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Homogeneous Label-Free Genotyping of Single Nucleotide Polymorphism Using Ligation-Mediated Strand Displacement Amplification with DNAzyme-Based Chemiluminescence Detection

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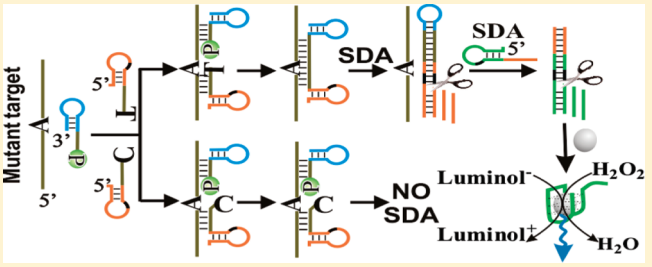
ABSTRACT: Genotyping of single nucleotide polymorphisms (SNPs) is a central challenge in disease diagnostics and personalized medicine. A novel label-free homogeneous SNP genotyping technique is developed on the basis of ligation-mediated strand displacement amplification (SDA) with DNAzyme-based chemiluminescence detection. Discrimination of single-base mismatches is first accomplished using DNA ligase to generate a ligation product between a discriminant probe and a common probe. The ligated product then initiates two consecutive SDA reactions to produce a great abundance of aptamer sequences against hemin, which can be probed by chemiluminscence detection. The developed strategy is demonstrated using a model SNP target of cytochrome P450 monooxygenase CYP2C19*2, a molecular marker for personalized medicines. The results reveal that the developed technique displays superb selectivity in discriminating single-base mismatches, very low detection limit as low as 0.1 fM, a wide dynamic range from 1 fM to 1 nM, and a high signal-to-background ratio of 150. Due to its label-free, homogeneous, and chemiluminescence-based detection format, this technique can be greatly robust, cost-efficient, readily automated, and scalable for parallel assays of hundreds of samples. The developed genotyping strategy might provide a robust, highly sensitive, and specific genotyping platform for genetic analysis and molecular diagnostics.



Single nucleotide polymorphisms (SNPs) are the most frequently occurring and stable forms of genetic variations found in human genome. Depending on their locations in the genome, SNPs may have different consequences at the phenotypic level.[1] SNPs present in regulatory sites of a gene will perturb the transcriptional rate, affecting the expression level of encoded protein. In the coding region, SNPs may alter the structure and, hence, function of encoded protein. These SNPs are often recognized as molecular markers of genetic disorders and disease predisposition. Another important implication of SNPs is alteration of the primary structure of a protein involved in metabolism of drugs or environmental toxins. These SNPs are useful targets for pharmacogenetic analyses. Genotyping of SNPs is, thus, a crucial step for disease diagnostics and personalized medicine.

Many technologies are known for SNP genotyping using a certain allele discrimination reaction combined with a specific detection strategy of the products.[2−5] Representative allele discrimination reactions include allele-specific probe hybridization,[6−8] allele-specific nucleotide incorporation and primer extension,[9,10] allele-specific probe cleavage,[11,12] and allele-specific probe ligation.[13,14] Hybridization-based methods discriminate single-base variations using differences in hybridization efficiency,[15−18] thermal stability,[19] and local surroundings[20−23] of double-stranded DNA hybrids between a designer probe and the target sequence. These techniques offer a unique advantage of eliminating the need of allele-discriminating enzymes, thus allowing the use of enzyme-impairing assay media and storage conditions. However, a critical issue toward these methods is the difficulty in controlling the assay conditions and the optimizing probe designs.[2] Also, the lack of efficient nonenzymatic approaches for target amplification has made preliminary amplification of genomic targets, typically polymerase chain reaction (PCR), necessary for these hybridization-based methods.[2]

On the other side, enzymatic methods use certain enzyme-aided biochemistry for SNP genotyping. Typical examples are single nucleotide incorporation and primer extension based on recognition of nucleotides by DNA polymerases[24,25] and specific probe cleavage of invader structure by flap endonuclease,[26,27] as well as probe ligation at perfectly matched ends by DNA ligases.[28,29] Because enzyme-catalyzed reactions exhibit high fidelity in single-base differentiation and facilitate efficient target amplification, these techniques can be highly preferable for clinical applications where reliable SNP scoring is required with very limited genomic targets available. Despite the success, all the

