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# Highly sensitive chemiluminescent point mutation detection by circular strand-displacement amplification reaction

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ABSTRACT

Single nucleotide polymorphism (SNP) genotyping is attracting extensive attentions owing to its direct connections with human diseases including cancers. Here, we have developed a highly sensitive chemiluminescence biosensor based on circular strand-displacement amplification and the separation by magnetic beads reducing the background signal for point mutation detection at room temperature. This method took advantage of both the T4 DNA ligase recognizing single-base mismatch with high selectivity and the strand-displacement reaction of polymerase to perform signal amplification. The detection limit of this method was $1.3 \times 10^{-16}$ M, which showed better sensitivity than that of most of those reported detection methods of SNP. Additionally, the magnetic beads as carrier of immobility was not only to reduce the background signal, but also may have potential apply in high through-put screening of SNP detection in human genome.



## 1. Introduction

Single-nucleotide polymorphisms (SNPs) are single-base mutation in DNA among individuals and can be stably inherited in biologic genome. Genotyping of such sequence variations are very important for the analysis of genetic disorders and pharmacological responses (Aouacheria et al., 2005; Sachidanandam, 2001; Wang et al., 2006). Therefore, the sensitive and high-throughput screening detection of single-base mutation and the biological molecular is an urgent need for clinical diagnosis and research uses (Fan et al., 2000; Zhu et al., 2009; Wang et al., 2010). However, current methods satisfied only part of needs and were distantly from clinical applications. At present, the reported allele-specific SNP discrimination methods include fluorescence resonance energy transfer (Nesterova et al., 2009; Wang et al., 2009; Xiao et al., 2009), electronic measurements (Liu and Lin, 2007; Wakai et al., 2004; Wu et al., 2007; Huang et al., 2009; Zhang et al., 2008), binary DNA probe (Kolpashchikov, 2008, 2006; Deng et al., 2008), oligonucleotide-modified gold nanoparticle (NP) probes (Liu et al., 2009; Moody and McCarty, 2009; Thompson et al., 2008; Cao et al., 2002; Lim et al., 2008; Xue et al., 2009; Xu et al., 2009), triple-stem DNA probe (Xiao et al., 2009), and chemiluminescence detection (Su et al., 2010; Willner et al., 2008). Among the existing methods, enzyme-aided allele discrimination was widely applied, such as allele-specific nucleotide incorporation, ligation, endonuclease cleavage, and primer extension (Kwok, 2001). To increase the specificity for SNP detection, many researchers have played more attention on oligonucleotide ligation-based methods by DNA ligase (Huang et al., 2009; Xue et al., 2009; Willner et al., 2008; Li et al., 2005; Huh et al., 2009; Shi et al., 2011). In a ligase reaction, two probes anneal onto the target at the site of a SNP. A perfect match allows the ligase to covalently join the two adjacent probes that flank the mutation site, while a mismatch does not. Therefore, the incorporation of DNA ligase offers additional advantages of high fidelity and improved sensitivity.

The amplified detection of DNA is an important concept since it can provide the highest analytical sensitivity (Wang et al., 2003; Niu et al., 2010; Bi et al., 2010a,b; Cao et al., 2010). Conventional method such as real-time PCR is a widely applied thermal cycling technique for DNA amplification and can detect trace amounts of DNA to provide high sensitivity. However, it suffers from several disadvantages that include easy contamination, complex sample handling, and expensive instrument (Wang et al., 2009; Grossmann et al., 2007). In contrast, isothermal, low cost, simple, and high sensitivity detection method for nucleic acids is an urgent demand (Guo et al., 2009; Compton, 1991; Van Ness et al., 2003; Guatelli et al., 1990). Isothermal strand displacement polymerase amplification of DNA has attracted extensive research efforts. This amplified detection of DNA is at constant temperature, so the time required of amplification is less than that of thermal cycling PCR. In addition,

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the isothermal detection of DNA needs no special instrument and is more suitable for "on the spot" detection of nucleic acids (Connolly and Tran, 2010).

