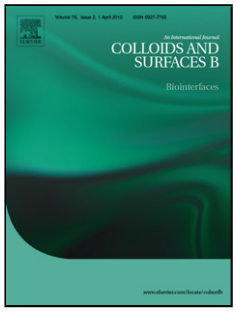

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PII: S0927-7765(14)00205-7
DOI: http://dx.doi.org/doi:10.1016/j.colsurfb.2014.04.007
Reference: COLSUB 6384

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 30-10-2013
Revised date: 9-4-2014
Accepted date: 14-4-2014

Please cite this article as: Y. Li, Y. Yan, Y. Lei, D. Zhao, T. Yuan, D. Zhang, W. Cheng, S. Ding, Surface plasmon resonance biosensor for label-free and highly sensitive detection of point mutation using polymerization extension reaction, *Colloids and Surfaces B: Biointerfaces* (2014), http://dx.doi.org/10.1016/j.colsurfb.2014.04.007

Surface plasmon resonance biosensor for label-free and highly sensitive detection of point mutation using polymerization extension reaction

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Highlights

- A simple SPR biosensor strategy has been developed for rapid and highly sensitive detection of point mutation.
- This is the first application based on polymerization extension reaction for point mutation detection.
- 100 pM mutant sequences can be detected within 15 min.
- Wild-type and mutant-type sequences can be successfully discriminated.

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Abstract

A novel biosensing technique was developed for label-free and highly sensitive detection of point mutation using surface plasmon resonance (SPR) biosensor coupled with polymerization extension reaction. In this work, 3'-thiolated DNA probes with complementary sequences to target DNA were immobilized onto the sensor surface via molecular self-assembly. In the presence of wild target sequences, the primers can be selectively extended by DNA polymerase to form double-stranded DNA. In contrast, mutant target sequences, containing one mutation site mismatched with the 3'-end base of the primer, can not be elongated. Thus, the extension reaction products can hybridize with the capture probes modified on the sensor surface to induce an SPR signal. The experimental results showed that the presented approach could detect the mutant sequences in *BRCA1* gene related to inherited breast cancer, and the wild-type and mutant-type sequences were successfully discriminated. Using synthetic DNA sequences as targets, 100 pM detection limits were achieved under the optimal reaction conditions. Hence, this highly sensitive and specific assay might have the potential to become an efficient alternative technique for point mutation detection in biomedical research and clinical diagnosis.

Keywords*:* Surface plasmon resonance biosensor, Polymerization extension reaction, Point mutation, BRCA-1, Breast cancer.

1. Introduction

Single nucleotide polymorphism (SNP) is one of the most common types of genetic diversity and occurs at a frequency of about one per 100-300 bases in human being [1]. SNPs are single-base mutations which can be stably inherited in human gene. Previous study indicated that the mutation in coding sequence of some tumor-related genes may disrupt gene functions and result in tumorigenesis [2]. Particularly, SNPs, as the promising biomarkers, play a key role in the early cancer diagnosis and monitoring of tumor progression [3]. Therefore, the identification of single-base mutations in target gene is crucial for cancer risk assessment and there is an urgent need to develop a simple, rapid, highly sensitive, and accurate method for point mutation assay.

Up to now, a variety of approaches have been developed for SNPs detection. Conventional techniques involved the implementation of denaturing gradient gel electrophoresis (DGGE) [4], mass spectrometry (MS) [5,6] and denaturing high performance liquid chromatography (DHPLC) [7,8] for the discrimination of allelic differences. However, various problems such as complex sample processing, laborious optimization and expensive instrumentation have restricted their application from meeting the demands of simple and rapid detection. Other approaches are

primarily built on allele-specific hybridization [9] or enzyme-aided allele discrimination，such as enzymatic cleavage [10]，oligonucleotide ligation [11,12] and primer extension mediated by the DNA polymerase [13,14]. Although hybridization-based methods are simple, they require complicated labeled process and stringent control of hybridization conditions in the assay. Mismatch recognitions with enzymatic reactions are attractive due to their high specificity, ease operation and quick detection. However, the limitation of these approaches is requiring the various radioactive or fluorescent labels.

In recent years, surface plasmon resonance biosensor has made great progress and can provide an alternative platform for detection of various biomolecules [15]. In this optical technique, the refractive index changes at the surface of a gold film induced by binding between target molecule and the immobilized ligand on the sensor surface can be measured, which is proportional to the amount of sensor surface-bound molecules [16]. SPR biosensors present a promising alternative to traditional techniques because they can obtain information of biomolecular interactions in real-time way and allow for rapid, sensitive and label-free detection [17]. Furthermore，SPR biosensors have been developed as a technique with great potential for high-throughput screening [18]. Significant efforts have been made to improve sensitivity and specificity of SPR biosensors, including the employment of peptide nucleic acids as a recognition element [19,20], DNA-modified gold nanoparticles utilized in a sandwich assay [21,22] and incorporation of enzyme reactions to achieve mismatch recognition [23,24]. In most of the published works, it has been demonstrated that primer

extension is a highly efficient reaction allowing a single set of analytic conditions for point mutation detection [25-27]. The properties of DNA polymerase in extension of a 3'-end match primer can be applied to discriminate mismatched configurations.