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Table 1. Synthesized Oligonucleotides (5′→3′) Used in the Experiments[a]

mutant target	5'-AAT TTT CCC ACT ATC ATT GAT TAT TTC CCA GGA ACC CAT AAC AAA TTA CTT AAA AA-3'
wildtype target	5'-AAT TTT CCC ACT ATC ATT GAT TAT TTC CCG GGA ACC CAT AAC AAA TTA CTT AAA AA-3'
probe **1**	5'-CCT GCT CAG CGT CCA CTC GTT TTG ACT CGG ACG CTG ATT TGT TAT GGG TTC CT-3'
probe **2**	5'-CCT GCT CAG CGT CCA CTC GTT TTG ACT CGG ACG CTG ATT TGT TAT GGG TTC CC-3'
probe **3**	5'-PO_3^{2-}-GGG AAA TAA TCA ATG ATA GAC ATC GGC CTT TTT TTT TTG GCC GAT G-3'
probe **4**	5'-CCC AAC CCG CCC TAC CCT TTT GAC TCG TAG GGC GGC CTG CTC AGC GTC CAC TCG-3'
probe **5**	5'-CCC AAC CCG CCC TAC CCT TTT GAC TCG TAG GGC GGA TTT GTT ATG GGT TCC T -3'
probe **6**	5'-PO_3^{2-}-GGG AAA TAA TCA ATG ATA GAC ATC GGC CCG CTA CTA-3'
probe **7**	5'-TAT GCT CGG TCT CCT TAA ATT TTG ACT CGG AGA CCG ATT TGT TAT GGG TTC CT-3'
probe **8**	5'-CCG AAC GGC GCC TAG CCT TTT GAC TCC TAG GCG CCC CTG CTC AGC GTC CAC TCG-3'
non-cognate target	5'-GAG CAT AAA CAG GTG GGA GTT GTC TTA CCA ACT CTG AGA GGC CAA TTA ATT AAG AGA AAA -3'

[a] Probes **1** and **2** are the discriminating probes, probe **3** is the common probe, probe **4** is used in the second-step SDA to produce hemin aptamer, probe **5** is used in place of probes **1** and **4** for the single-step SDA to produce hemin aptamer, probe **6** is different from probe **3** in that it has no hairpin structure at the 3′ terminus, probe **7** is different from probe **1** at 5′ end to alter the sequence of amplicons from the first-step SDA, probe **8** is obtained by altering the sequence of probe **4** at 5′ end in order to alter the sequence of amplicons from the second-step SDA. The recognition sequence for Nt.Bst NBI nickase is highlighted in blue. The SNP site is highlighted in red in target sequences and discriminant probes. Underlined sequences in a single probe are complementary to form hairpin structure.

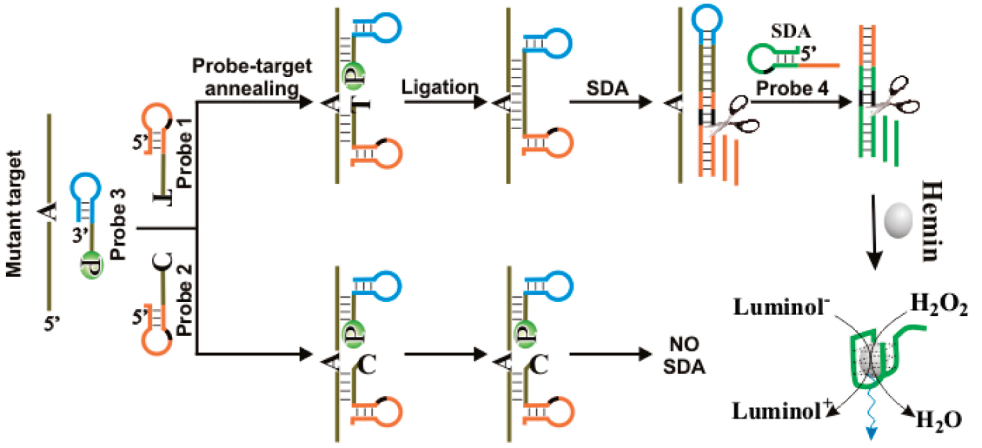
methods are known to have some disadvantageous operational attributes such as involving costly labeled reagents, sophisticate instrumentation, or multistep washing and separation. In the context, the development of selective and sensitive enzyme-aided genotyping techniques with desirable operational attributes has been a topic of intensive interest in analytical chemistry.

In the present study, a highly selective and sensitive label-free SNP genotyping technique is developed on the basis of ligase reaction and an isothermal signal amplification procedure, strand displacement amplification (SDA).[30] We have demonstrated that the fidelity of DNA ligases allows highly specific SNPs typing for molecular markers of genetic deseases[31] and cancers.[32] Following allele discrimination by DNA ligase, a nickase-based SDA is initiated, which produces a great amount of peroxidase-mimic DNAzyme sequences enabling label-free chemiluminescence-based ultrasensitive quantification of the allele-specific products. The developed technique can be implemented in homogeneous format with no need of washing and separation steps. This makes the assays more robust, easily automated, scalable for parallel assays of hundreds of samples, as desired in clinical applications. Moreover, chemiluminescence-based readouts permit a wide dynamic range and the use of simple instrumentation. The developed SNP typing strategy is demonstrated using a model SNP target of cytochrome P450 monooxygenase CYP2C19*2. Such a genetic polymorphism is known to lead to a truncated, nonfunctional enzyme with no drug-metabolizing activity, which has long been an important goal of biomedical research because of its clinical significance for personalized medicines.[33]

