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Enzyme-Mediated Single-Nucleotide Variation Detection at Room Temperature with High Discrimination Factor

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We demonstrate a new powerful tool to detect single-nucleotide variation in DNA at room temperature with high selectivity based on predetermined specific interactions between Lambda exonuclease and a chemically modified DNA substrate structure which comprises two purposefully introduced mismatches and a covalently attached fluorophore. The fluorophore not only acts as a signal reporter in the detection system, but also plays a notable role in the specific molecular recognition between the enzyme and the probe/target hybrid substrate. The method is single-step, rapid, and can be easily adapted to different high-throughput micro-devices without the need of temperature control.

Selective detection of rare base substitutions or low-abundance point mutations in a large background of DNA sequences with single-base difference is regarded as one of the major issues to be addressed for screening of single nucleotide polymorphism (SNP) in genomic study or tracking DNA damage in clinical diagnosis. A number of elegantly designed DNA probes, such as molecular beacon, binary probe, triple-stem probe and other hybridization probes have been reported in recent years for discrimination of single-base substitutions in DNA sequences. However, most of these hybridization-based DNA assays have limited power in specific identification of single-base variants because of the small difference between the stability of a perfectly matched duplex and a duplex containing only one mismatched base. By inserting an extra mismatch at certain positions of the probes, a significant increase of the specificity of DNA hybridization has been demonstrated by Smith and co-workers. A novel type of discontinuous double-stranded DNA (dsDNA) probes were also developed by Seelig et al. for specific DNA detection via a double-stranded toehold exchange mechanism. These probes showed promising results for a broad range of applications, though the sensitivity and the assay speed were not so competitive. Utilizing an isocycteine-mediated peptide nucleic acid (PNA) ligation reaction, Seitz et al. achieved a single-nucleotide selectivity as high as 3450:1. However, the assay has some practical limitations, such as the high cost of the PNA probes and the careful manipulation of the reactions to avoid exposure to oxygen. Many efforts have also been made to improve the selectivity and reduce the time and sample consumption by using various signal amplification reactions or PCR-based discrimination techniques such as allele-specific amplification PCR, coamplification at lower denaturation temperature PCR, wild-type blocking PCR and digital PCR. However, these approaches rely on precise temperature control and primer design.

Fig. 1 (A) Schematic depiction of the principle of the enzyme-mediated DNA detection at single-base difference level by using a double-mismatch/fluorophore modified DNA probe and lambda exonuclease. M represents the polymorphic base in the target strand. N is the base opposite M in the probe. (B) Comparison of the fluorescence response signals of two single-base different DNA strands. For the probe/target hybrid containing a matched N:M basepair, the probe was efficiently hydrolyzed by λ exo and gave out strong fluorescent signals. By contrast, when M was a mismatched base, the hydrolytic reaction by λ exo was almost completely prohibited.
In recent years, enzyme-mediated oligonucleotide-based fluorescent sensing methods have shown promising results in both DNA detection and nuclease measurement.\textsuperscript{21, 26-30} Recently, we have investigated the effects of a purposefully incorporated mismatch in the DNA duplexes on the discrimination ability of nucleases to the presence of a second mismatch,\textsuperscript{27, 29} which afforded remarkably enhanced selectivity in comparison to the hybridization-based probes. However, these established assays could not work efficiently at room temperature.

Herein, we present a novel strategy for single-nucleotide variation detection at room temperature with high selectivity. It is based on an extremely specific interaction between Lambda exonuclease (\textlambda\textsubscript{exo}) and a chemically modified DNA structure. As depicted in Figure 1A, a 5'-phosphorylated single-stranded DNA (ssDNA) probe is hybridized to a target strand that contains a polymorphic base (represented by M). A fluorophore (FAM) is covalently attached to the nucleoside at 5' side of N (the base site opposite M) and a quencher is attached at the 3' end of the strand. With the fluorophore-tagged nucleoside designated as position 0, two mismatched bases are intentionally inserted at position -1 (represented by X) and position +II (represented by Y), respectively. \textlambda\textsubscript{exo} is an exonuclease that prefers double-stranded DNA (dsDNA) with 5'-phosphorylated end as a substrate and catalyzes stepwise hydrolysis of the 5'-phosphorylated strand in 5' to 3' direction by forming a symmetrical toroid homotrimer\textsuperscript{32}.

