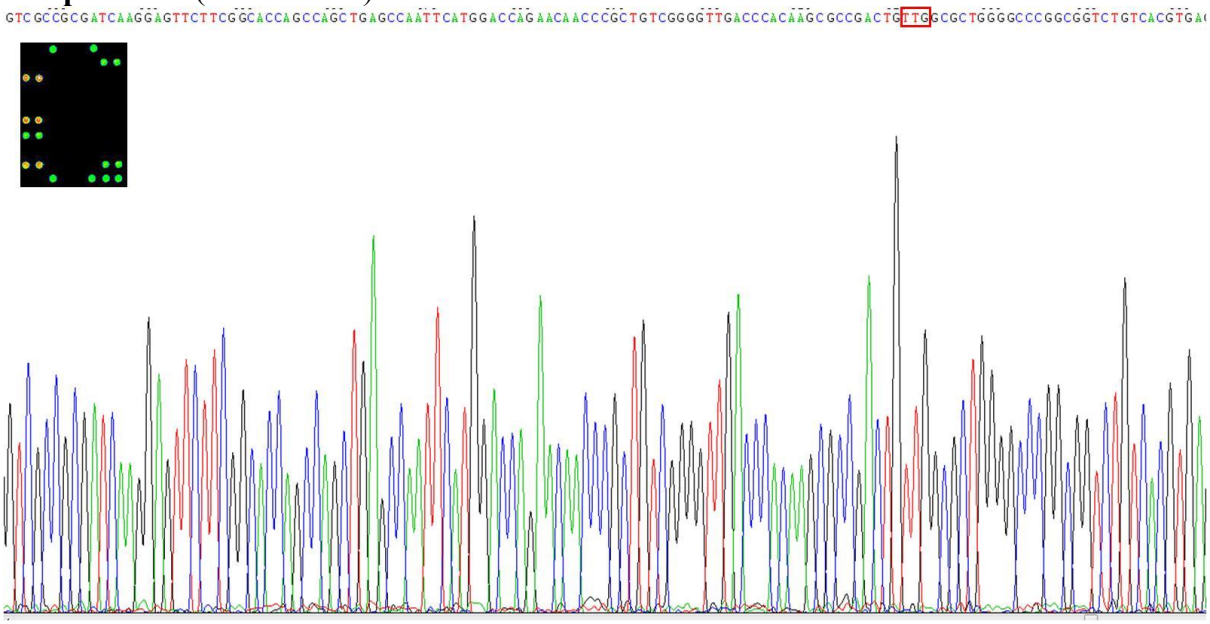


Figure S8: Spectra of sequencing analysis embedded with the fluorescence image for Sample2

Sample 3: 526 (CAC→CGC).

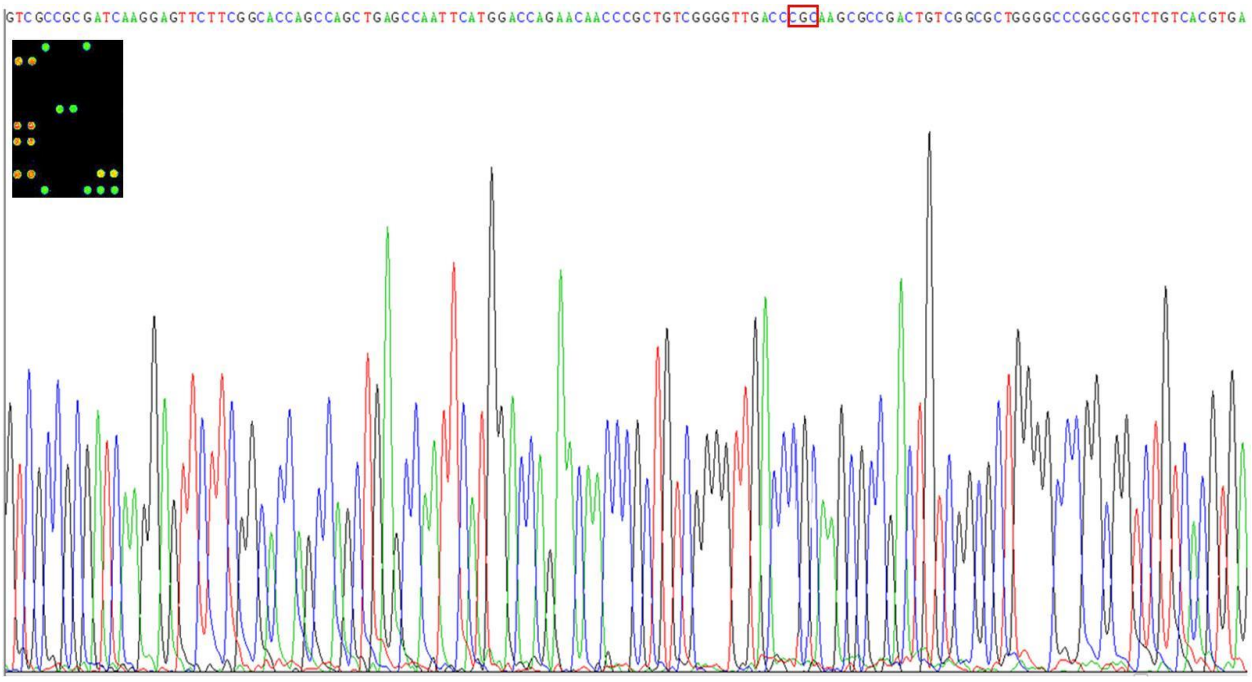


Figure S9: Spectra of sequencing analysis embedded with the fluorescence image for Sample 3

Sample 4: 516 (GAC→GTC)

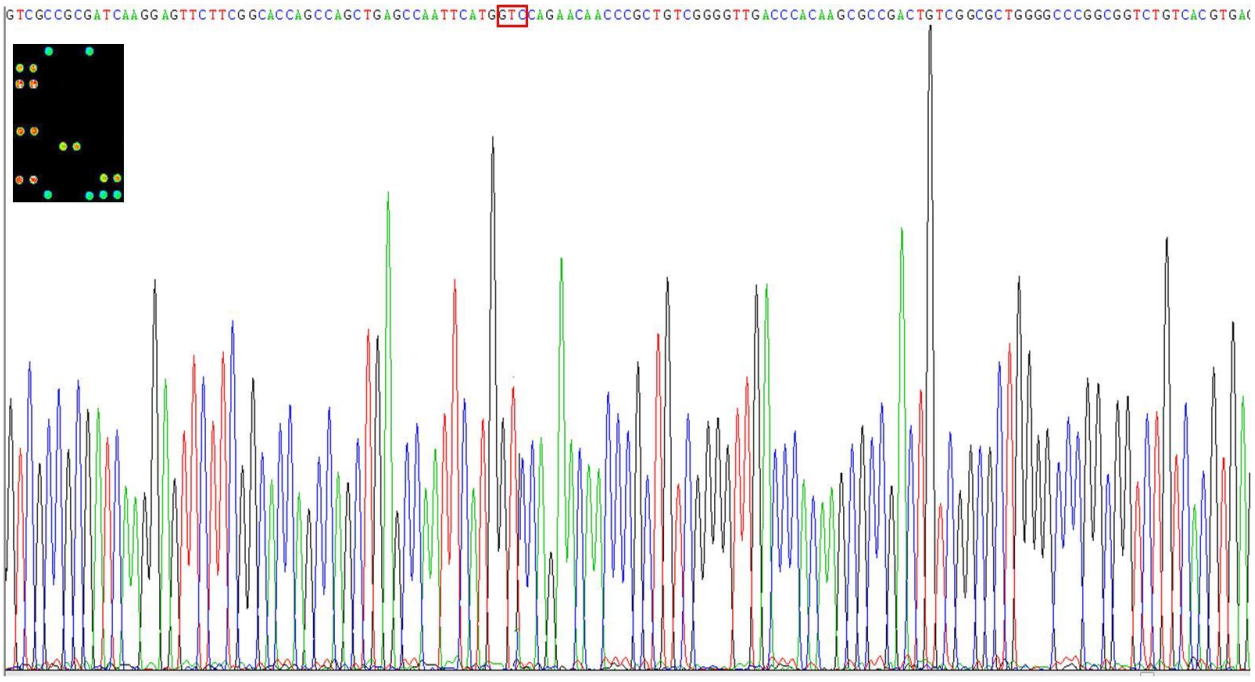


Figure S10: Spectra of sequencing analysis embedded with the fluorescence image for Sample 4