EXPERIMENTAL SECTION

Chemicals and Materials. Vent exo$^-$ polymerase, N.Bst NBI nickase, *E. coli* ligase, Thermopol buffer, 10× NEB buffer 3 (1 M NaCl, 500 mM Tris−HCl, 100 mM MgCl, and 10 mM dithiothreitol, pH 7.9), and four nucleotides (dNTPs) were purchased from New England Biolabs (Ipswich, MA, USA). Luminol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), nicotinamide adenine dinucleotide (NAD^+), hemin, and H_2O_2 were purchased from Sigma Aldrich Chemical Co. All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. Luminol solution (25 mM) was prepared using 0.5 mM NaOH. A stock solution of hemin (25 μM) was prepared with dimethysulfoxide (DMSO) and stored at −20 °C in the dark. All other solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance of >18.3 MΩ. Oligonucleotide DNA targets and probes were designed according to the model SNP target of cytochrome P450 monooxygenase CYP2C19*2.[33] These oligonucleotides were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). Thermodynamic parameters and secondary structures of all oligonucleotides were calculated using bioinformatics software

Scheme 1. Illustration of SNP Genotyping Strategy Based on Ligation-Mediated Strand Displacement Amplification with DNAzyme-Based Chemiluminescence Detection



(http://www.bioinfo.rpi.edu-/applications/). The sequences of the synthesized oligonucleotides are given in Table 1.

Ligation-Mediated SDA Reaction. The ligation reaction was performed at 37 °C for 0.5 h in 20 μL of reaction buffer (50 mM NaCl, 10 mM KCl, 45 mM Tris−HCl, pH 8.8, 2 mM $MgSO_4$, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% (w/v) Triton X-100, and 0.5 mM dithiothreitol (DTT)) containing 1 mM NAD^+, 10 nM probe **1** or **2**, 10 nM probe **3**, 10 nM probe **4**, 0.5 U/μL *E. coli.* ligase, and the mutant target of a given concentration.

After the ligation reaction, 2 μL of dNTPs (10 mM), 4 μL of Vent exo$^-$ polymerase (2 U/μL), and 2 μL of Nt.Bst NBI (10 U/μL) nickase were added, and then, the SDA reaction of 50 μL was performed at 60 °C for 2 h in a buffer solution consisting of 50 mM NaCl, 10 mM KCl, 45 mM Tris−HCl, pH 8.8, 2 mM $MgSO_4$, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, and 0.5 mM DTT.

Chemiluminescence Detection. In the mixture of SDA reaction, 16 μL of H_2O, 20 μL of 5× HEPES−NH_4OH buffer (125 mM HEPES, 100 mM KCl, and 1 M NaCl, pH 8.0), 2 μL of hemin (25 μM), and 2 μL of luminol (25 mM) were added. The mixture was incubated at 37 °C for 15 min to allow the folding of DNAzyme (hemin with its aptamer) produced in the SDA reaction. When 10 μL of H_2O_2 (300 mM) was added to the mixture, the time-dependent chemiluminescence signal was recorded on an FL3-P spectrofluorometer (Horiba Jobin YVON) with a time interval of 1 s and an emission slit of 2.5 at 425 nm. The chemiluminescence spectra were recorded immediately after the addition of H_2O_2 with an emission slit of 2.5 nm from 350 nm through 500 nm with an interval of 2.5 nm.

SNP Typing of Genomic Samples. Human genomes, which were isolated from seven individuals with SNP genotypes identified by DNA sequencing, were kindly provided by clinical pharmacogenetic center at Xiangya hospital. SNP typing of these genomic samples was also performed though ligation-mediated SDA reaction followed by chemiluminescence detection with a slightly modified protocol for ligation reaction: In 20 μL of reaction buffer (50 mM NaCl, 10 mM KCl, 45 mM Tris−HCl, pH 8.8, 2 mM $MgSO_4$, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% (w/v) Triton X-100, and 0.5 mM DTT) containing 1 mM NAD^+, 10 nM probe **1** or **2**, 10 nM probe **3**, and 10 nM probe **4**, genomic DNA (~1 μg) from one of seven samples was added.