Despite the existence of the two purposefully inserted mismatched bases, we found that the probe/target hybrid containing a matched N:M basepair at position +1 can still be efficiently hydrolyzed by \textlambda\textsubscript{exo} and gives out strong fluorescent signals. By contrast, when M is substituted by a mismatched base, the hydrolytic reaction by \textlambda\textsubscript{exo} was almost completely inhibited. More importantly, since \textlambda\textsubscript{exo} has little activity on ssDNA or non-5'-phosphorylated dsDNA substrate,\textsuperscript{33} excess amount of the probe can be added to the reaction system. Thus, after the hybridized probe was digested, the remaining intact target strand could hybridize to another probe and trigger next-round of enzymatic digestion, resulting in significantly amplified differential effects between the two single-base different target DNA strands (see Figure 1B).

The high discrimination power of \textlambda\textsubscript{exo} between a double mismatch containing DNA duplex and a triple mismatch containing DNA duplex in the presence of a covalently labelled fluorophore were unexpectedly observed recently in our lab in a systematic study on the fidelity of \textlambda\textsubscript{exo}.\textsuperscript{34} By using a 21-nt probe (P-3FAM-4C, see Table 1) in which the fluorophore (FAM) is attached to the third base from the 5' end, presence of double or triple mismatched bases adjacent to the fluorophore-tagged nucleoside resulted in different effects on the digestion rate of the probe/target hybrid by \textlambda\textsubscript{exo} (Figure 2A). In comparison with the perfectly matched probe/target hybrid, simultaneous presence of double or triple mismatched bases at 5' side of the fluorophore-tagged nucleoside all lead to increase of the digestion rates. By contrast, dramatic decline of the digestion rate was observed for probe/target duplexes containing mismatches at both 5' and 3' sides of the fluorophore-tagged nucleoside. By comparing the signal observed with (-I/+II)-triple-mismatch strand with that observed with (-I/0)-double-mismatch strand, substitution of the base at position +I with a mismatch in the presence of two existed mismatched bases induced an abrupt decrease of the digestion rate. Though a similar effect could be seen by comparing the signal of (-I/+I/0)-triple-mismatch strand with that of (-I/+II)-mismatch strand, the single-mismatch discrimination factor (DF, defined as the ratio of the rate of increase in fluorescence intensity observed with the target containing a matched base at the polymorphic site to that observed with the target mismatches with the probe at the polymorphic site) between (-I/+II)-double-mismatch and (-I/+I/0)-triple-mismatch was about two-fold of that between (-I/0)-double-mismatch and (-I/0/0)-triple-mismatch. These results implies great potential of the (-I/+II)-double-mismatch probe for ultra selective discrimination of single-nucleotide variation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{(A) Comparison of the rates of the fluorescence increase for the probe/target hybrid containing double or triple mismatches at different positions in comparison with the perfectly matched duplex. The Roman numerals in the brackets indicate the positions of the mismatch bases in the target strands when hybridized to probe P-3FAM-4C (The sequences is 5'PO\textsubscript{4}-TCT(T-FAM)\textsubscript{C}C\textsubscript{C}A\textsubscript{C}A\textsubscript{C}A\textsubscript{C}A\textsubscript{T}ACT\textsubscript{C}CTC\textsubscript{C}-BHQ1). The two red arrows denoted the data observed for the probe/target hybrids containing (-I/+II)-double-mismatch and (-I/+I/0)-triple-mismatch, respectively. (B) Comparison of the DFs between (-I/+II)-double-mismatch and (-I/0/0)-triple-mismatch at different temperatures. P-3FAM-4T(5'PO\textsubscript{4}-TCT(-FAM)\textsubscript{C}C\textsubscript{C}A\textsubscript{C}A\textsubscript{C}A\textsubscript{C}A\textsubscript{T}ACT\textsubscript{C}CTC\textsubscript{C}-BHQ1) was used for the test. The two purposefully inserted mismatched bases are underlined and the base opposite the SNP site is indicated in bold italics.}
\end{figure}
be due to the increase of the ionic strength of the solution, which
between the (PI/+II)PdoublePmismatched probe/target duplex and
probes.
To find out the main reasons for above discrimination effects, we
performed several comparison studies. First, we synthesized a
new 29-nt probe (P-15FAM-4C, see Table S1) which comprised
the same sequence as P-3FAM-4C (Table 1) near the 5'-end
while the fluorophore was attached to the fifteenth nucleoside
from the 5'-end of the probe. Then we measured the fluorescence
signals of the probe in the presence of three different target
strands. As shown in Figure S2A in the Supporting Information,
in comparison with the perfectly matched probe/target hybrid, the
(-I/+II)-double-mismatched probe/target duplex showed generally
similar declined signal as that observed with P-3FAM-4C. However,
the (-I/+I/+II)-triple-mismatched probe/target duplex didn’t show a significant reduced signal as that observed with P-
3FAM-4C (Figure 1B), suggesting a considerable contribution of
the fluorophore to the inhibition of the enzymatic digestion. To
confirm this, we further synthesized a probe P-3digoxin-15FAM-
4C, which had the same sequence as P-15FAM-4C but with an
additional label of digoxin at position 3 from the 5'-end. Figure
S2B shows considerable discrimination ability of this probe
between the (-I/+I)-double-mismatched probe/target duplex and the
(-I/+I/+II)-triple-mismatched probe/target duplex. We also
compared the reaction rates of perfectly-matched P-3digoxin-
15FAM-4C/target duplex with that of the perfectly-matched P-
15FAM-4C/target duplex, which were found to be at a ratio of
0.9:1.0, indicating that without the two inserted mismatches,
modification of the nucleotide with digoxin only slightly
influenced the reaction rate. Taken together, a synergistic effect
between the covalently modified fluorophore and two adjacent
mismatched bases seems to play a key role in the dramatic
change of the interactions between the probe/target hybrid and λ
exo. Further investigation on the exact mechanism of these
effects is undertaken and will be reported later.
The commonly used buffer for λ exo reaction was 1×Lambda
Exonuclease Buffer (67 mM Glycine-KOH, 2.5 mM MgCl₂, 50
µg/mL BSA, pH 9.4@25°C). However, above detection system
showed better performance in 1×ThermoPol Reaction Buffer (20
mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄,
0.1% TritonX-100, pH 8.8@25°C). We further tested different
concentrations of ThermoPol Reaction Buffer (from 0.5× to 5×).
As shown in Table S2, the largest DF was obtained in 3×ThermoPol Reaction Buffer. Then we tried to add (NH₄)₂SO₄-
KCl, MgSO₄, and Triton X-100 respectively to the 1×ThermoPol
Reaction Buffer to find out the major factor for the enhancement
effect. It was found that additional MgSO₄ in the buffer increased
the enzyme activity but decreased the DF, while additional Triton
X-100 slightly decreased both the enzyme activity and the DF.
Mg²⁺ was the essential ion in the active center of λ exo. With
more Mg²⁺, the digestion rate of (-I/+I/+II)-mismatched DNA
duplex by λ exo probably increased more than that of the (-I/+II)-
mismatched duplex, resulting in a decrease of the DF. By contrast,
additional (NH₄)₂SO₄ and KCl slightly decreased the enzyme
activity but obviously improved the DF (see Table S2). This may
be due to the increase of the ionic strength of the solution, which
decreased the digestion rate of (-I/+I/+II)-mismatched substrate
more than that of the (-I/+II)-mismatched substrate, thus lead to
an increase of the DF. Accordingly, we chose 1×ThermoPol
Reaction Buffer with 20-30 mM (NH₄)₂SO₄ and 20-30 mM KCl
as the reaction buffer.