In this work, we made good use of high fidelity of T4 DNA ligase and isothermal strand displacement polymerase amplification of DNA to realize the high specificity and sensitivity of point mutation detection. Hairpin DNA probe was immobilized onto magnetic beads and mixed with the biotinylated reporter DNA and the target DNA to form a sandwich. When the mutant target DNA (single base mutation in codon 12 of K-ras oncogene) was perfectly match with hairpin probe and reporter probe, the T4 DNA ligase covalently joined the two adjacent probes that flanked the mutation site by 5′-$PO_4$ of hairpin probe and 3′-OH of reporter probe. Then, a short primer was annealed to opened hairpin probe and triggered the polymerization reaction with Klenow Fragment (exo$^-$) polymerase and dNTPs, associated with the target DNA displaced. The displaced target DNA recognized and hybridized with another magnetic bead-captured hairpin DNA and the biotinylated reporter DNA, triggering the next round of polymerization reaction, and reaching signal amplification. This was followed by the biotin-streptavidin reaction with conjugated HRP, and then the conjugated HRP catalyzed the luminol-$H_2O_2$ reaction with *para*-iodophenol (PIP) as the enhancer. While a mismatch (wild type) could not perfectly hybridize with two probes, so that ligation reaction was not successful for high specificity and fidelity of T4 DNA ligase. Then a primer annealed with opened hairpin probe and extended, which resulting in the reported probe displaced from magnetic beads. So the chemiluminescent signal did not occur, thereby the background signal was greatly reduced.

## 2. Experimental

### 2.1. Reagents and materials

The carboxylated magnetic beads (3–4 μm, diameter) were obtained from BaseLine Chromtech Research Centre (Tianjin, China). T4 DNA ligase, dNTPs, and Klenow Fragment (exo$^-$) polymerase were purchased from MBI Fermentas Crop. N-hydroxysuccinimide (NHS), *para*-Iodophenol (PIP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma. Streptavidin-horse-radish peroxidase (HRP) was acquired from Boster biotechnology Co. Ltd. Luminol was obtained from ABCR GmbH & Co. Imidazole was purchased from Guoyao Chemical Co. All other compounds were purchased from Sigma and used as received. All oligonucleotides were supplied and HPLC purified by SBS Genetech. Co. Ltd.

### 2.2. Apparatus

The chemiluminescent measurements were performed with a BPCL Ultra-Weak Luminescence Analyzer (Biophysics Institute of Chinese Academy of Science, China). UV visible spectra were carried out on a Cary 50 UV vis NIR spectrophotometer (Varian) for oligonucleotide quantitation.

### 2.3. Immobilization of hairpin DNA onto magnetic bead

50 μL (10 mg/mL) carboxylated magnetic beads were washed three times with 200 μL imidazole buffer (0.1 M, pH 6.0). 200 μL of 0.8 M EDC and 200 μL of 0.2 M NHS were added to the washed magnetic beads and shaked for 2 h at 37 °C. Next, 300 pmol $NH_2$-modified hairpin DNA in 100 μL 10 mM PBS buffer (0.3 M NaCl, pH7.4) was added to the activated magnetic beads for 12 h. Afterwards, 100 μL of 2% bovine serum albumin (BSA) were added to the magnetic bead/DNA suspension and incubated for 1 h at 37 °C with shaking. Ultimately, the modified magnetic beads by hairpin DNA were washed two times with 200 μL water and stored at 4 °C to use.

### 2.4. Single-base mutation detection

Single-base mutation detection was performed in 50 μL solution consisting of $4.8 \times 10^{-9}$ M report DNA, $6.0 \times 10^{-9}$ M primer, 200 μM dNTPs, 10 U T4 DNA ligase, 5 U polymerase Klenow Fragment (exo$^-$), and 5.0 μL modified magnetic beads by hairpin DNA in T4 DNA ligase reaction buffer (40 mM Tris–HCl, 10 mM magnesium chloride, 10 mM dithiothreitol and 0.5 mM ATP, pH7.8). A series of targets (mutant or wild type) at different concentrations were added to the described above mixture solution at 25 °C for 2 h and the chemiluminescence measurements were performed.

