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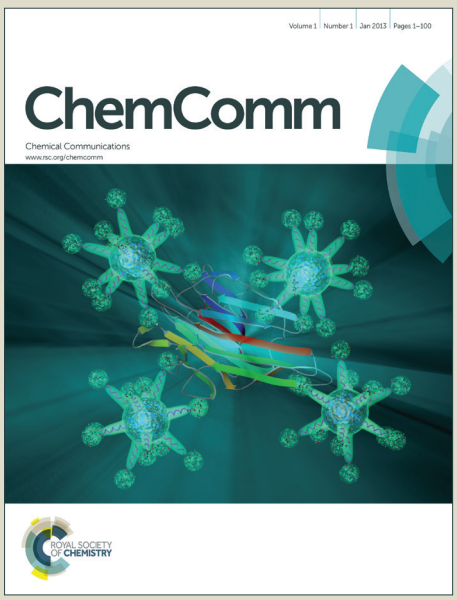

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COMMUNICATION

Detection of Multiple Mutations in the Single Codon of Genomic DNA

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

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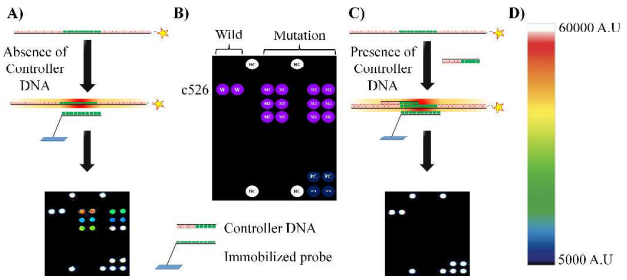
The controller DNA technology allows the detection of multiple mutations in the single codon of genomic DNA. In this technology, the controller DNA is used to control the hybridization of target DNA's with the immobilized DNA's. The controller DNA technology is rapid, specific, and cost-effective for following reasons, i) final results in 40 min after PCR, ii) detection and discrimination of the six mutations at single codon, iii) high sensitivity.

Antibacterial drug resistance is often correlated to the single-nucleotide polymorphisms (SNP's).[1] The multidrug resistance tuberculosis (MDR-TB) strains have several mutations including the multiple mutations at codon 526 (c526). The codon CAC (c526) of the wild TB is altered to well-known six mutations such as TAC, AAC, GAC, TGC, CGC, and CTC, each resulting in the MDR-TB strain. It is well known that the mutation in the codon of the genomic DNA may change the sequence of the target protein that it codes for, resulting in the poor binding of the drug to target protein.[2] Hence, it is inevitable that these mutations are responsible for the resistance to the antibacterial drugs.[3] Thus, monitoring the SNP's is as an alternative to monitoring the drug resistance.[4] Therefore, a technique, which can facilitate the detection and discrimination of multiple mutations in a specific codon with a simple experimental protocol, is essential for the timely detection of the drug resistant strains and selection of drugs for the treatment.[5]

Conventional culture-based method is reliable only for the discrimination of wild and mutant strains. DNA sequencing is considered as a gold standard but it is labour-intensive, time consuming, expensive, and low-throughput.[6,7] Real-time PCR is another approach, however due to the nonlinear amplification it fails to detect multiple mutations at single codon.[8] DNA microarrays, on the other hand, provide a high-throughput platform for the detection of SNP's.[9] However, the requirement of elevated temperature (40–60 ^{0}C) with the longer hybridization time (3-12 hrs.) impose a critical limitation for the detection of multiple SNP's at single codon.[10] Furthermore, the major issues of the DNA microarrays are low signal to background ratio (3:1) and very low target-specific hybridizations.[11]

The platform technology, which can detect multiple mutations at a single codon with a simple experimental protocol, is not available for wide clinical applications.