**Table 1.** The sequences and discrimination factors (DF) of
duplexes containing different types of 5’,3’ mismatches.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Type of mismatched base (N:M)</th>
<th>Discrimination factor (DF)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-3FAM-4C</td>
<td>C:C</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>C:A</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>C:T</td>
<td>140</td>
</tr>
<tr>
<td>P-3FAM-4G</td>
<td>G:T</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>G:A</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>G:G</td>
<td>17</td>
</tr>
<tr>
<td>P-3FAM-4T</td>
<td>T:C</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>T:T</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>T:G</td>
<td>4.0</td>
</tr>
<tr>
<td>P-3FAM-4A</td>
<td>A:C</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>A:A</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>A:G</td>
<td>4.2</td>
</tr>
</tbody>
</table>

¹The probe sequence is 3’PO₄-TC(T-FAM)NCACAGACAC
ATACTCCA-BHQ1; The target sequences are 5’GTITTAAAA
TTATGGAGTATGTGTCTGTTMAACGAGAGTAAG; The
two purposefully inserted mismatched bases are underlined. M
represents the SNP site. N is the base opposite M in the probe,
while C, G, T and A for P-3FAM-4C, P-3FAM-4G, P-
3FAM-4T and P-3FAM-4A, respectively. ²1×ThermoPol
Reaction Buffer with additional (NH₄)₂SO₄ and KCl was used
as the reaction buffer. For reactions with P-3FAM-4G, the
concentration of (NH₄)₂SO₄ and KCl was 30 mM. For other three
probes, the concentration of (NH₄)₂SO₄ and KCl was 20 mM.