### 2.5. Chemiluminescence measurements

After the circular strand-displacement polymerization reaction, the modified magnetic beads by DNA double helix were separated. Next, 1 μL of 80 nM HRP in 10 mM PBS buffer were added to the final volume 100 μL and incubated for 30 min at 37 °C with gentle shaking. Afterwards, the conjugated magnetic beads were washed four times with 200 μL PBS and then resuspended in 300 μL PBS buffer. Ultimately, the conjugated magnetic beads were transferred into the 300 μL chemiluminescence (CL) reaction solution (0.2 mM luminol, 8.0 mM PIP, 2.0 mM $H_2O_2$) in 10 mM PBS (pH7.4) and the CL intensities were measured with the luminometer.

## 3. Results and discussion

### 3.1. Principle of amplified point mutation detection method

The operation of our isothermal amplified detection point mutation method was depicted in Scheme 1. This DNA detection system consisted of hairpin DNA probe, a short primer, reporter DNA probe, T4 DNA ligase, and polymerase. Hairpin DNA probe was immobilized onto carboxylated magnetic beads activated by EDC and NHS. The region with the dotted line below it identified the complementary sequence to the primer that was a 9-nt sequence long. A 48-mer single strand DNA (ssDNA) target is given and the 3′-end of the target molecule that had four C was not complementary with the reporter probe for blocking target extension. The base at position 25 from the 5′-end of the 48-mer ssDNA target was A (mutant) or C (wild type). The reporter DNA probe was modified at 5′-end by biotin. The boldface region of the reporter probe at 3′-end was complementary to the italic portion of the target. The italicized region of the hairpin DNA probe indicated in the loop and stem sequences was complementary to 5′-end of the target and the hairpin probe was opened and the primer annealed to it and amplified. The report probe was terminated at its 3′-end with 20-mer that were all complementary to the 19 bases at the 3′-end of wild type target but differed only by the last nucleotide at their 3′-termini (see Table 1). In the presence of wild type target DNA, the hairpin probe was opened and the primer annealed to it. Because of the specificity of T4 DNA ligase, 5′-$PO_4$ of hairpin probe and 3′-OH of reporter probe were unable to be ligated. The primer triggered a polymerization reaction in the presence of dNTPs and polymerase and the target was displaced from hairpin probe followed by the reporter probe separated from magnetic beads. The CL signal did not occur by the HRP-catalyzed luminol–PIP–$H_2O_2$ reaction system. However, in the presence of mutant target DNA, the T4 DNA ligase could covalently join the two adjacent probes that flanked the mutation site by 5′-$PO_4$ of hairpin probe and