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Sample 5: Susceptible

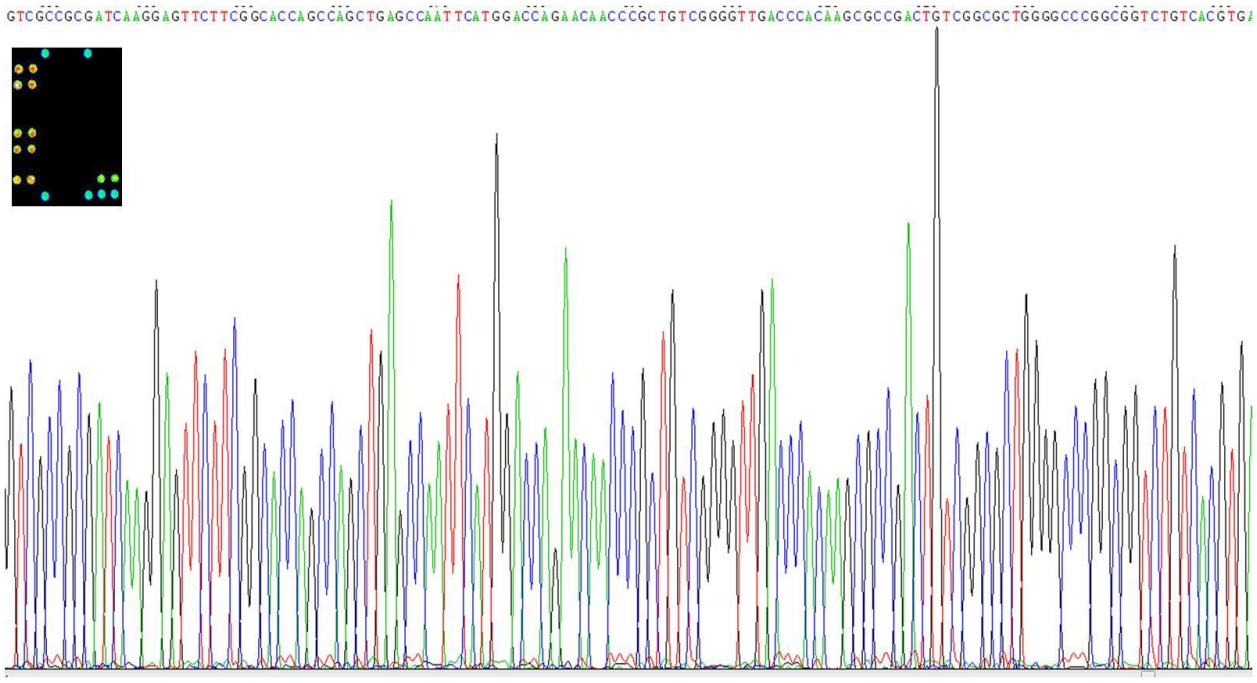

Figure S11: Spectra of sequencing analysis embedded with the fluorescence image for Sample 5

Sample 6: 526 (CAC→TGC)

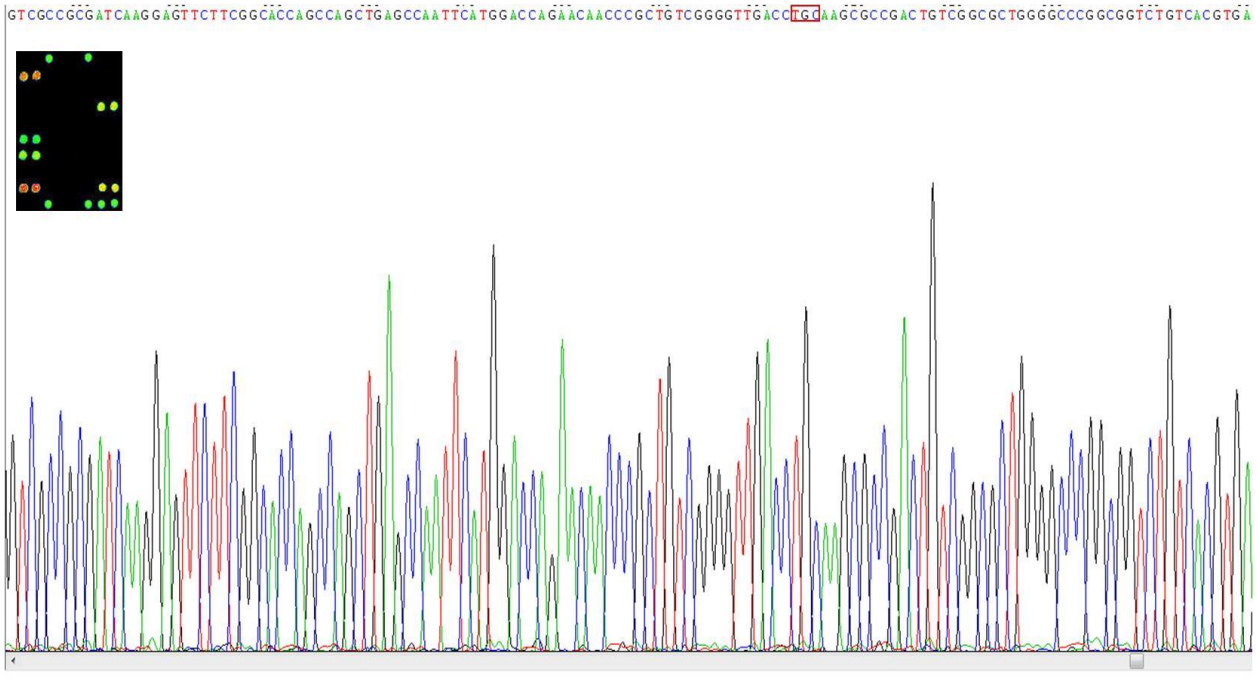

Figure S12: Spectra of sequencing analysis embedded with the fluorescence image for Sample 6

Sample 7: 526 (CAC→TAC)

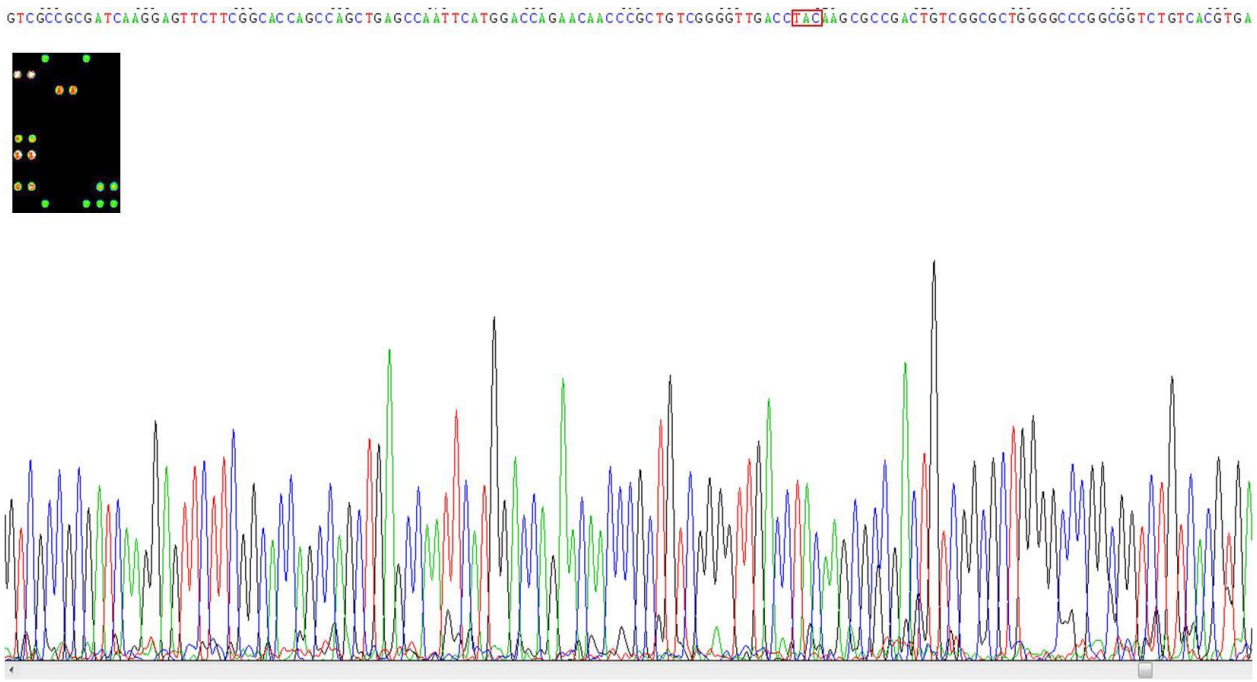

Figure S13: Spectra of sequencing analysis embedded with the fluorescence image for Sample 7