The influences of temperature on the discrimination effects
were examined in the range from 25°C to 48°C. From Figure 2B,
the assay shows high DFs in the temperature range from 25°C to
37°C, suggesting a widely adaptable working temperature of the
method. At temperatures higher than 42°C, the enzyme activity
significantly decreased. Considering the potential use of the
method in high-throughput microfluidic chips or other micro-
devices without temperature-control system, we chose 25°C (the
room temperature condition) to perform the detection.

Under the optimized reaction conditions, we tested twelve
types of targets with match or mismatched bases at the +1
position. As shown in Table 1, the DFs obtained by our new
probes are remarkably higher than those obtained by the simple
hybridization approaches. As an example, the highest DF (320)
observed with the probe/target hybrid containing a C:C mismatch
is more than 10-fold higher than that obtained by the triple-stem
probe for the same type of mismatch (28.4)³.

The specificity of hybridization-based probe was usually
believed to be determined by its thermodynamic stability, and
insertion of an additional mismatch has been found to cause
significant decrease the melting temperatures (Tₘ) of the hybrid¹².
But as aforementioned, without the covalently attached
fluorophore, presence of double mismatches in the probe-target
hybrid didn’t offer significant discrimination power. To clarify
whether the exceptionally large DFs of the new detection system was mainly due to the difference of melting temperatures (T_m) or the specific interaction between the enzyme and the modified structure of the probe, we measured the T_m of the perfectly-matched probe/target duplexes containing different mismatches. The differential in melting temperature for (-I/+II)-double-mismatch vs. (-I/+I/II)-triple-mismatch (57.5°C vs 56.8°C) was observed to be much smaller than that for (-I)-double-mismatch vs. (-I/0/I)-triple-mismatch (62.5°C vs 59.5°C), suggesting that the stability of the hybrid is not the major factor for the high discriminate effect between the (-I/+II)-double-mismatch and (-I/+I/II)-triple-mismatch. We also measured the T_m of the perfectly-matched probe/target duplexes with and without the fluorophore label, which were found to be 65.0°C and 63.5°C, respectively. Clearly, the difference of T_m was not the major cause of the large DFs of the new method. We conferred that the modification of the chemical structure of the probe by the fluorophore and the two mispaired bases might have altered the interactions between the enzyme and the probe/target hybrid.

It is worth mentioning that the fluorophore in our probe not only acts as a signal reporter in the detection system, but also plays a notable role in the specific molecular recognition between the enzyme and the probe/target hybrid. Moreover, the amplification process of our assay is not a simple enzyme-driven amplification process. Actually, each time the probe strand was digested by λ exo, the enzyme will precisely differentiate the special structure of the probe/target hybrid. Thus the amplification process resulted in an accumulated effect of multiple differentiation processes. So the high discrimination power actually originates from a more complicated protein/DNA interaction rather than a simple hybridization between two single strands.

To demonstrate the applicability of the system in SNP detection, we used P-3FAM-4T to detect different types of bases at position +1. From the fluorescence intensity responses over time shown in Figure 3A, the four hybrids formed with normal and single-nucleotide-variant DNA targets are clearly differentiated. Then we further applied the method to detect low-abundance mutations in the presence of different amounts of single-base different wild-type strands. The probe was designed to be matched with the mutant type at +1 position, thus the wild type will mismatch with the probe at position +1. From Figure 3B, the C:C mismatched target (G→C & C→G mutant types) can be clearly differentiated from the matched target with an abundance down to 0.05% (S/N=4.3-3) as shown in the inset of Figure 3B) at room temperature within 20 min. The assay sensitivity was determined by using P-3FAM-4C as the probe and 5'-GTTTTAATATTGAGTATGTGCTGTGAAACGAGAGTAAG as the target sequence. From the results shown in Figure S3, the limit of detection of the assay was 30 pM (1.5 fmol in 50 µL) (S/N=4.9-3). Other mutant types were also measured and the detailed results were summarized in Figure S4 in the Supporting Information.