**Table 1**
DNA probes and oligonucleotides used in this work.

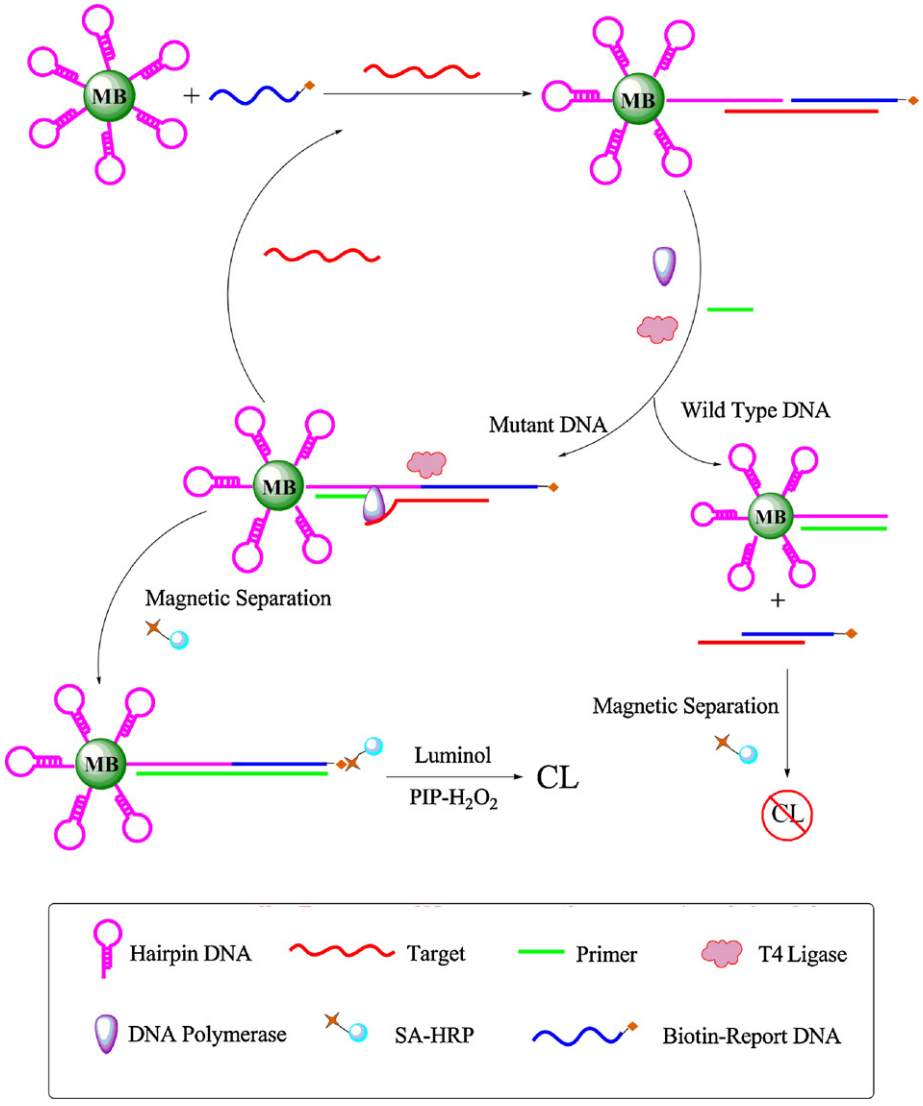
| Oligonucleotide | Sequence (5′ to 3′) |
|---|---|
| Hairpin probe[a] | $PO_4$-*TGGCGTAGGCAAGAGTGCCC*TGCCTACGCCATTCC-$(T)_{13}$-$(CH_2)_3$-$NH_2$ |
| Reporter probe | Biotin-TTT TTT TTT**CTTGTGGTAGTTGGAGCTGT** |
| Primer | GGAATGGCG |
| Mutant DNA[b] | TTTT**GGGCACTCTTGCCTACGCCA***A**CAGCTCCAACTACCACAAG*CCCC |
| Wild-type DNA | TTTTGGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAGCCCC |

[a] 5′-$PO_4$ denoted a 5′-phosphorylation. The stem and loop were highlighted in drop shadow and broken underline, respectively.
[b] The mutation position was highlighted in box and the boldface was the complementary nucleotides to hairpin DNA in italic portion. The italic portion was complementary with the boldface of reporter DNA.

3′-OH of reporter probe. Following this, the primer annealed to opened hairpin probe and originated a polymerization reaction with polymerase and dNTPs. A complementary DNA was synthesized onto magnetic beads, followed by the biotin-streptavidin reaction with conjugated HRP. The CL signal was obtained by the HRP-catalyzed luminol–PIP–$H_2O_2$ reaction system. The target was displaced during the primer extension and triggered new polymerization reaction.

### 3.2. Optimization of assay conditions

To optimize the detection method, the amount of the magnetic beads and HRP were investigated. The hairpin probes ($6.0 \times 10^{-7}$ M) were mixed with 30 μg, 50 μg, 100 μg, or 150 μg carboxylated magnetic beads, respectively. In Fig. 1, the CL intensities of different amount magnetic beads with (gray bars) or without (black bars) mutant targets were monitored at room temperature. It could




**Scheme 1.** Schematic outline of chemiluminescence biosensor based on circular strand-displacement amplification and the separation by magnetic beads reducing the background signal for point mutation detection.

