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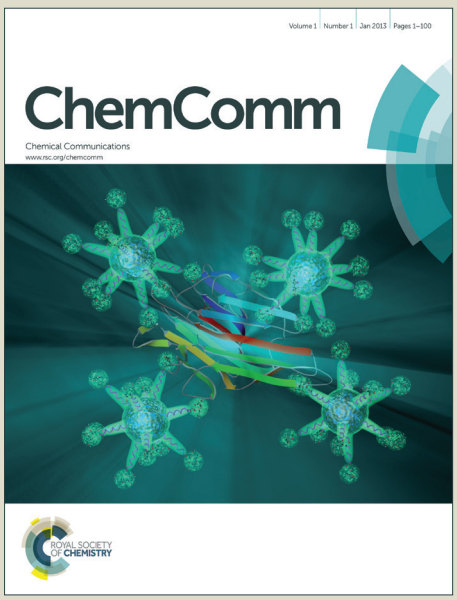

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COMMUNICATION

Multiplex SNP Detection in Multiple Codons for Accurate Drug Therapy

Danishmalik Rafiq Sayyed,[a] Satish Balasaheb Nimse,[a] Keum-Soo Song,[b] Nackmoon Sung,[c] Taisun Kim*[a]

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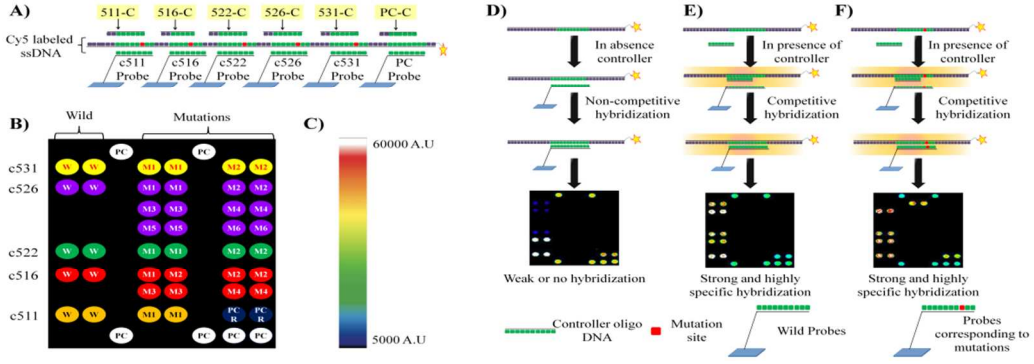
The multiplex SNP discrimination in the multiple codons of the genomic DNA is demonstrated by applying controller DNA technology (CDT) on the MDR-TB 9G DNAChips. The CDT allows efficient detection of 20 SNP's in five codons of the genomic DNA in 40min. CDT can distinguish SNP targets to as low as 1 copy of the genomic DNA. The 100% agreement with the sequencing analysis of clinical samples ensures the clinical applicability of MDR-TB 9G DNAChips

The multi-drug resistant tuberculosis (MDR-TB),[1] drug resistant hepatitis B virus (DR-HBV),[2] and drug resistant pneumonia (DR-pneumonia), are posing a great threat to the human health worldwide.[3] The drug resistant strains contain multiple single nucleotide polymorphisms (SNP's) in the multiple codons of the genomic DNA's as compared to the wild strains.[4] In case of the DR-HBV, the information on the drugs and drug-resistance mutation is available. Therefore, by identifying the particular mutation physicians can avoid the drug and chose another drug for the accurate therapy.[5] Moreover, many clinicians recommend drug-resistance testing prior to initiation of therapy.[6] However, the optimized information on the drugs and drug-resistance mutation is not available for MDR-TB. Unfortunately, there are no research tools to detect multiple SNP's with simple experimental protocol.[7] According to the WHO Global Tuberculosis Control Report 2012, there are more than 400,000 cases of the MDR-TB worldwide each year.[8] Hence, for the accurate drug therapy it is important to detect the mutations so that physicians can choose specific drug.[9,10]