The controller DNA technology (CDT) presented in this article can identify multiple mutations at the c526 of the genomic DNA of TB with a simple experimental protocol on 9G DNAChips. In controller DNA technology, the controller DNA 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**, hybridization of the Cy5 labelled PCR product of wild TB strain in absence of the controller DNA results in the specific as well as non-specific hybridization. The non-specific hybridization is indicated by the appearance of the spots corresponding to the mutant strains. However, as depicted in the **Scheme 1C**, the hybridization of the Cy5 labelled PCR product of wild TB strain in presence of the controller DNA results in the highly specific hybridization without any non-specific hybridizations. The highly specific hybridization is indicated by the appearance of the spots corresponding to the wild strain without any spots for mutant strains. Thus, controller DNA controls the hybridization of the immobilized probes with the PCR product, thereby eliminating the non-specific hybridization. The development of CDT is explained in details. Further the sensitivity and specificity of the CDT were evaluated for its applicability in clinical samples.



Scheme 1 A) and C) Hybridization of the Cy5 labelled PCR product of wild TB strain in absence and presence of the controller DNA, respectively, B) Scheme depicting the positions of the immobilized probes corresponding to wild (W) and mutant strains (M1-M6), D) fluorescence scale.

The probes (Probe1 – Probe10, **ESI† Table S1**) for the detection of the multiple mutations at c526 in the TB were selected according to the well-established generalized probe selection method.[12] The selected probes were immobilized on the 9G DNAChips (**ESI† Scheme S2**) according to previously reported method.[13] The 128mer Cy5 labelled single stranded PCR product (Cy5 labelled ssDNA) was obtained by the asymmetric PCR amplification using Cy5 labelled reverse primer and forward primer with the ratio of 2:1 (**ESI† Figure S2**). It is well known that the PCR product containing ssDNA shows higher hybridization efficiency than dsDNA with the immobilized probes on the DNA chips.[14] It is important to note that, the Cy5

labelled ssDNA in the PCR product of wild TB strain after asymmetric PCR amplification undergoes self-dimerization[15] due to the complementary base pairs in its sequence (**ESI† Scheme S1**). Therefore, to separate the strands of the Cy5 labelled ssDNA in the dimerized form, a splitter DNA (5'-GCTGGGGCCTGGCGGTCTGT-3') was used.