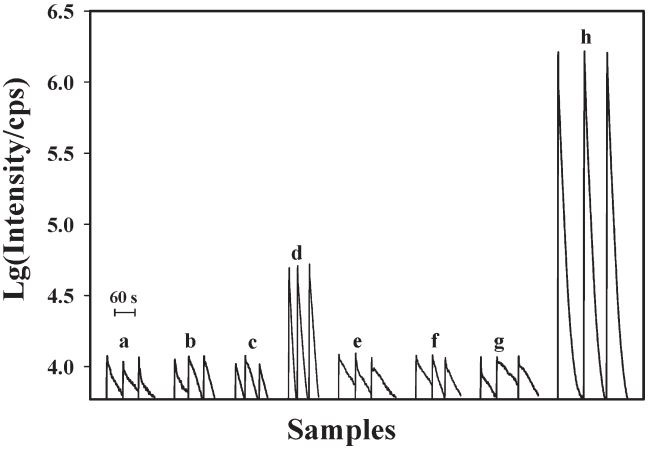


Figure 1. Time-dependent chemiluminescence responses (in logarithmic scale) in triplet repetitive assays for SNP typing. Luminescence signals were recorded at 425 nm with an emission slit of 2.5 nm: (a) control experiment using probes **1**, **3**, and **4** with no DNA target; (b) control experiment using probes **1**, **3**, and **4** with 10 pM wild-type DNA target; (c) control experiment using probes **1**, **3**, and **4** with 10 pM noncognate DNA target; (d) control experiment using probes **3** and **5** with 10 pM mutant target; (e) control experiment using probes **1**, **6**, and **4** with 10 pM mutant target; (f) control experiment using probes **7**, **3**, and **4** with 10 pM mutant target; (g) control experiment using probes **1**, **3**, and **8** with 10 pM mutant target; (h) responses using probes **1**, **3**, and **4** with 10 pM mutant target.

The mixture was heated to 95 °C for 5 min followed by annealing at 37 °C for 15 min. Following the addition of 0.4 U/μL *E. coli.* ligase, the ligation reaction was performed for another 30 min at 37 °C.

RESULTS AND DISCUSSION

Probe Design and Analytical Principle. The SNP typing strategy comprises a ligase-based allele discrimination reaction followed by two consecutive SDA reactions that are able to produce a great abundance of aptamer sequences against hemin, as illustrated in Scheme 1. Two discriminating probes, **1** and **2**,

are designed to have a downstream sequence (near 3′ end) complementary to the mutant and the wild-type targets, respectively, with 3′ termini overlapping the polymorphic bases. At the 5′ end, probe **1** and **2** both have a barcode sequence upstream to a half recognition site (5′-GACTC-3′) for Nt.Bst NBI nickase. A common probe, **3**, is designed with the 5′-terminal sequence complementary to target DNA upstream to the SNP site. At the 3′ end, probe **3** has two complementary sequences to form a hairpin structure. Probe **4** is designed to have a reporter sequence complementary to hemin aptamer on the upstream region and the same barcode sequence as probe **1** on the downstream part with a half recognition site for Nt.Bst NBI nickase in-between.

In genotyping of DNA target, discriminating probe **1** or **2** is separately annealed with probe **3** on DNA target. When the discriminating probe forms a perfect complement with the target at the junction, it can be covalently joined with probe **3** by *E. coli* ligase, while no ligation product is obtained for discriminating probe mismatching with the target at the junction. The 3′ end of probe **3** can behave as a primer to initiate an extension reaction in the presence of vent exo$^-$ DNA polymerase and dNTPs. For ligated product, such a self-primed extension reaction can proceed across the half recognition site of Nt.Bst NBI nickase on the discriminant probe, yielding a full recognition site for nickase with a nicking site generated on the replicated strand. Hence, a cycle of nickase cleavage, polymerase extension, and subsequent replicated strand release is created, which renders a SDA of the ligated product. The released amplicons are then annealed to the downstream sequence of probe **4**, launching another SDA for the ligated product to produce abundant aptamer sequence against hemin. Thus, the target-specific ligation product is greatly amplified by two consecutive SDA into a large amount of hemin−aptamer complex, a peroxidase-mimic DNAzyme that can be readily probed from its strong chemiluminescence signal.[34−36] Note that a perfect complement is achieved only with probe **1** for mutant target and probe **2** for wild-type target. Hence, the mutant target can be determined by chemiluminescence signal using probe **1**, and the wild-type can be quantified by luminescence readout using probe **2**, thereby allowing immediate SNP typing of DNA target.

It was important to note that probes **1** and **2** were designed to have hairpin structure with a 9-basepair stem region. These probes, used in excess (10 nM), were complementary to the product sequences generated in the first SDA reaction, so they could capture the product sequences via hybridization, preventing them from annealing on probe **4** to prime the second SDA reaction. When such a hairpin structure was designed, capture of the product sequences generated in the first SDA was inhibited, thereby improving the amplification efficiency of consecutive SDA reactions. Similarly, probe **4** was also designed to have hairpin structure with a 9-basepair stem region. Such hairpin structure was beneficial for inhibiting the capture of aptamer sequences by probe **4** present in excess.