Several research labs have identified 15 mutations in the genomic DNA of TB to discriminate the TB and MDR-TB strains.[11] The gene sequencing analysis is tedious and expensive which is not good for clinical applicability. The widely used real-time PCR technology,[12] Xpert MTB/RIF test cannot obtain the information on the multiple SNP's.[13] Unfortunately, the platform technology, which can detect the multiple SNP's, is not available for the wide clinical applications.[14] Recently, we have reported the controller DNA technology (CDT) for the detection of multiple mutations in the single codon of the genomic DNA.[15] In CDT, controller DNA (CD) is a small oligo DNA that has sequence complementary to the sequence on the genomic DNA in the probe binding region as shown in the **Scheme 1A**. Here, we demonstrate the application of CDT for the identification of 15 mutations in the five codons (c511, c516, c522, c526, and c531) of the genomic DNA of wild the TB strain to discriminate it from MDR TB strains. Each of these five codons have at least 2 – 6 mutations. It is the first ever attempt to detect each and every mutation irrespective of their position on the genomic DNA using single PCR primer set with the primer ratio of 2:1 (Cy5-reverse: forward). The detection of the 15 mutations in 40 min after PCR will boost up the clinical study worldwide on the MDR-TB for the optimized or personalized drug. The CDT can assist to optimize the accurate drug therapy resulting in the rapid cure and the prevention of the spread of the lethal diseases.



Scheme 1 A) Respective positions of the probe and controller DNA (CD) binding regions, mutation site on the genomic DNA, B) Scheme depicting the positions of the immobilized probes corresponding to wild and mutant strains, C) fluorescence scale, D) hybridization with the Cy5 ssDNA of wild TB strain in absence of CDs, E) and F) hybridization with the Cy5 ssDNA of wild TB and MDR TB strain, respectively, in presence of CDs.

As shown in the **Scheme 1D**, the hybridization of PCR product of wild TB strain with immobilized probes in absence of CDs results

in the weak hybridization. The weak hybridization is confirmed by the low spot intensities of wild probes. However, as depicted in the **Scheme 1E** and **1F**, the use of CDs increase hybridization efficiency indicated by the corresponding spot fluorescence intensities in the range of 50000-60000. The control of CDs on probe hybridization results in the strong and highly specific hybridization. The CDs used in this study were designed based on the recently reported method.[15] The application of CDT technology in the identification of 15 mutations for the detection and discrimination of the wild TB and MDR TB strains is justified by using clinical samples.

For the multiple SNP discrimination of the wild TB and MDR TB strains, the MDR-TB 9G DNAChips were produced[16] (see the **ESI† Scheme S1** for chip map) by using Probe1 – Probe22 (**ESI† Table S1**). All controller DNA's15 and probes were selected according to the well-established generalized probe selection method.[17] The MDR-TB 9G DNAChip is targeted to discriminate the multiple SNP's at the codons c511, c516, c522, c526, and c531 in the wild TB and MDR TB strains. It is well known that the PCR product containing ssDNA shows better hybridization yield than the ds DNA with the probes on the microarray.[18,19] Therefore, the Cy5 labelled single stranded PCR products (Cy5 ssDNA) were obtained by the asymmetric PCR amplification using Cy5 labelled reverse primer and forward primer with the ratio of 2:1 (**ESI† Figure S2**).[20] The higher hybridization yield of ssDNA than the dsDNA was also confirmed on the MDR-TB 9G DNAChips (**ESI† Figure S3**).

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 1** (NO).