Sample 8: 526(CAC→AAC)

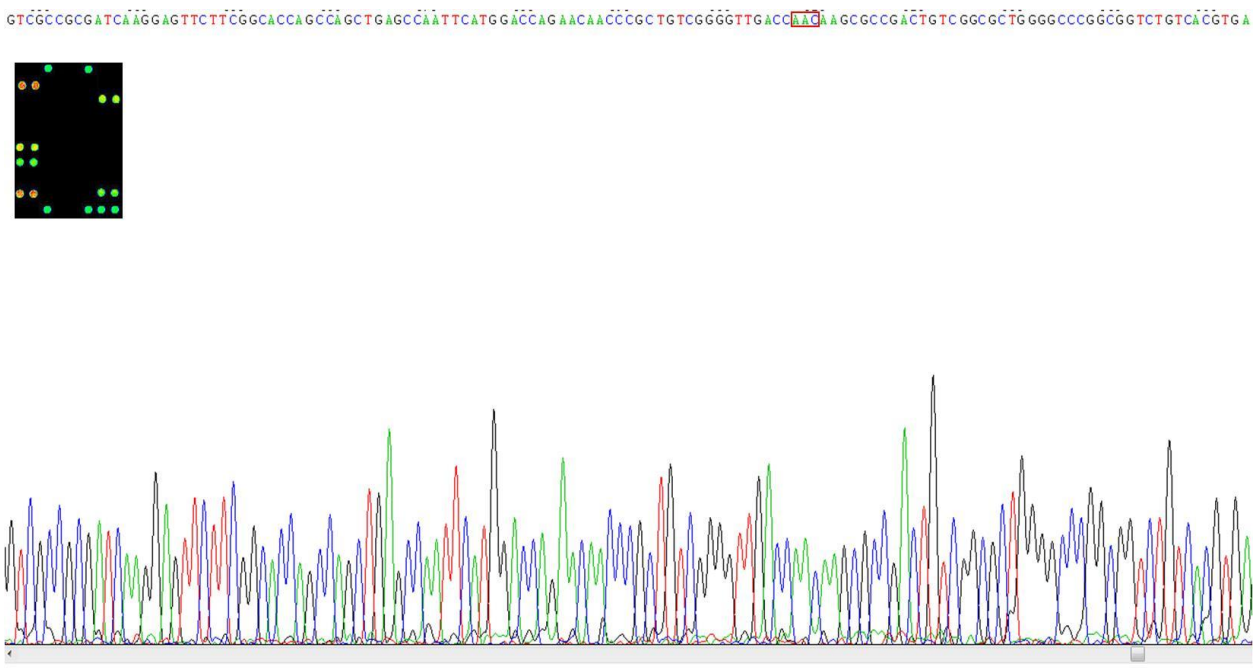

Figure S14: Spectra of sequencing analysis embedded with the fluorescence image for Sample 8

Sample 9: 526 (CAC→GAC)

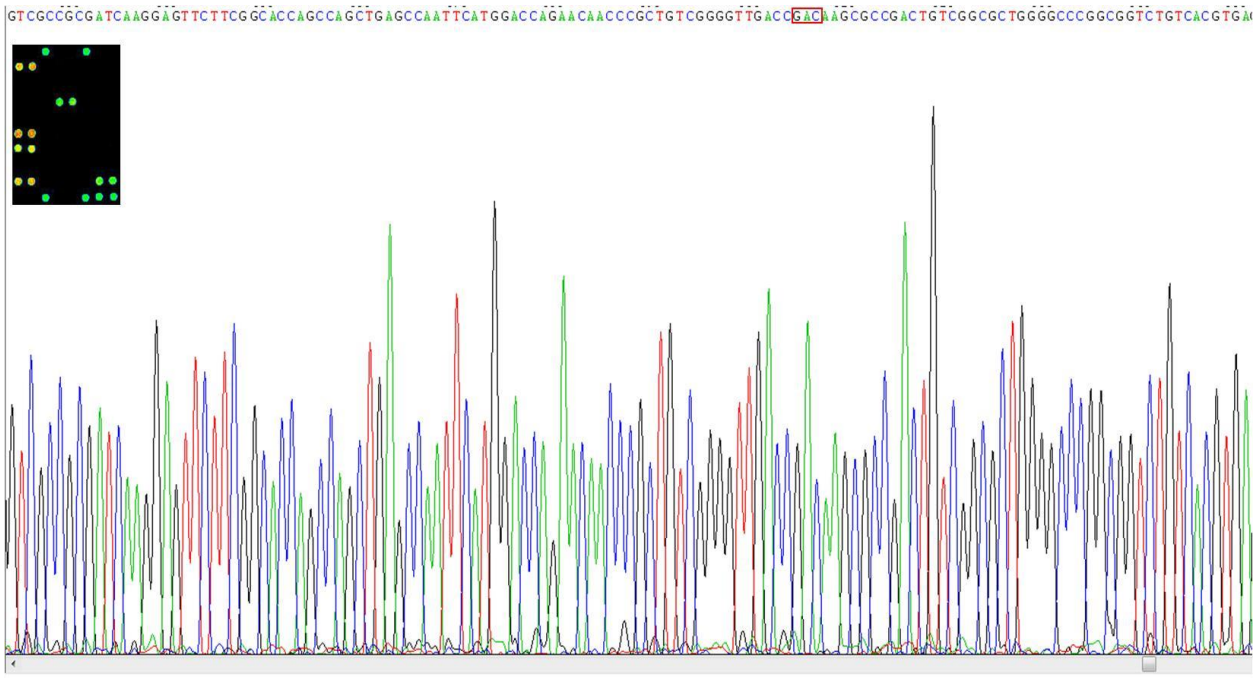

Figure S15: Spectra of sequencing analysis embedded with the fluorescence image for Sample 9

Sample 10: Susceptible

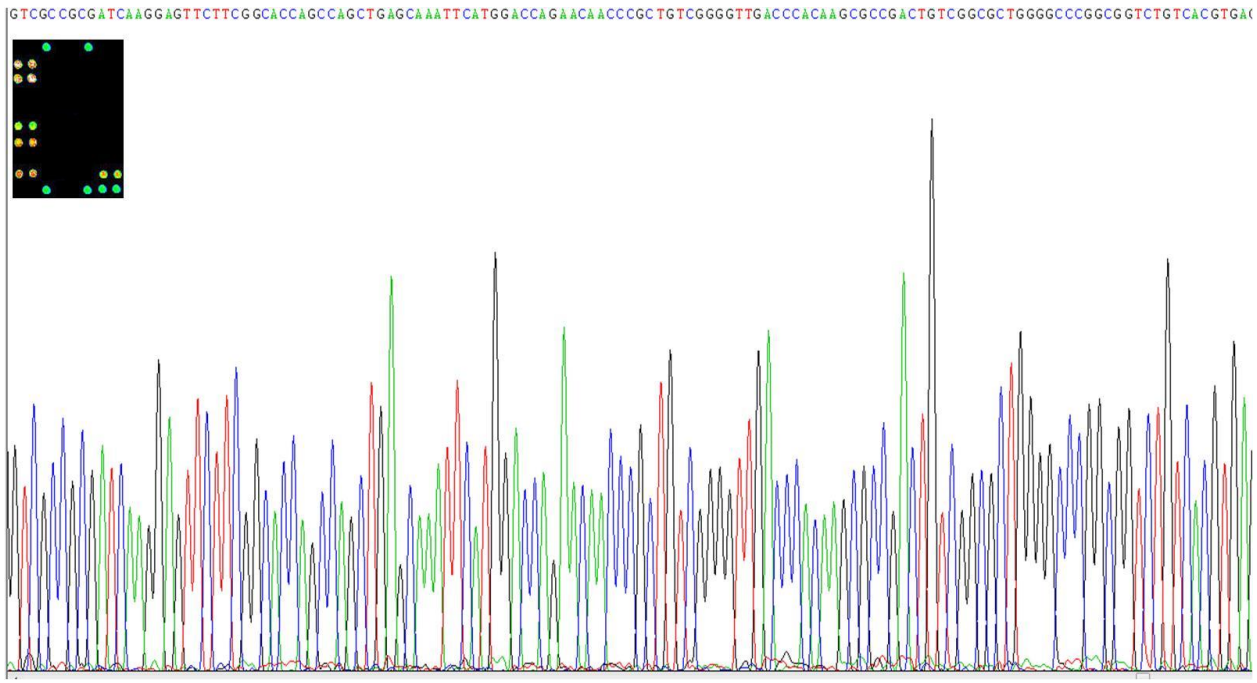

Figure S16: Spectra of sequencing analysis embedded with the fluorescence image for Sample 10

