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Single-base mismatch discrimination by T7 exonuclease with target cyclic amplification detection†

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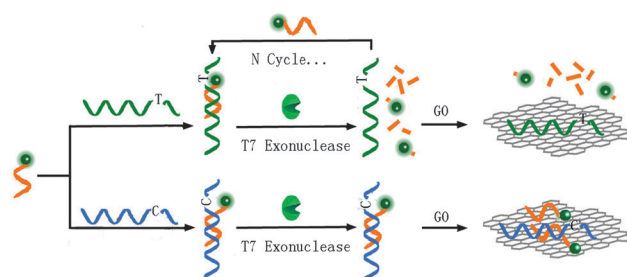
T7 exonuclease is reported for the first time to have high specificity in discriminating single-base mismatch and utilized for developing a target cyclic amplification biosensor strategy for sensitive SNP detection based on graphene oxide quenching of uncleaved probes.

Single-nucleotide polymorphism (SNP) detection is of great importance for clinical diagnostics because of its close association with many diseases such as tumours and genetic disorders.¹ SNP detection typically relies on a mechanism for allele discrimination. While allele-specific hybridization for SNP detection, complicated optimization and stringent control of the assays is required.² Hence, enzymatic discrimination of single-base mismatch becomes a prevailed option for SNP detection due to its robustness and specificity. Existing mechanisms for allele discrimination include ligase based ligation,³ polymerase based nucleotide incorporation,⁴ primer extension,⁵ proof-reading⁶ and endonuclease based cleavage.⁷ Our group⁸ and others⁹ have adapted these mechanisms to design a variety of biosensor strategies for SNP genotyping. However, a low-cost, robust and selective strategy for SNP detection still remains an unmet challenge.

T7 exonuclease hydrolyzes duplex DNA in the direction of 5' to 3' from 5' phosphoryl or 5' hydroxyl nucleotides. It shears from the 5' termini or at gaps and nicks of double-strand DNA. It has also been reported to degrade DNA and RNA from DNA-RNA hybrids in the 5' to 3' direction.¹⁰ Herein we report for the first time that T7 exonuclease has very high specificity in discriminating single-base mismatch with a reaction rate ratio of ~26 for perfect match over single-base mismatch. This new allele discrimination mechanism is exploited for developing a target cyclic amplification biosensor strategy for sensitive SNP detection. By using graphene oxide (GO) based selective quenching of uncleaved probes, the biosensor

strategy provides a low-cost platform for sensitive and selective SNP detection.

The allele-specific discrimination mediated by T7 exonuclease was firstly investigated (Fig. S1, ESI†). Probe 1 (Table S1, ESI†) with a 5-carboxy fluorescein (FAM) fluorophore at the 5' terminal and a dabcyI quencher in adjacent nucleotides was designed and was a perfect match to the mutant-type target while forming single-base mismatch to the wild-type target at the 5' terminal. For the perfect match system, probe 1 will be hydrolysed successively into mononucleotides from the 5' terminal by T7 exonuclease and the fluorescence of FAM will be recovered. For the single-base mismatch system, the mismatched base will prevent T7 exonuclease from degrading probe 1 and the fluorescence will remain quenched by dabcyI. Because cyclical degradation of input probe 1 affords efficient amplification of the fluorescence signal, we are able to achieve high sensitivity in detecting the target DNA. Based on the above mechanism, a novel fluorescence SNP detection strategy was proposed by using GO as a "nanoquencher". It was reported that GO bound and quenched fluorophore-labeled single-stranded DNA but had less affinity to double-stranded DNA.¹¹ As illustrated in Scheme 1, probe 2 with a FAM tag at the 5' end is designed to perfectly complement the mutant-type target while forming single-base mismatch to the wild-type target at the 5' terminal. For the mutant-type target, T7 exonuclease facilitates the process of target recycling that rapidly



Scheme 1 Scheme of SNP detection based on T7 exonuclease with target cyclic amplification.

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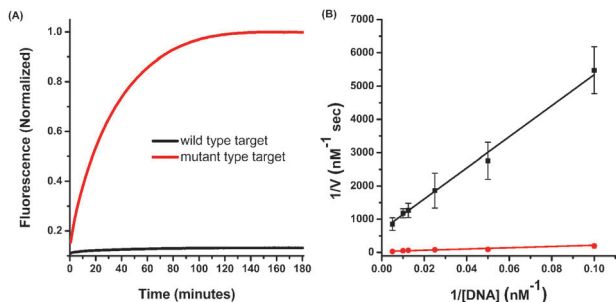


Fig. 1 Comparison of the digestion rates of the mutant-type target and the wild-type target. (A) Fluorescence-based progress curves of the T7 exonuclease digestion reaction as a function of time. (B) Double reciprocal plot of the initial reaction velocity as a function of DNA concentration.

shears probe 2, and produces numerous mononucleotides and short oligonucleotide fragments, and after adding GO, the product of digestion will not be adsorbed by GO, leading to the recovery of fluorescence; while for the wild-type target, probe 2 will not be digested by T7 exonuclease, resulting in fluorescence quenching after adding GO.