Typical Characteristics of SNP Assay. Figure 1 depicts typical time-dependent response curves using the developed SNP assay strategy in triplet repetitive assays. Typically, the chemiluminescence system of luminol−H_2O_2 and peroxidase or its analogues is a flash-type reaction in which the luminescence signal rises very sharply at the start of the reaction and decays quickly within seconds. Therefore, we obtained sharp peak-shaped time-dependent luminescence profiles in chemiluminescence measurements. In the absence of target DNA, we obtained a chemiluminescence

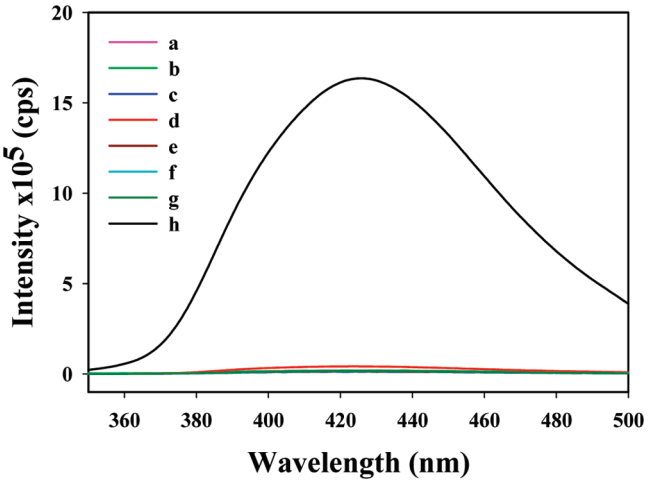


Figure 2. Chemiluminescence spectral responses in SNP typing assays. Luminescence spectra were recorded immediately after addition of H_2O_2 with an emission slit of 2.5 nm from 350 nm through 500 nm with an interval of 2.5 nm: (a) control experiment using probes **1**, **3**, and **4** with no DNA target; (b) control experiment using probes **1**, **3**, and **4** with 10 pM wild-type DNA target; (c) control experiment using probes **1**, **3**, and **4** with 10 pM noncognate DNA target; (d) control experiment using probes **3** and **5** with 10 pM mutant target; (e) control experiment using probes **1**, **6**, and **4** with 10 pM mutant target; (f) control experiment using probes **7**, **3**, and **4** with 10 pM mutant target; (g) control experiment using probes **1**, **3**, and **8** with 10 pM mutant target; (h) responses using probes **1**, **3**, and **4** with 10 pM mutant target.

peak of ∼1.52 × 10^4 cps with a relative standard deviation (RSD) of 4.5% in triplet repetitive assays (curves a). This luminescence background was originated from oxidation of luminol by H_2O_2 occurring at a relatively low rate due to the weak catalytic activity of hemin.[35] In the system of mutant target (10 pM) and probe **1**, a much stronger chemiluminescence signal (∼1.64 × 10^6 cps with a RSD of 5.8% in triplet repetitive assays) was observed with a signal to background ratio of ∼108 (curves h). Such an enhanced luminescence signal implied the formation of an abundant hemin−aptamer complex with substantially enhanced catalytic activity, evidencing successful ligation of probe **1** with probe **3** followed by the generation of aptamer sequences via two consecutive SDA reactions. In contrast, a luminescence response as weak as the background (∼1.54 × 10^4 cps with a RSD 3.7% in triplet repetitive assays) was obtained for the system with wild-type target (10 pM) and probe **1** (curves b), an obvious indicator of the presence of a very small amount of aptamer in the system as a result of unsuccessful SDA reactions. This signified that the developed SNP typing strategy was highly specific, which was able to identify single mismatch between the discriminant probe and target DNA. A further control was performed using noncognate DNA target (noncomplementary sequence for probes **1**, **2**, and **3**) in place of mutant target. A weak chemiluminescence signal (∼1.45 × 10^4 cps with a RSD 5.5% in triplet repetitive assays) was also observed in this control experiment (curves c), confirming the specificity of the genotyping method. Similar observations were obtained for the assay of DNA target using probe **2**, in which only a strong luminescence was achieved for wild-type target while a weak signal was achieved for mutant target. Combining these observations together, it was concluded that the presence of mutant or wild-type target could be identified with high specificity by a strong chemiluminescence