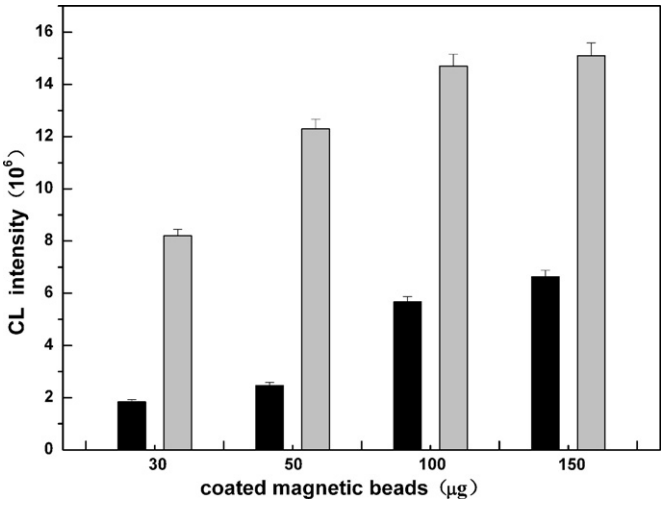



**Fig. 1.** Effect of the amount of magnetic beads on CL intensity. Testing solution consisted of $4.8 \times 10^{-9}$ M report DNA, $6.0 \times 10^{-9}$ M primer, 200 μM dNTPs, 10 U T4 DNA ligase, 5 U polymerase Klenow Fragment (exo$^-$), and different concentrations of modified magnetic beads by hairpin DNA in presence (gray bars) or absence (black bars) of $1.0 \times 10^{-13}$ M mutant target at 25 °C for 2 h. The illustrated error bars represented the standard deviation of three measures obtained at the same amount of magnetic beads.

be observed that the CL intensity increased with increasing the amount of magnetic beads. As shown in Fig. 1, 50 μg of magnetic beads provided a relatively high CL intensity enhancement in the presence of mutant target with a low background. Therefore, 50 μg of magnetic beads were used for the following experiments.

To optimize the concentration of HRP, we determined the effect of different concentrations of HRP on the CL intensity. As seen in Fig. 2, the CL signal was rapidly generated upon the addition of mutant target (gray bars), and the CL intensity maintained its increase with increasing the amount of HRP (6 μL, 8 μL, 12 μL, and 16 μL of $10^{-8}$ M). In control experiments without mutant target, it could be observed from Fig. 2 that the CL intensity was increased proportional with the amount of HRP (black bars). 8 μL of $10^{-8}$ M HRP provided a relatively high CL intensity enhancement in the presence of mutant target with a low background. Therefore, 8 μL of $10^{-8}$ M HRP were used for the following experiments.