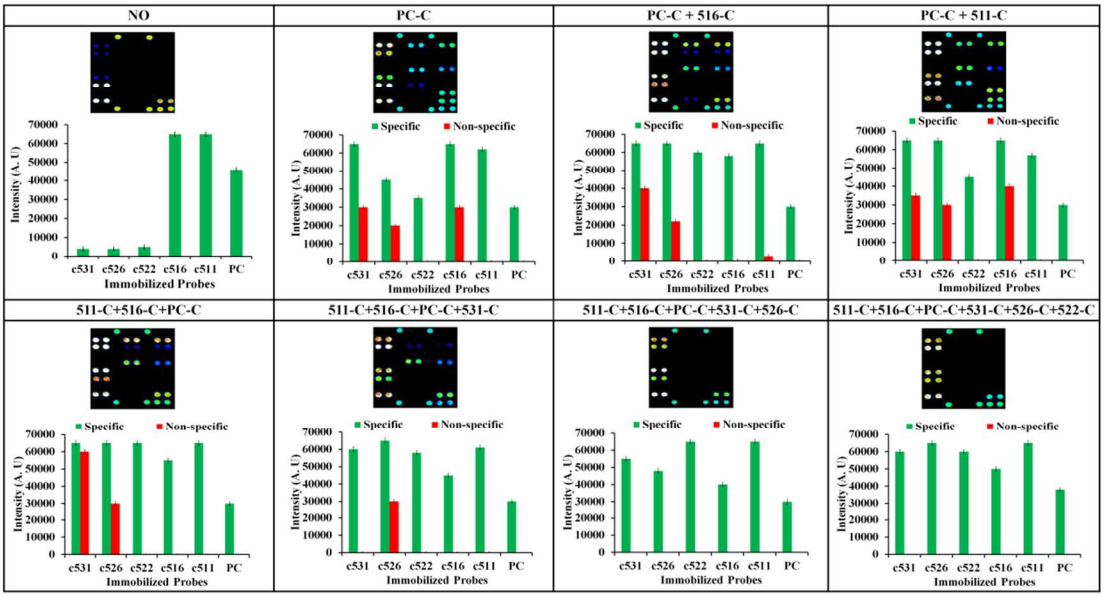


Fig. 1 Fluorescence images and corresponding graphs for the hybridization of the probes with the Cy5 ssDNA of wild TB strain in absence (NO) and in presence of CDs corresponding to the PC (PC-C), c511 (511-C), c516 (516-C), c522 (522-C), c526 (526-C), c531 (531-C), PMT gain = 53.

The probes corresponding to the PC, c511, and c516 showed strong hybridization indicated by the spot intensities of 46000 - 65000. Moreover, the probes corresponding to the c522, c526, and c531 also showed hybridization but with a very low signal intensities of 4000 - 4800. It is very clear from the **Figure 1** (NO) that, the probes corresponding to the positive control (PC), c511, and c516 bind more strongly than the probes corresponding to the c522, c526, and c531 to the Cy5 ssDNA of the wild TB strain. It was considered that, decreasing the hybridization of PC probe with Cy5 ssDNA may help in increasing the hybridization of c522, c526, and c531 probes, respectively. Therefore to control the hybridization of the PC probe, a 100 pmol of the controller DNA for PC (PC-C) was mixed with the 100fmol of the Cy5 ssDNA right before hybridization. After hybridization for 30min the chips were scanned. The obtained result is depicted in the **Figure 1** (PC-C).

The PC-C competes with the PC probe for hybridization with the Cy5 ssDNA, because it has the sequence complementary to the genomic DNA in the PC probe binding region. Therefore, the PC-C significantly decreases the hybridization of the PC probe indicated by the decrease in the spot intensity from 46000 to 30000. Interestingly, the addition of PC-C to the Cy5 ssDNA resulted in the improved hybridization of the probes corresponding to the c522, c526, and c531 indicated by the increase in fluorescence intensities from 4000 to 35000, 45000, and 65000, respectively. However, the addition of the PC-C also resulted in the non-specific hybridization of the Cy5 ssDNA with probes corresponding to the mutations at c531, c526, and c516. The signal intensity for the non-specific hybridization of the mutation probe corresponding to c516 (M4) was increased from 0 to 30000. These results indicate that, the addition of PC-C not only control the hybridization of the Cy5 ssDNA with the PC probe but also result in uncontrolled hybridization of Cy5 ssDNA with probes corresponding to the mutations at c531, c526, and c522. Therefore to control the hybridization of the c516 probe, the 100 pmol's of controller DNA for c516 (516-C) and PC-C were mixed with the Cy5 ssDNA. As depicted in the **Figure 1** (PC-C+ 516-C), the addition of PC-C and 516-C resulted in the decrease in signal intensity for the c516 probe from 65000 to 58000. Moreover, the non-specific hybridization with the