To assess the ability of allele-specific discrimination by T7 exonuclease, we synthesized two model DNA sequences of the human β -thalassemia gene around the IVSII-654 mutation site (mutant-type target and wild-type target), a known C > T mutation highly associated with β -thalassemia.¹² Probe 1 is separately annealed with the mutant-type target and the wild-type target followed by addition of T7 exonuclease, and the degradation reactions of probe 1 were studied *via* real-time fluorescence analysis. As shown in Fig. 1A, for the wild-type target, a very weak fluorescence signal was observed after incubating it for 3 hours, and it did not show appreciable time-dependent fluorescence intensity changes. In contrast, for the mutant-type target, the fluorescence signal increased rapidly, suggesting that probe 1 was effectively degraded by T7 exonuclease. The maximum cleavage velocity ($\nu_{\max}$) was calculated by the standard Michaelis-Menten equation and is shown in Fig. 1B. The $\nu_{\max}$ of the perfect match system is about 26 times faster than that of the single-base mismatch system, indicating that T7 exonuclease has very high specificity in discriminating single-base matches. This single-base mismatch discrimination efficiency was comparable against those using high-specific ligases.¹³

The feasibility of the developed strategy for single-base mismatch discrimination was investigated. GO nanosheets used in this experiment were characterized by atomic force microscopy (AFM). GO nanosheets showed a topological height of ~ 1.1 nm (Fig. S2, ESI†), a typical thickness for single-layered GO nanosheets.¹⁴ As shown in Fig. 2A, when incubating probe 2 with the wild-type target and the mutant-type target separately, subsequently followed by addition of GO nanosheets, only weak fluorescence was observed, suggesting that the fluorescence of FAM labelled to probe 2 was efficiently quenched by the GO nanosheets. When adding T7 exonuclease into the systems above, the fluorescence was remarkably enhanced for the perfect match system of probe 2 and the mutant-type target, evidencing that the FAM fluorophore was not adsorbed on the surface of GO nanosheets, and confirming that probe 2 was digested by T7 exonuclease. However, no substantial fluorescence increase

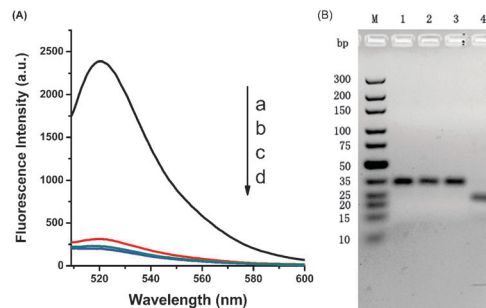


Fig. 2 (A) Fluorescence spectrum of SNP analysis. Line a, 20 nM mutant-type target and 80 nM probe 2 plus 0.26 U μL^{-1} T7 exonuclease and 5 ng μL^{-1} GO nanosheets; line b, 20 nM wild-type target and 80 nM probe 2 plus 0.26 U μL^{-1} T7 exonuclease and 5 ng μL^{-1} GO nanosheets; line c, 20 nM mutant-type target and 80 nM probe 2 plus 5 ng μL^{-1} GO nanosheets; and line d, 20 nM wild-type target and 80 nM probe 2 plus 5 ng μL^{-1} GO nanosheets. (B) Agarose gel electrophoresis images for hybridization of DNA probe 2 and target DNA. Lane M, DNA marker; lane 1, 1 μM probe 2 and 1 μM wild-type target; lane 2, 1 μM probe 2 and 1 μM mutant-type target; lane 3, 1 μM probe 2 and 1 μM wild-type target with 1 U μL^{-1} T7 exonuclease; and lane 4, 1 μM probe 2 and 1 μM mutant-type target with 1 U μL^{-1} T7 exonuclease.

was achieved for the single-base mismatch system of probe 2 and the wild-type target, implying that the FAM fluorophore was adsorbed on the GO surface. Thus, the assumption that the single-base mismatch at the 5' terminus can efficiently prevent the T7 exonuclease from degrading probe 2 is proved and confirmed.

Further verification regarding the single-base mismatch discrimination mechanism was conducted by agarose gel electrophoresis. As shown in Fig. 2B, double-stranded DNAs generated by the hybridization of probe 2 with the wild-type target and the mutant-type target, respectively, exhibited two bright bands with the same mobility (lanes 1 and 2). Upon adding T7 exonuclease into the systems above, almost no change occurred to the bright band (lane 3) compared with that in lane 1, repeatedly implying that probe 2 was not digested by T7 exonuclease due to the single-base mismatch with the wild-type target at the 5' end. In contrast, a bright band with obvious migration shifts (lane 4) was observed, suggesting that probe 2 was degraded by T7 exonuclease and this bright band is only from the mutant-type target.