Sample 11: 526 (CAC→CTC)

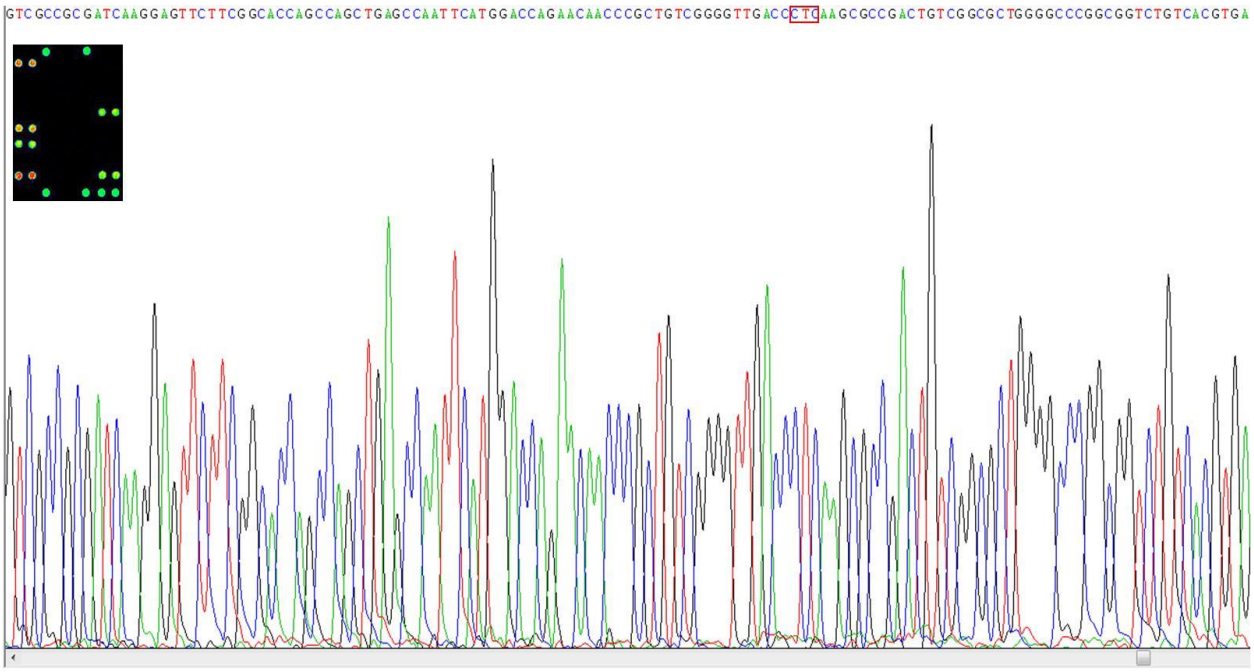

Figure S17: Spectra of sequencing analysis embedded with the fluorescence image for Sample 11

Sample 12: 516 (CAC→TAC)

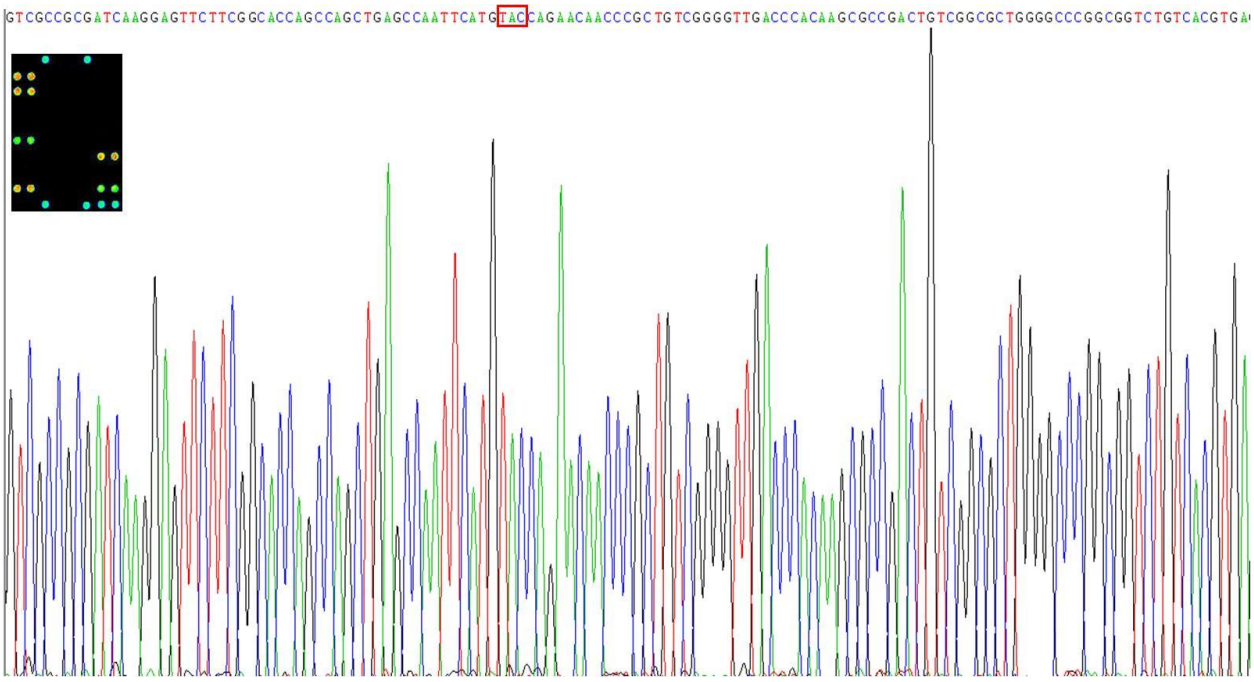

Figure S18: Spectra of sequencing analysis embedded with the fluorescence image for Sample 12

Sample 13: 516 (GAC→TAC)

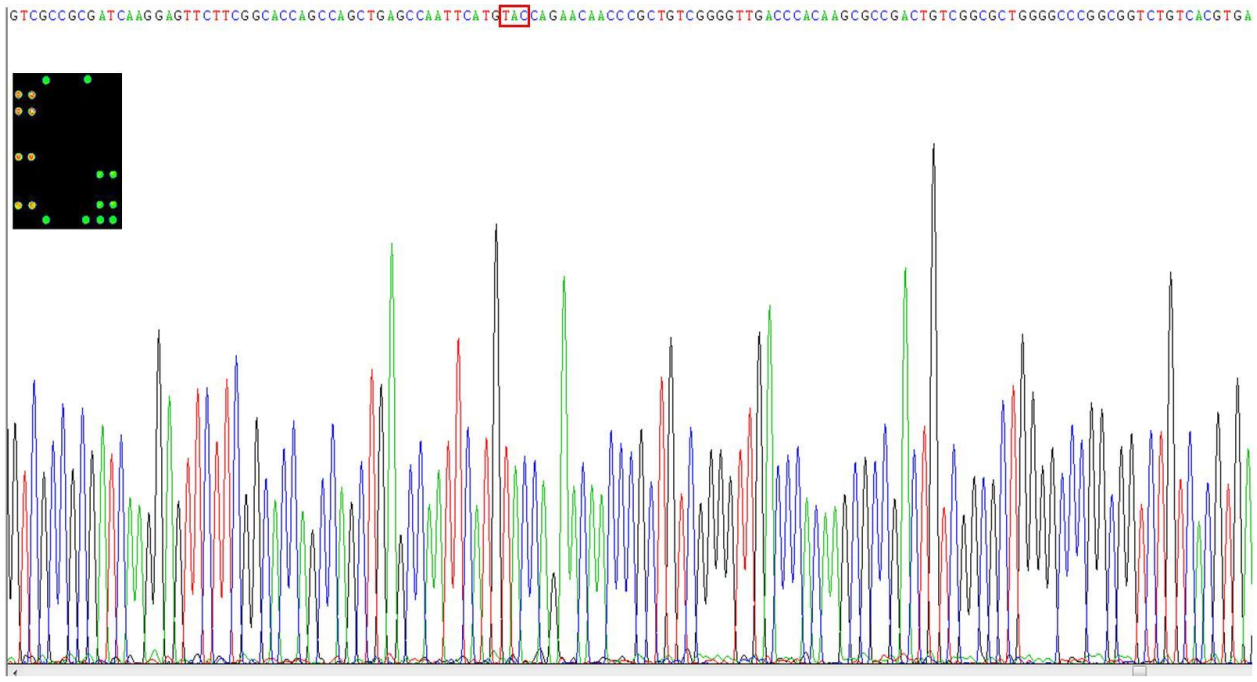

Figure S19: Spectra of sequencing analysis embedded with the fluorescence image for Sample 13