mutation probe corresponding to c516 were eliminated. Similarly, mixing the controller DNA's PC-C, 516-C, and 511-C with the Cy5 ssDNA resulted in the improved binding of all probes corresponding to the PC, c511, c516, c522, c526, and c531 as depicted in the **Figure 1** (511-C + 516-C + PC-C). However, the mutation probe corresponding to c526 and c531 showed strong non-specific hybridizations with the signal intensity of 30000 and 60000, respectively. As shown in the **Figure 1** (511-C + 516-C + PC-C + 531-C), the addition of 531-C along with the PC-C, 516-C, and 511-C resulted in the elimination of the non-specific hybridization of the mutation probe corresponding to c531 only. However, there was no effect on the non-specific hybridization of the mutation probe corresponding to c526. Therefore, to control the hybridization ability of the wild and mutation probes corresponding to the c526 the controller DNA 526-C was added to the Cy5 ssDNA along with the PC-C, 516-C, 511-C, and 531-C. Obtained result indicates that, all non-specific interactions were eliminated successfully as depicted in the **Figure 1** (511-C + 516-C + PC-C + 531-C + 526-C). However, the signal intensity for the probe corresponding to the c526 was decreased from 65000 to 48000 and the signal intensity for the probe corresponding to the c522 was increased from 58000 to 65000. Therefore, now to control the hybridization of the probe corresponding to the c522 the controller DNA 522-C was mixed with the Cy5 ssDNA along with the PC-C, 516-C, 511-C, 531-C, and 526-C. As depicted in the **Figure 1** (511-C + 516-C + PC-C + 531-C + 526-C + 522-C) obtained results indicate that, there were no non-specific interactions at all. Moreover, the signal intensities for the probes corresponding to PC, c511, c516, c522, c526, and c531 were in the range of 40000 to 65000. These results also suggested that the specific/ non-specific hybridization ratio was higher than 100, which is equal to the signal to background ratio. It is clear from these results that the use of CDs can be used for the detection and discrimination of multiple SNP's in multiple codons of the genomic DNA. To determine the sensitivity of the CDT, the hybridization solutions containing PCR products of the 1 – 35 copies of the wild and mutant TB mutant TB strains were used for hybridization. The obtained results clearly indicate that the CDT can distinguish SNP targets to as low as 1 copy of the genomic DNA. Comparison with the results published elsewhere indicates that the CDT as sensitive as that of the reported methods.[21] It was important to confirm the clinical applicability of the CDT for the identification of 15 mutations in the five codons (c511, c516, c522, c526, and c531) of the genomic DNA of wild the TB strain to discriminate it from MDR TB strains. Therefore, to evaluate the clinical applicability of the CDT, known clinical samples comprising wild and MDR TB strains were examined.

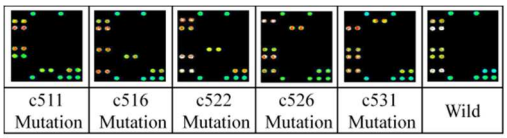


Fig. 2 Detection and discrimination of multiple mutations in the genomic DNA's of wild TB and MDR TB strains, PMT gain = 53.

The Cy5 ssDNA's of the wild TB and MDR-TB strains (mutations at c511, c516, c522, c526, and c531) were mixed with the 100 pmol each of the 511-C, 516-C, 522-C, 526-C, 533-C, and PC-C. Then the mixture was loaded on the MDR-TB 9G DNAChips to allow hybridization. The obtained results are depicted in the **Figure 2**. The **Figure 2** (**ESI† Figure S4, S5**) demonstrate that, the use CDs allows the highly sensitive and highly specific identification of the multiple mutations for the detection and discrimination of the wild TB and MDR TB strains. The results of MDR-TB 9GDNAChip and the sequencing analysis of 24 clinical samples demonstrate 100% agreement with each other (**ESI† Figure S6-S30**). These results confirm the clinical applicability of the CDT and assure the boost in the clinical study worldwide to define the optimized drugs in the treatment of the drug-resistant bacterial and viral infections.

The CDT is successfully applied in the clinical samples for the detection and discrimination of the multiple mutations at multiple codons. Therefore, CDT makes the MDR-TB 9G DNAChips rapid, specific, and cost-effective platform for the detection of the multiplex SNP's in multiple codons. CDT endows following advantages to the MDR-TB 9G DNAChips, i) final results in 40 min after PCR, ii) discrimination of the 15 mutations in multiple codons, iii) specific/ non-specific hybridization ratio higher than 100, iv) 100% agreement with the sequencing analysis for detection of mutations in the clinical samples. In turn, CDT will boost up the clinical study worldwide to define the optimized drugs in the treatment of the drug-resistant bacterial and viral infections.

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Notes and references

[a]Institute for Applied Chemistry and Department of Chemistry, Hallym University, Chuncheon, 200-702, Korea.
E-mail: tskim@hallym.ac.kr; Fax: +82-33-256-3421
[b]Biometrix Technology, Inc., 202 BioVenture Plaza, Chuncheon, 200-161, Korea.
[c]National Masan Hospital, Clinical Research Center, Changwon, Korea.

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