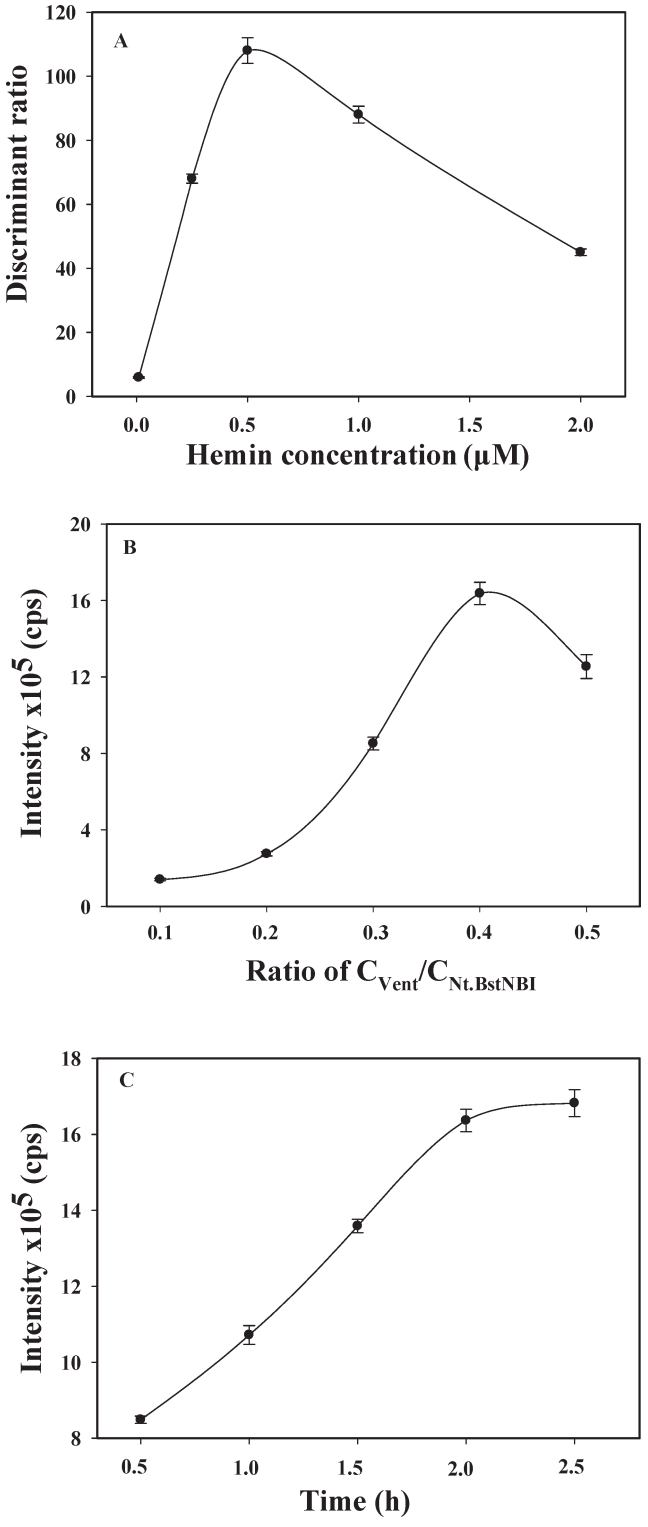


Figure 3. (A) Dependency of discriminant ratio values on hemin concentration using 10 pM mutant and wild-type target. Discriminant ratio is defined as the ratio of luminescence signal from the mutant target to that from the wild-type. (B) Dependency of chemiluminescence signals on polymerase concentration. DNA target concentration is 10 pM. (C) Dependency of chemiluminescence signals on SDA reaction time. DNA target concentration is 10 pM. Error bars are standard deviation of three repetitive experiments.

signal (relative to the background) obtained using probes **1** and **2**, respectively.

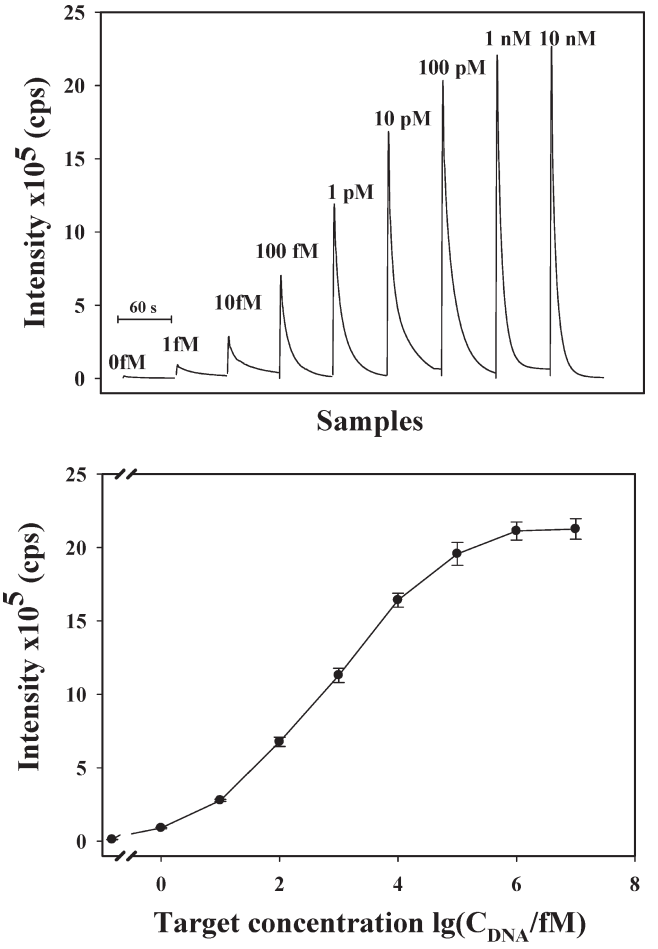


Figure 4. Time-dependent chemiluminescence signals of the SNP typing strategy in response to mutant target of varying concentrations. Luminescence signals were recorded at 425 nm with an emission slit of 2.5 nm.