Sample 14: 516 (GAC→TAC)

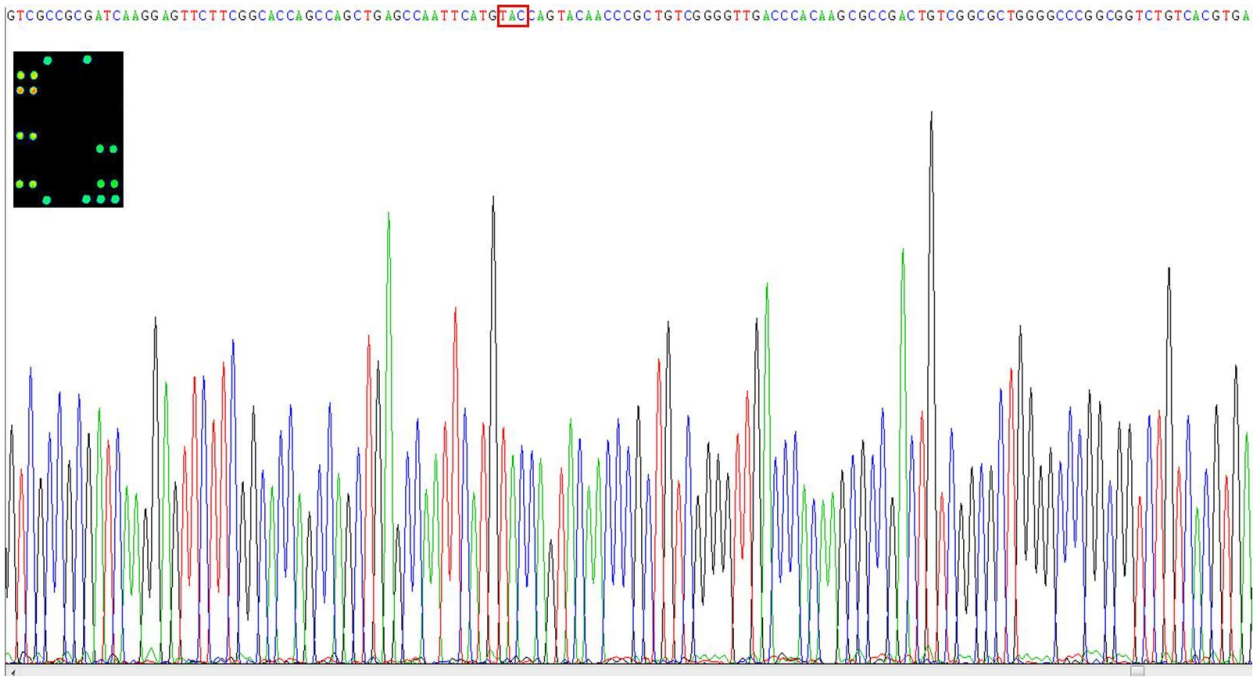

Figure S20: Spectra of sequencing analysis embedded with the fluorescence image for Sample 14

Sample 15: 516 (GAC→TTC)

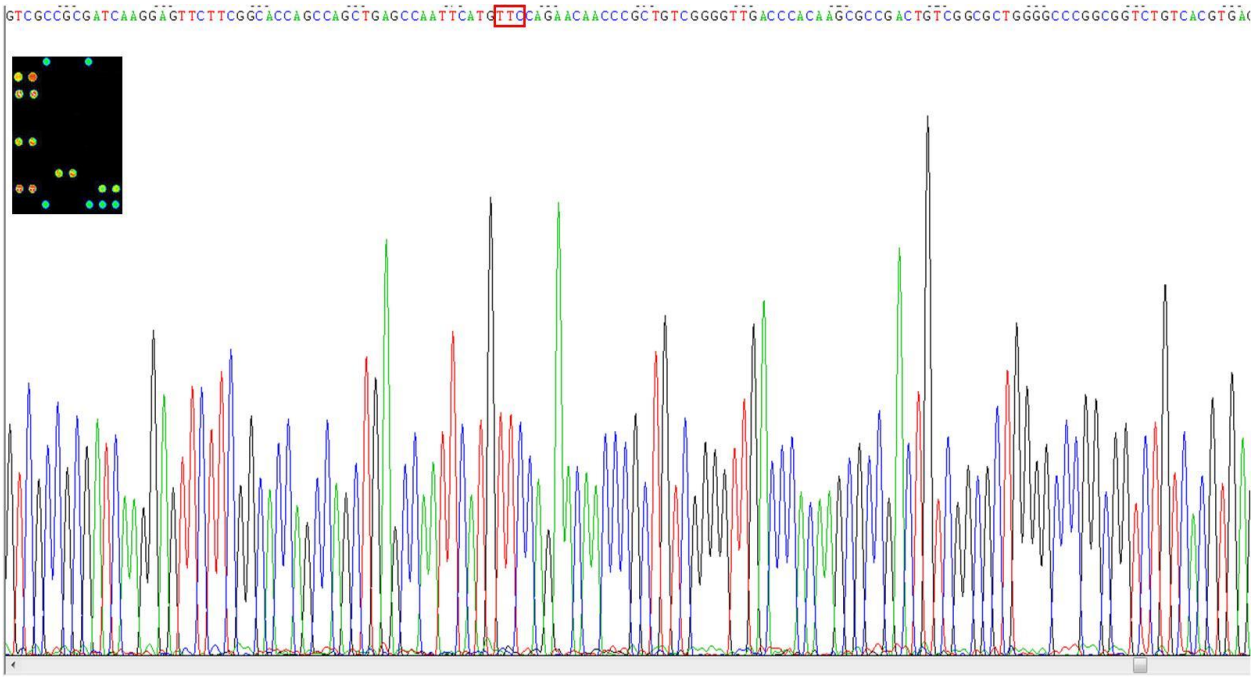

Figure S21: Spectra of sequencing analysis embedded with the fluorescence image for Sample 15

Sample 16: 511(CTG→CCG) 516(GAC→GGC)