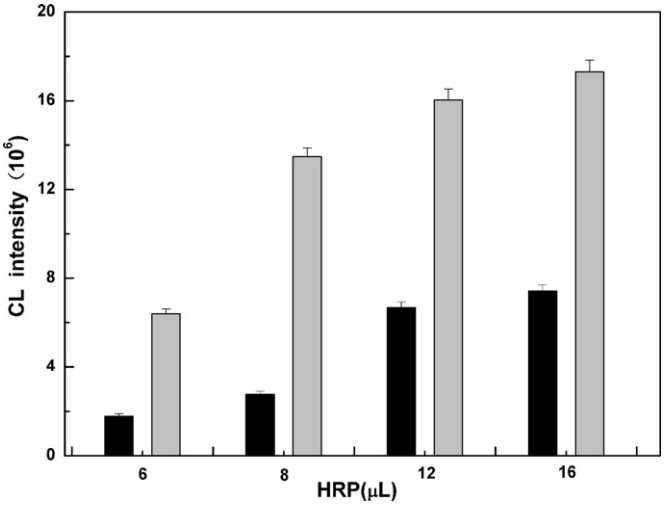



**Fig. 2.** Effect of the concentration of HRP on CL intensity. Testing solution consisted of $4.8 \times 10^{-9}$ M report DNA, $6.0 \times 10^{-9}$ M primer, 200 μM dNTPs, 10 U T4 DNA ligase, 5 U polymerase Klenow Fragment (exo$^-$), and 50 μg modified magnetic beads by hairpin DNA in presence (gray bars) or absence (black bars) of $1.0 \times 10^{-13}$ M mutant target at 25 °C for 2 h.

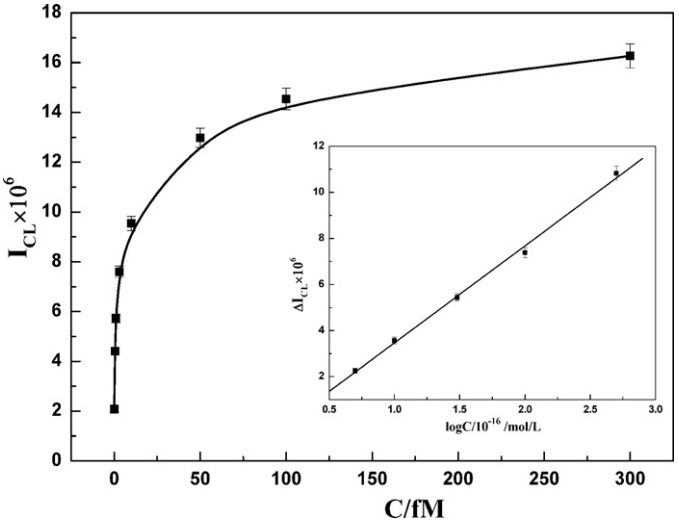



**Fig. 3.** Correlation between the CL intensity and the different concentration of mutant targets. The concentrations of mutant target DNA were 0, $5.0 \times 10^{-16}$, $1.0 \times 10^{-15}$, $3.0 \times 10^{-15}$, $1.0 \times 10^{-14}$, $5.0 \times 10^{-14}$, $1.0 \times 10^{-13}$, and $3.0 \times 10^{-13}$ M, respectively. The inset showed the linear relationship between the relative CL intensity and the logarithm of mutant target concentration.

### 3.3. Characteristics of point mutation detection with CL biosensor

Under the optimized experimental conditions, experiments were carried out by adding different concentrations of mutant target to verify whether the relative CL intensity could be used for mutant target quantitation according to the protocols described above. The CL intensity was observed on the addition of different concentrations of mutant target. The relationship between the relative CL intensity and the concentration of mutant target is determined in Fig. 3. As shown in Fig. 3, the relative CL intensity ($\Delta I$) revealed a good linear relationship with the logarithm of mutant target concentration ranging from $5.0 \times 10^{-16}$ to $5.0 \times 10^{-14}$ M. The regression equation could be expressed as $\Delta I = 4.212 \log C - 0.74811$ ($C$ was the concentration of mutant target, $10^{-16}$ M) with a correlation coefficient of 0.9985 for the linear calibration curve. A detection limit of $1.3 \times 10^{-16}$ M of mutant target could be further estimated using $3\sigma$ ($\sigma = S_b/s$; $S_b$, standard deviation of blank sample, $S_b = 0.1613$ ($n = 11$) in this experiment; $s$, the slope of the standard curve). This high sensitivity of mutant target detection was achieved. The most important was circular strand-displacement amplification to realize signal amplification and the amplification reaction was very significant to improve detection sensitivity. Second, the CL system of the HRP-catalyzed luminol–PIP–$H_2O_2$ reaction as attractive high sensitivity method can further improve the assay sensitivity. This sensitivity is comparable to or better than that of reported detection method for SNP listed in Table S1.