In order to optimize the concentration of T7 exonuclease, we adopted the fluorescence signal ratio (F/F_0 , a signal from the mutant target divided by that from the wild type) as the measure (Fig. S3, ESI†). It was observed that the fluorescence signal ratio displayed a peak-shape dependency on the concentration of T7 exonuclease with a maximum achieved using 0.26 U μL^{-1} T7 exonuclease. Thus, this concentration of T7 exonuclease was used throughout the subsequent experiments.

In order to investigate the quantitative nature of the single-base discrimination technique, the mutant-type target of varying concentrations was measured using fluorescence spectra. As shown in Fig. 3A, the fluorescence peaks dynamically increased with the increasing concentration of the mutant-type target within the range from 0 to 20 nM. The fluorescence intensity *versus* mutant-type target concentration showed a linear correlation

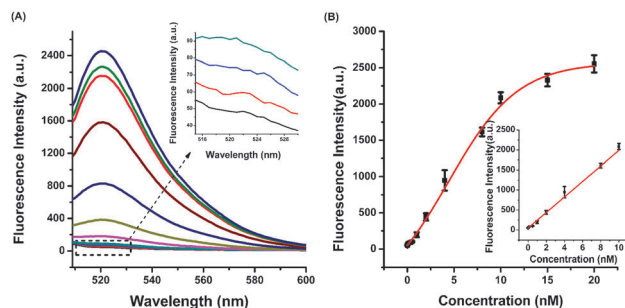


Fig. 3 (A) Fluorescence spectrum of probe 2 (80 nM) with different concentrations of the mutant-type target (0, 0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 10, 15, and 20 nM, from bottom to top) was measured with 494 nm excitation wavelength. (B) Fluorescence intensity at 520 nm versus the concentration of the mutant-type target of this SNPs assay. Inset: the linear relationship between fluorescence intensity and mutant-type target concentration. Error bars are standard deviations of three repetitive experiments.

with the mutant-target concentration range from 10 pM to 10 nM, as shown in Fig. 3B. The detection limit was estimated to be 4 pM according to the 3σ rule. The performance of the proposed method was compared with other reported methods (Table S2, ESI[†]), demonstrating that this biosensor exhibited low detection limit and achieved high sensitivity. The relative standard deviations (RSDs) of fluorescence intensity were 3.6%, 2.8%, 5.3% and 3.7%, in four repetitive assays of 10 pM, 100 pM, 500 pM and 10 nM mutant-type targets, exhibiting excellent reproducibility due to simple operations. The developed strategy offered advantages of a high signal-to-background ratio and a wide dynamic response range and it could be more robust, cost-efficient, and readily automated, because of its homogeneous and fluorescence-based detection format.

Further results from detecting mixtures of mutant-type targets and wild-type targets were investigated (Fig. S4, ESI[†]). The mixed DNA samples were prepared by mixing the mutant-type target and the wild-type target of different ratios from 0% to 100%, and the total concentration of wild-type and mutant-type analytes is 20 nM. One can observe that the fluorescence signals were dynamically enhanced with the increase of the mutant-target from 0% to 100% in mixed DNA samples. These experimental results demonstrated that the technique afforded extremely high specificity, which might be a potential alternative as an effective mutation detection technology for solid tumor-based cancer research.

The single-base mismatch discrimination technique was further validated using the human genomic DNA sample with a point mutant (C > T) at the IVSII-654 site in the beta-thalassemia gene. Six genomic samples were collected. Polymerase chain reaction (PCR) was performed on these samples to obtain 373-base-pair amplicons. The PCR amplicons were confirmed by agarose gel assay (Fig. S5, ESI[†]) and subsequently utilized for mutation detection by using the developed technique (Fig. S6, ESI[†]). It was observed that for samples 3, 4, 5 and 6, fluorescence signals were significantly greater than the background response, implying that those four samples are of mutant-type. While samples 1 and 2 gave fluorescence signals comparable to the background response, suggesting

that those two samples are of wild-type. The identified gene types were consistent with the sequence data, demonstrating that the developed method holds the potential for SNP detection of genomic DNA samples.

In conclusion, we reported for the first time that T7 exonuclease can efficiently discriminate the perfectly matched base pairs from the single-base mismatched pairs with a discrimination ratio of ~ 26 . On the basis of this new allele discrimination mechanism, a target cyclic amplification biosensor strategy was developed for SNP detection. This strategy is validated to exhibit superb robustness, a high signal-to-background ratio, desirable sensitivity and selectivity, low cost, and simple. Furthermore, different sequence DNA probes with distinct fluorescence tags can be designed, which should allow the strategy to be useful for multiplex assays. In light of these advantages, this new allele discrimination mechanism can be expected to hold considerable potential for genomic research.

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