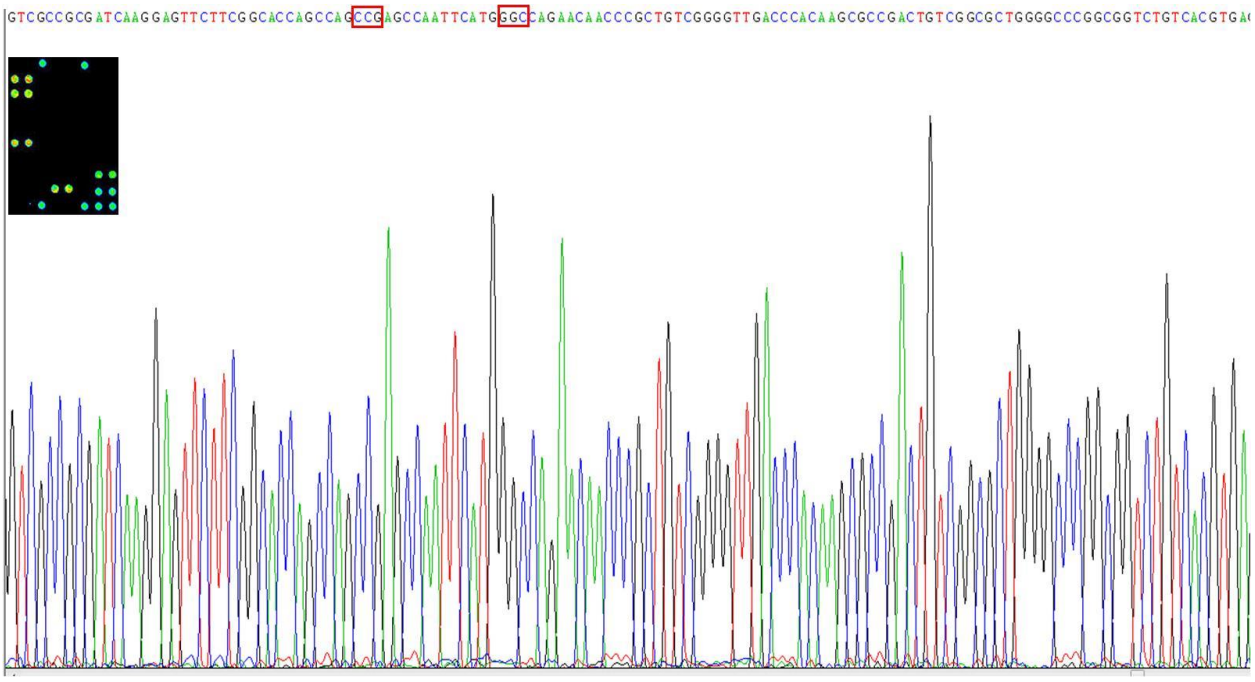

Figure S22: Spectra of sequencing analysis embedded with the fluorescence image for Sample 16

Sample 17: 516 (GAC→GGC)

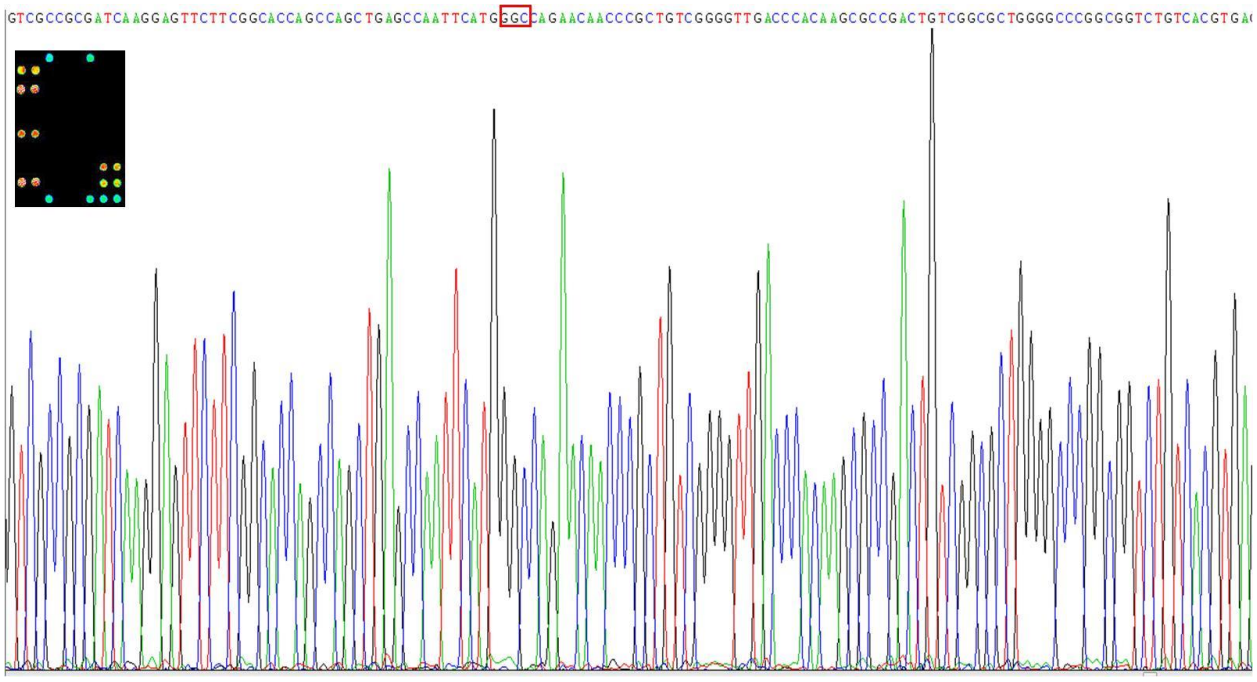

Figure S23: Spectra of sequencing analysis embedded with the fluorescence image for Sample 17

Sample 18: 531(TCG→TGG)

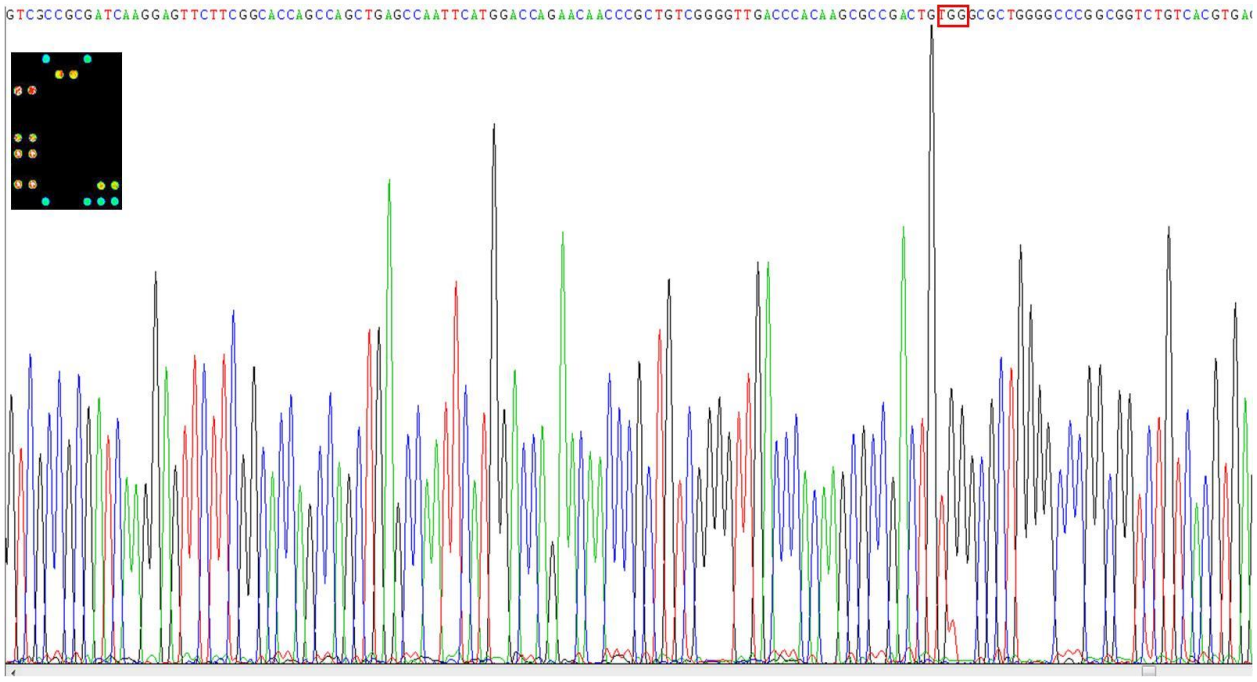

Figure S24: Spectra of sequencing analysis embedded with the fluorescence image for Sample 18

Sample 19 : 511(CTG→CCG) 526(CAC→CAA)