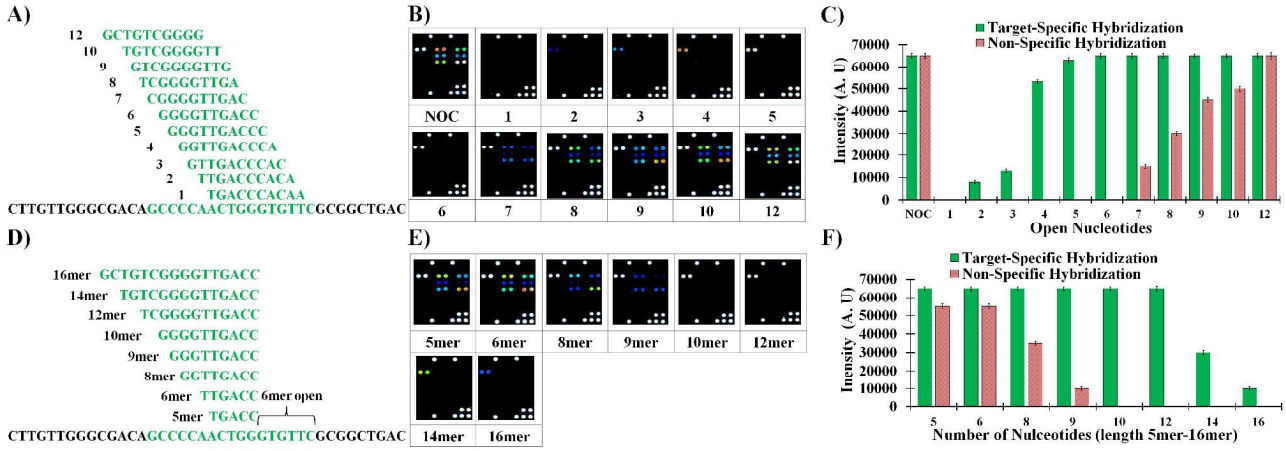


Fig. 1 A) Controller DNA's (with a starting position of 2-11 and 13 leaving 1-10and 12 nucleotides open, respectively) and the partial sequence of Cy5 labelled ssDNA, B) Fluorescence images after hybridization of the Cy5 labelled ssDNA of wild TB strain with the immobilized probes in absence (NOC) and presence of controller DNA's (with a starting position of 2-11, 13), and C) Corresponding graph, D) Controller DNA's (with a starting position of 7, and length 5mer-16mer) and partial sequence of Cy5 labelled ssDNA, E) Fluorescence images after hybridization of the Cy5 labelled ssDNA of wild TB strain with the immobilized probes in presence of controller DNA's (length 5mer-16mer), and F) Corresponding graph, PMT= 53.

The immobilized probes on the 9G DNAChips were allowed to hybridize with the 100fmol of the Cy5 labelled ssDNA of the wild TB strain in presence of the 100pmol of a splitter DNA at 25 ^{0}C for 30 min. The result of hybridization is depicted in the **Figure 1B** (No Controller, NOC). The Probe1 corresponding to the wild strain is the only one which should have shown hybridization with the Cy5 labelled ssDNA. Unfortunately, all probes corresponding to the mutant strains showed non-specific hybridization with the Cy5 labelled ssDNA of the wild TB strain. The ratio of the target specific to the non-specific hybridization was found to be 1:1. Similar non-specific hybridizations were also observed upon hybridization of the Cy5 labelled ssDNA of the mutant strain (c526 CAC -> CGC, **ESI† Figure S3**). From these results it was clear that, there was no control on the hybridization of the immobilized probes with the corresponding Cy5 labelled ssDNA.

Therefore, to control the hybridization of the immobilized probes with the Cy5 labelled ssDNA, a controller DNA (CD) was designed. It was assumed that the controller DNA can strongly compete with the immobilized probes for the hybridization with the Cy5 labelled ssDNA if it has a complementary sequence with Cy5 labelled ssDNA in the probe binding region.

Therefore, to choose the optimum binding position and optimum length of the controller DNA's, a nucleotide in the Cy5 labelled ssDNA, which is complementary to the first nucleotide in the immobilized probe was assigned as number 1. The other complementary nucleotides were assigned with numbers in the ascending order. To optimize the binding position of the controller DNA, 11 DNA's (CD1-CD11) with the length of 10 nucleotides (**ESI† Table S2**) were chosen as shown in the **Figure 1A**. The CD1-CD10, CD11 leave 1-10, 12 nucleotides open, respectively, on the Cy5 labelled ssDNA for the hybridization with the for immobilized probes as demonstrated in the **Figure 1A**. The results of hybridizations of the immobilized probes with the Cy5 labelled ssDNA in presence of 100pmol of these controller DNA's are depicted in the **Figure 1B** and **C**.

As demonstrated in the **Figure 1B** and **C**, the CD1 leaving only one nucleotide open with the binding position of 2, completely prohibits the hybridization of the immobilized probes with the Cy5 labelled ssDNA. However, CD2, CD3, CD4, and CD5 with the binding positions of 3, 4, and 5, respectively, allow controlled hybridization of immobilized probes with the Cy5 labelled ssDNA. The controlled hybridization is indicated by the absence of non-specific hybridizations. The controlled hybridization is also indicated by the low fluorescence intensity in case of CD2, CD3, and CD4. It is very important to note that, if controller DNA covers most of the nucleotides in the probe binding region (CD1, CD2, CD3) it is very difficult for the immobilized probe to displace the controller DNA bound to the Cy5 labelled ssDNA's. Therefore, the probes show only specific hybridization with very low fluorescence intensity.