To further disclose the advantage of two consecutive SDA over one-step amplification, another probe **5** was designed to have the reporter sequence complementary to hemin aptamer and used in place of probes **1** and **4** in the SNP assay. In this case, only a one-step SDA reaction was accomplished, and the released amplicons from the ligated product, hemin aptamer sequences, could directly deliver the chemiluminescence signal. It was observed that the corresponding luminescence response became much weaker ($\sim 4.25 \times 10^4$ cps with a RSD of 6.2% in triplet repetitive assays) than that obtained via two SDA reactions (curves d), suggesting that two consecutive SDA reactions offered substantial enhancement in detection sensitivity. Additional control experiments were further performed to validate the mechanism of the proposed SNP assay. In the control with probe **3** replaced by probe **6** that had no hairpin structure at the 3′ terminus, a luminescence signal comparable to the background was obtained (curves e). This confirmed that the hairpin structure was necessary to self-prime the SDA reaction. In another control, probe **1** was replaced by probe **7** that had a sequence different from that of probe **1** at the 5′ end. This sequence change made the amplicons generated in the first SDA reaction noncomplementary to probe **4**. In this case, we also observed a luminescence signal as weak as the background (curves f), which verified that the second-step SDA is primed by the product generated in the first-step SDA. In the third control experiment, we replaced

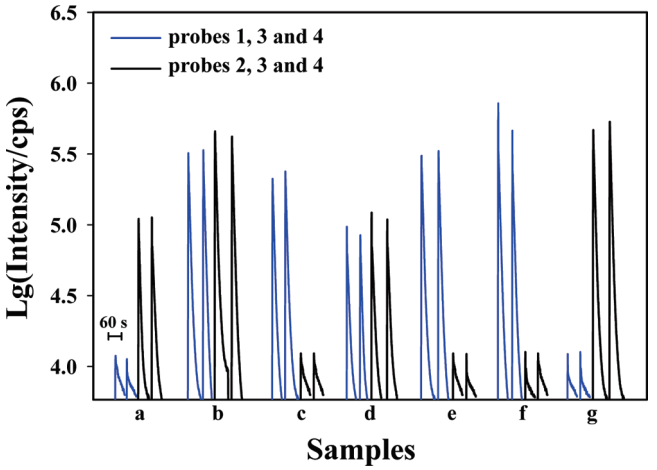


Figure 5. Time-dependent luminescence signals of the SNP typing strategy in response to seven genomic DNA samples. The samples are numbered alphabetically. Each sample is assayed using probes **1**, **3**, and **4** as well as probes **2**, **3**, and **4**, respectively, in order to determine the genotypes. Each assay is performed twice in parallel.

probe **4** by probe **8** that was obtained by slightly altering the sequence of probe **4** at 5′ end. Such sequence alterations made the sequence of the amplicons generated in the second SDA reaction different from that of hemin aptamer. In this case, we again obtained a weak chemiluminescence response (curves g), evidencing that hemin aptamer was generated in the second-step SDA reaction.

A further inspection of the corresponding luminescence spectra for the SNP assay was performed. As shown in Figure 2, in the perfect complement system of probe **1** and mutant target, a strong, broad emission peak was observed in wavelength range from 350 to 500 nm with a maximum at 428 nm, typical luminescence spectrum for the luminol−H_2O_2 reaction. This clearly verified the production of abundant hemin−aptamer complex in the system though two consecutive SDAs. In contrast, single-base mismatched, noncomplementary, and blank target DNA all gave very small luminescence peaks, revealing that amplification of hemin aptamer sequences by two consecutive SDAs was specific for DNA target mediating ligation between the discriminant and the common probes.

Optimization of SNP Assay Conditions. Luminescence responses of the SNP assay were greatly dependent upon the assay conditions such as concentrations of hemin, polymerase, and nickase and reaction time of SDA. Figure 3 depicts typical luminescence responses in correlation to these assay conditions.

It was observed that with increasing hemin concentrations luminescence signals in assay of both targets, mutant and wild-type, were enhanced substantially, signifying hemin-dependent catalytic kinetics for the luminol−H_2O_2 reaction. In order to optimize the concentration of hemin, we chose discriminant ratio, signal from the mutant target divided by that from the wild-type, as the measure. This measure reflected the selectivity of our SNP assay in detecting DNA target perfectly complementary to the discriminant probe. Figure 3A depicts the dependency of discriminant ratio values of the SNP assay on hemin concentration. It was observed that the discriminant ratio displayed a peak-shape dependency on the concentration of hemin with a maximum achieved using 0.5 μM hemin. Thus, this concentration of hemin was used throughout subsequent experiments.