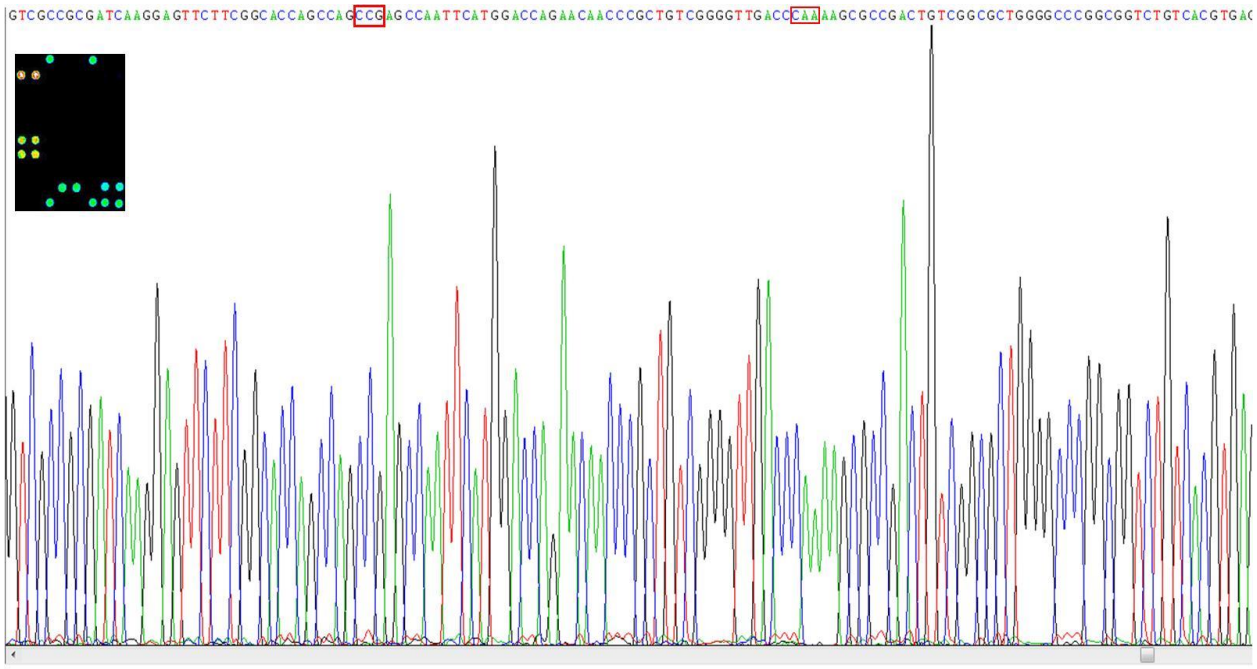

Figure S25: Spectra of sequencing analysis embedded with the fluorescence image for Sample 19

Sample 20: 522 (TCG→CAG)

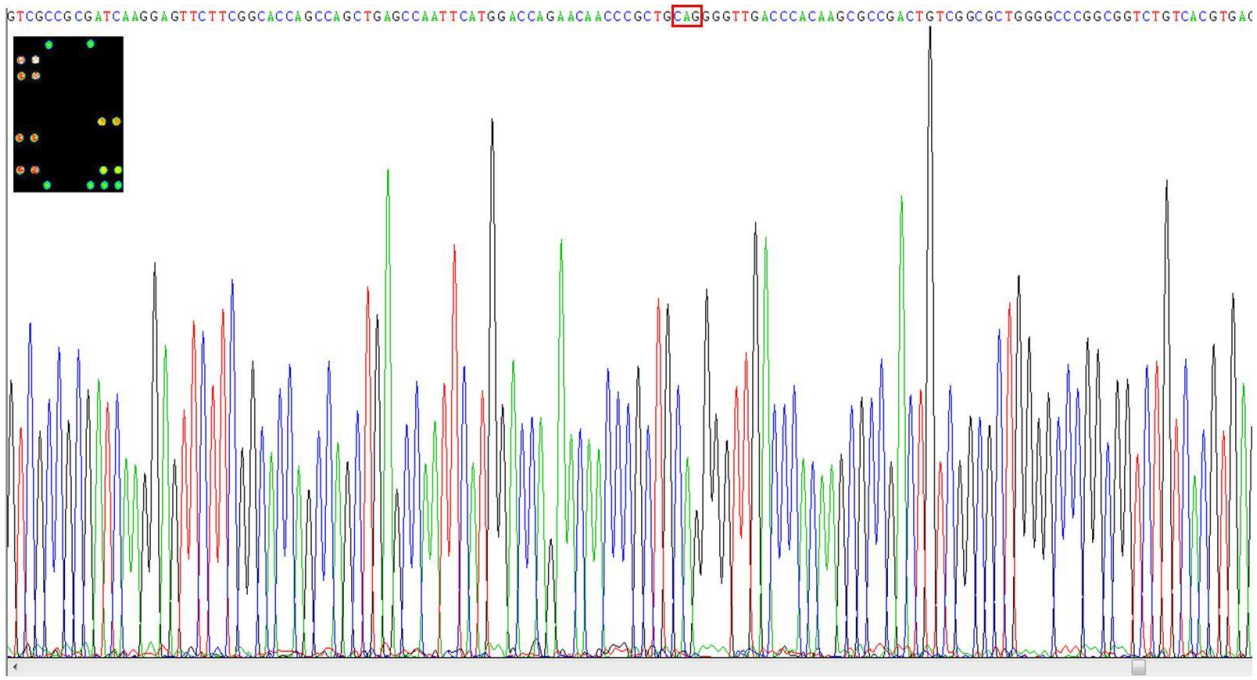

Figure S26: Spectra of sequencing analysis embedded with the fluorescence image for Sample 20

Sample 21: 511(CTG→CCG)

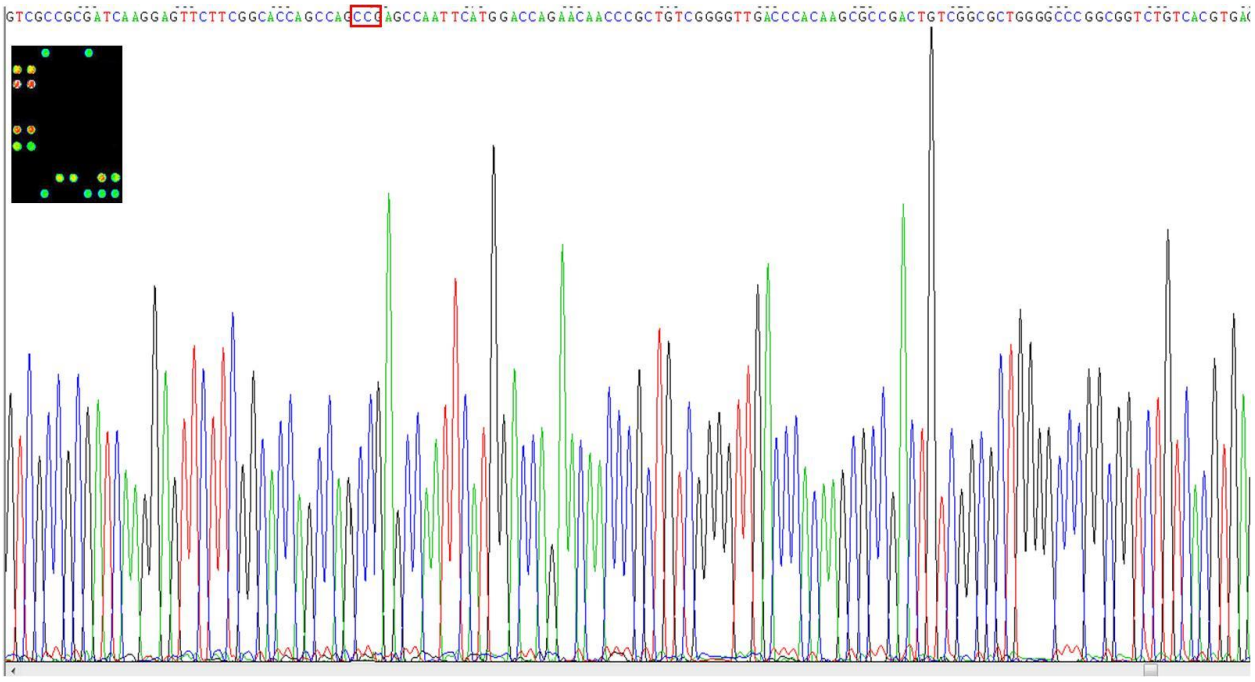

Figure S27: Spectra of sequencing analysis embedded with the fluorescence image for Sample 21