The CD6 with the binding position of 7 leaving six nucleotides open demonstrates highest fluorescence intensity of 65000 without any non-specific interaction. The competitive control of CD6 on the hybridization of immobilized probes with the Cy5 labelled ssDNA allows the target specific to non-specific hybridization ratio more than 100:1.

However, CD7 with the binding position of 8 leaving seven nucleotides open loses control over hybridization, which results in the non-specific hybridization. Moreover, CD8, CD9, and CD10 with the binding position of 9, 10, and 11, respectively, constantly lose control over hybridization resulting in the increase in non-specific hybridization intensity from 15000 to 50000. The CD11 with the binding position of 13 leaving twelve nucleotides open, completely lose control over the hybridization resulting in the target specific to non-specific hybridization ratio of 1:1. From this data it is clear that, if the controller DNA leaves most of the nucleotides open (CD7 - CD11, 7 to 12 nucleotides, respectively) in the probe binding region, the immobilized probes can easily displace the controller DNA for hybridization with the Cy5

labelled ssDNA's. Therefore, the probes corresponding to the wild as well as mutation can hybridize with the Cy5 labelled ssDNA's resulting in the specific as well as non-specific hybridization. However, when the controller DNA leaves only six nucleotides open (CD6) for hybridization of immobilized probes with the Cy5 labelled ssDNA's both controller as well as probes start to have a competition for the hybridization with the Cy5 labelled ssDNA's resulting only in the target specific hybridizations. Therefore, CD6 was considered to be optimum and used for further experiments.

To optimize the length of the controller DNA, 7 DNA's (CD12-CD18) starting from the binding position of 7 with the lengths 5mer, 6mer, 8mer, 9mer, 12mer, 14mer, 16mer, respectively, (**ESI† Table S3**) were chosen as demonstrated in the **Figure 1D**. The results of the hybridizations with the Cy5 labelled ssDNA in presence of 100pmol these controller DNA's (CD12-CD18) are depicted in the **Figure 1E** and **F**.

As demonstrated in the **Figure 1E** and **F**, the CD12 and CD13 with the length of 5mer and 6mer, respectively, did not show any control over hybridization. CD12 and CD13 are too short to compete with the immobilized probes for hybridization with Cy5 labelled ssDNA resulting in very high non-specific hybridization. The target specific to non-specific hybridization ratio was only 1.2:1. However, with the increase in the length of controller DNA to 8mer and 9mer as in case of CD14 and CD15, respectively, the target specific to non-specific hybridization ratio increased to 2:1 and 6:1. The data for CD14 and CD15 demonstrate that they show partial control over hybridization of immobilized probes with the Cy5 labelled ssDNA as compared to CD12 and CD13. Interestingly, controllers CD16 and CD17 with the length of 10mer and 12mer, respectively, gain the complete control over the hybridization of the immobilized probes with the Cy5 labelled ssDNA. Both of these controller DNA's allowed to achieve the target specific to non-specific hybridization ratio more than 100:1.

Thus, it is clear that the longer controller DNA's effectively compete with immobilized probes for the hybridization with the Cy5 labelled ssDNA resulting in the decrease in non-specific hybridizations. However, the controller DNA's CD18 and CD19 with the length of 14mer and 16mer, respectively, competitively prohibit the binding of the immobilized probes with the Cy5 labelled ssDNA. The competitive prohibition of hybridization was indicated by the decrease in fluorescence intensity for target specific hybridization from 65000 to 10000. Therefore, the controller DNA CD16 with the length of 10mer was considered as optimum and used for further experiments.

To determine the sensitivity of the CDT, the hybridization solution containing the PCR products of the 1 – 35 copies of wild TB strain were used for hybridization. The **Figure 2A** (**ESI† Figure S4, S5**) clearly indicates that the present technology can distinguish the SNP target to as low as 1 copy of the genomic DNA. Similar results were also obtained for the mutation sample. The sensitivity of the CDT is comparable with that of the reported methods.[16]

The **Figure 2B** demonstrates that, the use controller oligo DNA's allow highly sensitive and highly specific detection of the all six mutations in the c526 of the genomic DNA of TB. To evaluate the clinical applicability of the CDT in the detection of multiple mutations at c526 of the genomic DNA of TB, known clinical samples comprising wild and six mutant strains were examined (**ESI† Figure S6-S15**). These results showed 100% agreement with the results of sequencing analysis for each clinical sample.