### 3.4. Selectivity of the DNA biosensor

Because of most clinical samples are the heterozygous state, the mutant DNA and wild-type DNA were mixed together to assess the selectivity of our proposed method. These heterozygous samples were prepared by mixing $1.0 \times 10^{-13}$ M wild-type DNA with mutant target of concentration varying from $2.5 \times 10^{-16}$ M to $1.0 \times 10^{-14}$ M. The proposed method to quantitatively detect such heterozygous samples was examined. Fig. 4 shows the effect of the wild-type to mutant DNA ratio on the detection of point mutation. As seen in Fig. 4, the CL intensity increased with the increased mutant DNA in the heterozygous sample solution. It was noticeable that as low as 1:400 (the mutant target concentration

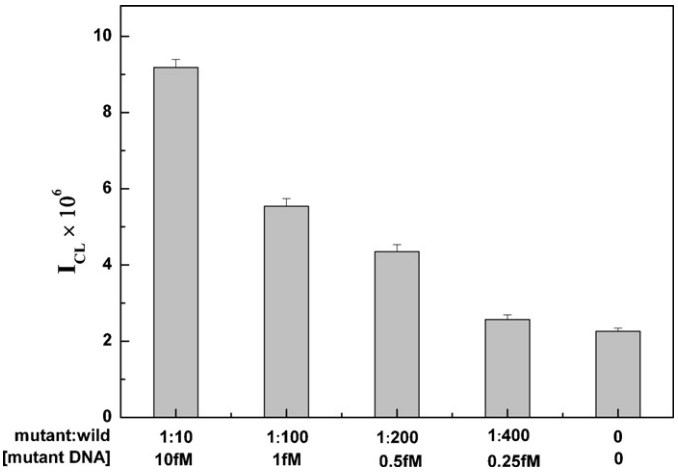



**Fig. 4.** The CL intensity of a series of heterozygous solutions containing mutant DNA of different concentrations from $2.5 \times 10^{-16}$ M to $1.0 \times 10^{-14}$ M and wild type DNA of $1.0 \times 10^{-13}$ M. The results were the average of three experiments.

is $2.5 \times 10^{-16}$ M) allele frequency was also detected. This was contributed to magnetic separation used to avoid CL of wild type DNA in heterozygous samples, which could greatly improve the sensitivity of the assay. In the presence of wild type target DNA, 5′-$PO_4$ of hairpin probe and 3′-OH of reporter probe were unable to be ligated because of the specificity of T4 DNA ligase. The target was displaced from hairpin probe followed by the reporter probe separated from magnetic beads after the primer triggered a polymerization reaction. So the CL signal did not occur. The result indicated the presented method had the high specificity for detecting a scarce mutation among abundant wild-type targets owing to the high fidelity of T4 DNA ligase.

The proposed method can also work with genomic DNA at isothermally 25 °C after the pretreatment of the samples. Firstly, the genomic DNA for point mutation detection usually was pretreated by restriction endonuclease, ultrasonication or DNAase to produce short DNA fragments. Then, the short DNA fragments were denatured and hybridized with hairpin DNA on magnetic beads, immediately removing the supernatant to separate the unhybridized DNA fragments. Next, circular strand-displacement amplification was originated for SNP detection by the proposed method.

## 4. Conclusions

In conclusion, a strategy that combined advantages of circular strand-displacement amplification and chemiluminescence was presented for point mutation detection at room temperature. The circular strand-displacement amplification to realize signal amplification was to significantly improve detection sensitivity. The CL system of the HRP-catalyzed luminol–PIP–$H_2O_2$ reaction can also further improve the assay sensitivity. In addition, the separation by magnetic beads was used to significantly reduce the background signal in heterozygous state and T4 DNA ligase to produce good selectivity, so 1:400 (the mutant target concentration is $2.5 \times 10^{-16}$ M) allele frequency was also detected. More importantly, this strategy can be expected for the simultaneous detection of various SNPs when different hairpin probes and report probes are used in a microarray. Therefore, this highly sensitive detection method of point mutation may have great potential applications, especially in high throughput detection of SNPs in human genome.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.05.017.

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