Sample 22: Susceptible

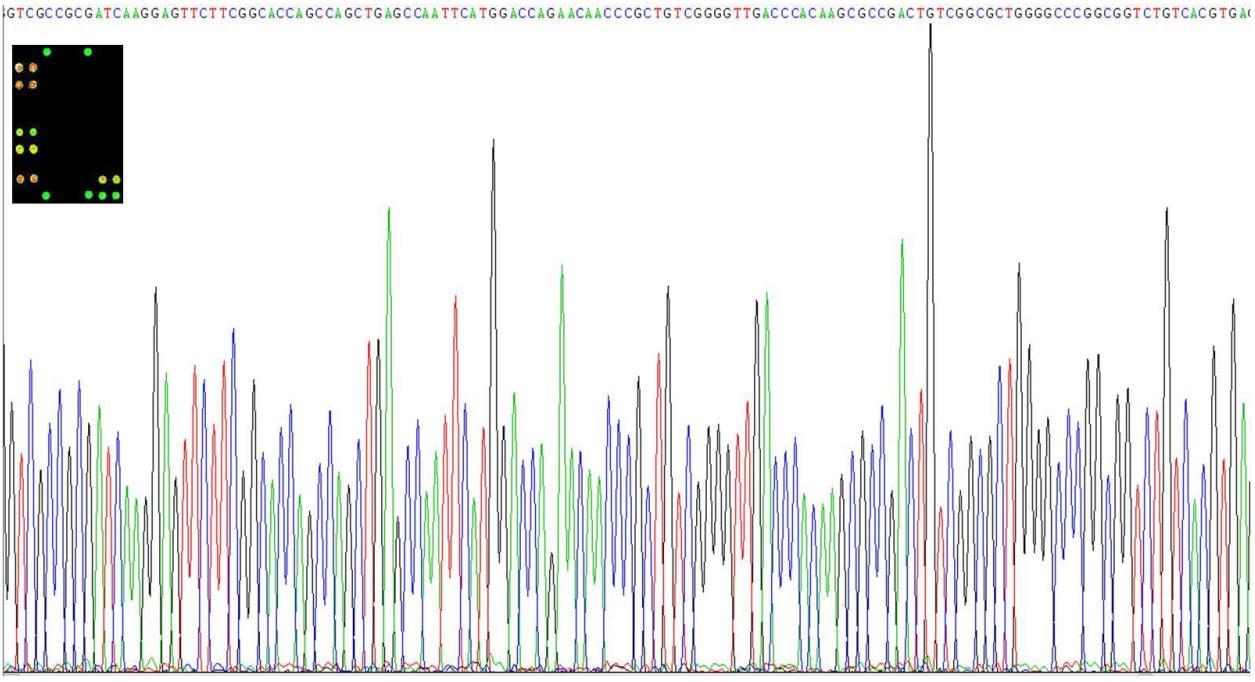

Figure S28: Spectra of sequencing analysis embedded with the fluorescence image for Sample 22

Sample 23: Susceptible

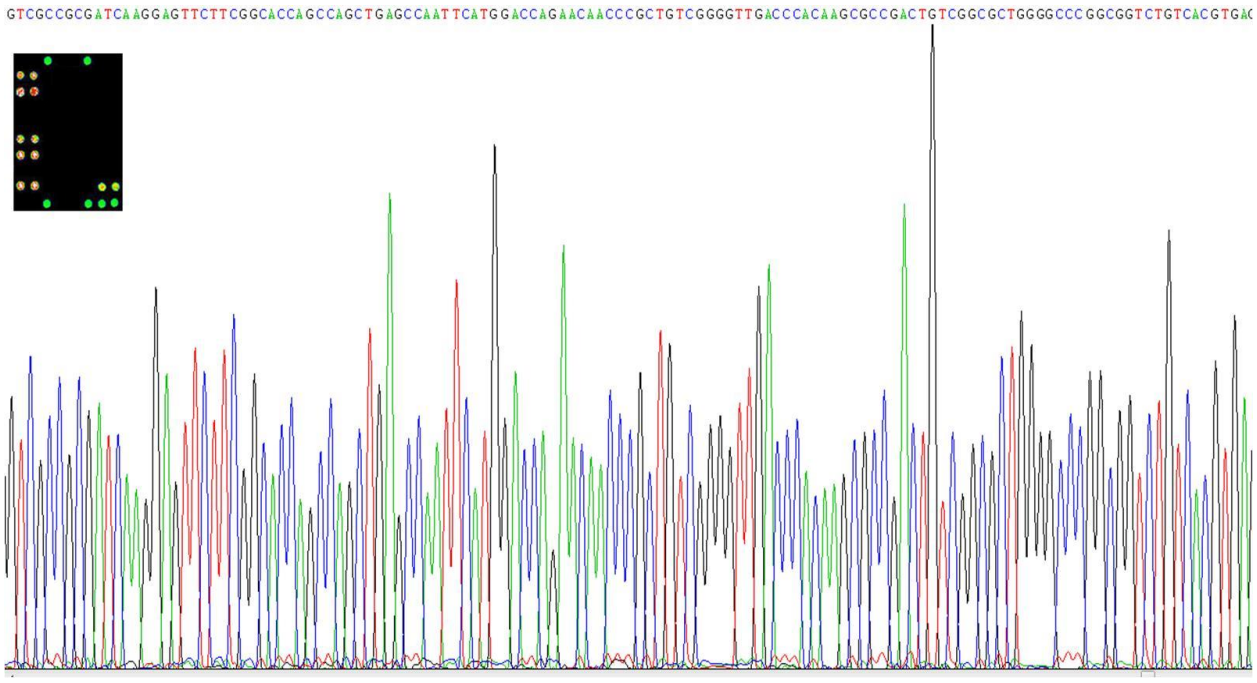

Figure S29: Spectra of sequencing analysis embedded with the fluorescence image for Sample 23

Sample 24: Susceptible

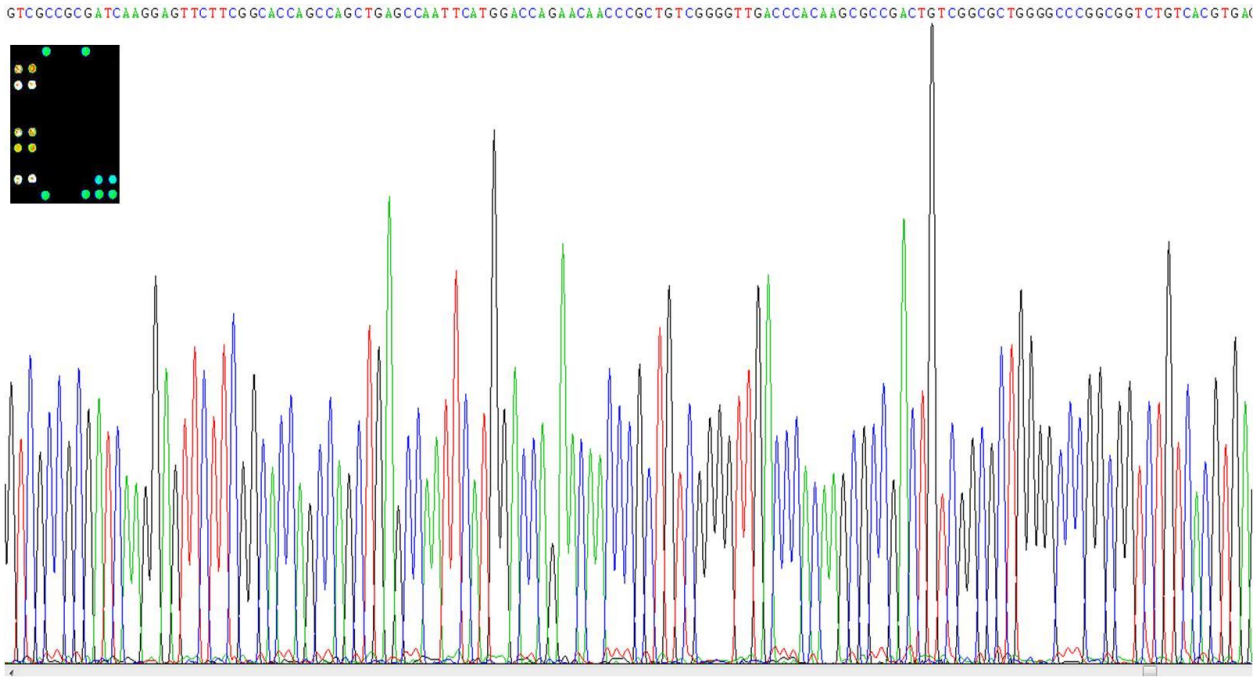

Figure S30: Spectra of sequencing analysis embedded with the fluorescence image for Sample 24

11. References:

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