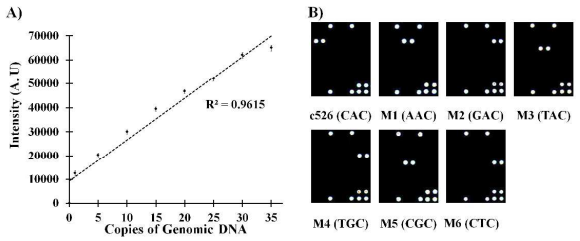


Fig. 2 A) Determination of sensitivity by using 1 – 35 copies of genomic DNA of the wild TB strain, B) Detection and discrimination of multiple mutations in the c526 of the genomic DNA of TB in presence of CD6, PMT gain = 53.

Conclusions

The controller DNA technology allows rapid, specific, and cost-effective detection of multiple SNP's in the clinical samples for following reasons, i) detection and discrimination of the multiple mutations at single codon, ii) high sensitivity in terms of the specific to non-specific hybridization ratio higher than 100:1, and iii) final results in 40 min after PCR (**ESI† Scheme S3**). The 100% agreement of the clinical results with sequencing analysis demonstrates the high sensitivity and specificity of the CDT. In turn, this technology can boost the ongoing efforts on monitoring the drug resistance for the rapid cure and the prevention of the spread of the disease.

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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*
[b]*Biometrix Technology, Inc. 202 BioVenture Plaza, Chuncheon, 200-161, Korea Fax: +82-33-256-3421*

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1 S. H. Abbadi, et al., *Int. J. Infec. Dis.,* 2009, **13**, 673.
2 P. C. Ng and S. Henikoff, *Nucl. Acids Res.* 2003, 31, 3812.
3 N. Woodford and M. J. Ellington, *Clin. Microbiol. Infe.* 2007, **13**, 5.
4 H. Wang et al, *Anal. Chem.* 2011, **83**, 1883; Y. Zhang et al. *Nanoscale* 2013, **5**, 5027.
5 J. A. Caminero et al., *Lancet. Infect. Dis.* 2010, **10**: 621; M. Yagui, et. al., *Int. J. Tuberc. Lung. Dis.* 2006, **10**, 838.
6 K. Sato, et al., *Anal. Biochem.* 2006, **350**, 162.
7 B. Veigas, et al., *Nanotechnol.* 2010, **21**, 1.
8 L. van der Weyden and D. J. Adams, *Biochimica. Et. Biophysica. Acta.*, 2007, **1776**, 58.
9 S. B. Nimse, et. al., *Chem. Soc. Rev.,* 2013, **42**, 366.
10 L. Wang and P. C. H. Li, *J. Agri. Food Chem.,* 2007, **55**, 10509; A. Sedighi and P. C. H. Li, *Int. J. Mater. Sci. Eng.,* 2013, **1**, 45.
11 Y. D. Choi, et al., *Gynecol. Oncol.*, 2007, **98**, 369.
12 S. B. Nimse, et. al., *Chem. Commun.* 2011, **47**, 12444.
13 K. Song, et. al., *Chem. Commun.* 2011, **47**, 7104; S. B. Nimse, et. al., *Tetrahedron Letters*, 2010, **51**, 6156; S. B. Nimse, et. al., *Tetrahedron Letters*, 2009, **50**, 7346.
14 X. Tang, et. al., *BioTechniques* 2006, **40**, 759.
15 M. Deng, et al., *J. Clin. Microbiol.* 2012, **50**, 3443.
16 W. Shen, et al., *Chem. Commun.,* 2013, **49**, 8114