The cooperation of two enzymes, vent exo$^-$ polymerase and Nt.Bst NBI nickase, had crucial effect on the efficiency of SDA. With a fixed concentration (0.4 U/μL) of Nt.Bst NBI nickase reported previously,[30] the effect of polymerase concentration was investigated, as shown in Figure 3B. A peak-shape dependency of luminescence response on polymerase concentration was also observed. The maximum luminescence signal was obtained with a polymerase concentration of 0.16 U/μL, which was taken throughout subsequent experiments.

Figure 3C depicts the dependency of luminescence signal of the SNP assay on SDA reaction time. With prolonged reaction time for SDA, the observed luminescence intensity gradually increased and became almost leveled off at 2 h. Thus, the reaction time for SDA was set to 2 h throughout subsequent experiments.

Quantitative Analysis and SNP Typing of Genomic DNA. Figure 4 shows typical time-dependent luminescence signals obtained in response to varying concentrations of mutant target. A dynamic increase in luminescence peak intensity was observed with increasing target concentrations within a seven-decade range from 1 fM to 10 nM. This wide dynamic range might be derived from the use of chemiluminescence for signal detection. A high-dose sensitivity was obtained in a logarithmic concentration scale within a six-order of magnitude concentration range from 1 fM to 1 nM with a detection limit of 0.1 fM, a concentration readily available in genomic analysis. In addition, the developed technique provided a high signal-to-background ratio of 150 at a target concentration of 1 nM, implying its excellent robustness in SNP assay. Note that the chemiluminescence signal obtained with 1 fM DNA target is approximately equal to that obtained using 0.5 nM hemin aptamer, which indicated that the amplification fold of the two consecutive SDA reactions was $\sim 5 \times 10^5$. It was also observed that the strategy exhibited excellent reproducibility due to its homogeneous assay format with simple operations. RSDs of luminescence peak intensities were 4.9%, 3.2%, 5.8%, and 4.3% in three repetitive assays of 1 fM, 100 fM, 10 pM, and 1 nM DNA target. Besides its high sensitivity compared to the most existing sensitive SNP typing techniques,[12,24,31] the developed strategy offered advantages in high signal-to-background ratio and wide dynamic response range. Also, it could be more robust, cost-efficient, readily automated, and scalable for parallel assays of hundreds of samples, because of its label-free, homogeneous, and chemiluminescence-based detection format.

The SNP typing technique was further validated using genomic DNA samples. Seven genomic samples were collected and directly analyzed without preliminary PCR amplification using the developed SNP assay strategy. The time-dependent response curves for these samples using discriminant probes **1** and **2**, respectively, are depicted in Figure 5. It was observed that, for samples *a* and *g*, the use of discriminant probe **2** gave luminescence signals significantly greater than the background response while the use of discriminant probe **1** showed luminescence signals comparable to the background response, indicating that target DNA was perfectly complementary to probe **2** at the 3′ terminus. In other words, the genotype of target DNA could be identified as homozygous wild-type in samples *a* and *g*. For samples *c*, *e*, and *f*, only the use of discriminant probe **1** generated significantly stronger luminescence signals than the background response, implying that the genotype of target DNA was homozygously mutant in samples *c*, *e*, and *f*. For samples *b* and *d*, the use of discriminant probes **1** and **2** both yielded appreciably stronger luminescence signals than the background response, suggesting that the genotype of target DNA was heterozygous (DNA is mutant on one chromosome and not on the other chromosome) in samples *b* and *d*. These SNP types were in good

agreement with the sequencing data, which clearly reveal that the developed genotyping strategy might become a promising technique for genomic research.

CONCLUSIONS

A highly selective and sensitive label-free SNP genotyping technique was developed on the basis of ligase reaction and an isothermal amplification reaction of SDA. The use of DNA ligase furnished this technique with desirable specificity in identification of SNPs, and the utilization of two consecutive nickase-based SDAs allowed highly efficient amplification to create a great abundance of peroxidase-mimic signal-reporter sequences. The developed strategy was demonstrated to display superb selectivity in discriminating single-base mismatches, very low detection limit as low as 0.1 fM, and a wide dynamic range from 1 fM to 1 nM. Due to its label-free, homogeneous, and chemiluminescence-based detection format, this technique could be greatly robust, cost-efficient, readily automated, and scalable for parallel assays of hundreds of samples, which supported its considerable potential in clinical applications. When a downstream cascade of SDA reactions with the addition of low-cost label-free DNA probes was introduced, this technique could be further improved to show extremely high sensitivity for direct assay of trace-level genomic samples. In view of these advantages, the developed genotyping strategy was expected to provide an intrinsically robust, highly sensitive, and specific genotyping platform for genetic diagnosis and association studies.

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