For an arbitrary analyte, the double-mismatch/fluorophore modified DNA probe (P-3FAM-4T) in the detection of different SNP at position +1; (B) Fluorescence intensity responses of P-3FAM-4C in the detection of DNA point mutations at different abundances. The inset shows the mean values and standard deviations of the rate of fluorescence increase in the time period from 100 s to 600 s for the background solution and the two tested solutions with the lowest abundances (i.e. 0.05% and 0.2%). The probe sequence is 5’PO4-TCT[-FAM]NCACAGACACATACCTCA-BHQ1. The target sequence is 5’GGTTTTAATGATGAGTATGCTGTGAAACGAGAGTAAG. 100% means the tested strands are all mutant type (N:M = C:G). 0% means the tested strands are all wild-type (N:M= C:C).

V617F point mutation (1849G→T) in the Janus kinase 2 (JAK2) gene has been shown to be associated with several myeloproliferative disorders. The sequences of the JAK2...
wild-type and V617F mutant were listed in Table S1. Employing the strand complementary to the sequence of V617F mutant type as the target, we designed P-JAK2-DD in which 5-nitroindole (represented by D) was inserted at -I/+II positions as the universal mismatch base (see Table S1). Thus, in addition to the purposely inserted two mismatches, the third T:C mismatch at position +I between P-JAK2-DD and the wild-type would significantly prohibit the reactions between the probe and the wild-type DNA. For comparison, we also synthesized P-JAK2-CC in which we used the native base C to form mismatches at positions -I/+II (see Table S1). The reaction buffer was chosen to be 1×ThermoPol Reaction Buffer with 30 mM (NH₄)₂SO₄ and 30 mM KCl after optimization.

From the results shown in Table S3, P-JAK2-DD achieved a DF of 26, which was higher than that of P-JAK2-CC (DF=9.3), indicating better performance of the artificial base than native bases in the enzyme-mediated single-base difference discrimination test. To confirm this, we further tested two other target strands in which the two bases opposite the two artificial bases at positions -I/+II in the probe were changed from C/A to T/A and T/T, respectively (see Table S3). Interestingly, the DFs by P-JAK2-DD were both higher than those by P-JAK2-CC.

Next, we used P-JAK2-DD to detect the JAK2V617F mutant-type at different abundances. From the data shown in Figure S5, V617F mutant-type targets could be successfully identified in the presence of wild type strands at an abundance as low as 0.5%. Since the ThermoPol Reaction Buffer used for above detection is suitable for many polymerases such as Taq polymerase, our method can also be readily coupled to various PCR procedures. As shown in Fig. 4, the assay could also be applied for the low-abundance mutation detection in PCR amplicons with high selectivity.

Fig. 4 Fluorescence intensity responses of P-JAK2-DD to detect JAK2V617F mutant-type at different abundances in PCR amplicons. 100% means the tested strands are all JAK2V617F mutant-type. 0% means the tested strands are all JAK2 wild-type. The inset shows the mean values and standard deviations of the rate of fluorescence increase within 300-1200 s for the two tested solutions with the lowest-abundance (i.e. 0.5% and 2%).

The above results clearly demonstrate the high single-base substitution selectivity of the new system. It offers a very simple and rapid tool for highly selective single-base mutant detection at room temperature. The probe sequence can be flexibly adjusted to various target sequences without any strict length constraints, as long as the fluorophore and the two adjacent auxiliary mismatched bases are located at the proper positions. Moreover, we found that 5-nitroindole could be utilized as a universal mismatch base for different target sequences, which further simplified the probe design work. As no temperature control is required, the assay can be easily adapted to different high-throughput micro-devices without the need of complicated instrument design.

Conclusions

In this work, we have demonstrated that combination of two purposefully introduced mismatches and a fluorophore tag in the probe sequence offers a special chemical structure that shows specific interactions with Lambda exonuclease (λ, exo). By taking advantages of this new property, a simple and powerful system for the detection of single-nucleotide variation has been developed with extraordinarily high DFs (320±4.0). The method is single-step, rapid, and applicable at room temperature with minimum-sample consumption. It can be used individually or flexibly coupled to microfluidic instruments for sensitive detection of SNP and low-abundance point mutations without the need of temperature control.

Acknowledgements

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Notes and references