In this work, we presented a novel method that combines the high specificity of primer extension analysis with the label-free detection of surface plasmon resonance (SPR) biosensors to discriminate single-base mutation. Moreover, the incorporation of DNA polymerase is favorable for improving SPR detection sensitivity. The $Vent_R$ (exo^-) DNA polymerase employed in the research was genetically engineered to eliminate the 3' to 5' proofreading exonuclease activity associated with $Vent_R$ DNA Polymerase [28], which selectively extends the primers with a complementary base at the 3'-end to the target DNA. Although the extension reaction mediated by DNA polymerase was also applied in polymerase chain reaction (PCR) to obtain amplification product for point mutation detection [29], PCR technique cannot directly distinguish the mutation site. To our knowledge, this is the first application of SPR biosensor based on the mutation recognition of polymerization extension reaction for point mutation detection. All steps of the sample preparation are simple and analysis process can be completed within 15 min. The new established platform has been successfully applied for the detection of mismatched sequences corresponding to R1443X mutations of *BRCA1* gene [30]. Hence, this highly sensitive and specific assay might have the potential to become an efficient alternative technique for point mutation detection.

2. Materials and methods

2.1. Reagents

In our experiments, synthetic oligonucleotide sequences were used as models for PCR samples of the *BRCA*-1 gene obtained from real patients carrying the selected mutation (R1443X). All oligonucleotides used in the work were synthesized by Sangon Biotech (Shanghai, China) and the corresponding sequences are shown in Table 1. The 30-base DNA probes were modified with the insertion of a 15-T nucleotidic sequence and a six carbon thiol (C6-SH) at the 3′-end. All oligonucleotides were dissolved with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) into stock solutions and diluted to the desired concentration with Milli-Q water before use. $Vent_R$ (exo−) DNA polymerase was purchased from New England Biolabs (Beijing, China). 6-Mercapto-1-hexanol (MCH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions used for SPR experiments were prepared in Milli-Q water(≥ 18 MΩ, Milli-Q, Millipore) and filtered prior to use.

2.2. Probe immobilization

The method employed herein for the immobilization of oligonucleotide probes on the SPR sensor chip is based on the attachment of thiolated molecular (Probe-C6-SH) to the surface of the gold film. The gold chip surface was first cleaned with fresh piranha solution (70% H_2SO_4, 30% H_2O_2) for 10 min to remove organic adsorbed impurities, then rinsed with Milli-Q water several times and allowed to dry. The chip was then docked into the instrument socket, HEPES buffer saline-EP (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) was used as running buffer. 1 μM thiolated probes were dissolved in immobilization solution (1 M

KH_2PO_4, pH 3.8) and the immobilization procedure was carried out in flux at a rate of 2 μl min^{-1} for 30 min until the baseline reached a steady state. After undocked from the Biacore X^{TM} instrument, the sensor chip was covered with MCH (6-mercapto-1-hexanol) solution and kept for 4 h at room temperature in the dark, in order to minimize non-specific adsorption. Finally, the sensor chip was rinsed with deionized water and ready for hybridization reaction.

2.3. Primer extension reaction

Prior to SPR testing, polymerization extension reactions were carried out to obtain two allele-specific products for hybridization with the immobilized capture probes. The reaction volume of 50 μl contained various concentration of target sequences, 1 μM primers, 50 μM dNTPs, 0.1 U L^{-1} $vent_R$ (exo−) DNA polymerase and 1×thermopol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8) provided by the manufacturer. The mixtures of target sequences and primers were denatured at 95 □ for 10 min and cooling to room temperature. Then, dNTPs, DNA polymerase and reaction buffer were added into the mixture solution and reacted at 75 □ for 1 h. After the completion of the reaction, the product solution was immediately placed on ice. To investigate optimal reaction conditions for DNA polymerase activity, a series of concentrations of $MgSO_4$ (1.0, 1.5, 2.0, 2.5, 3.0 mM) were added into the thermopol (Mg^{2+}-free) reaction solution. Following, according to the optimized concentration of Mg^{2+} ion, different reaction temperature (60 □, 65 □, 70 □, 75 □, 80 □) and different reaction time from 30 min to 75 min were also studied.

2.4. SPR experiments

In this work, the Biacore X^{TM} system and a bare gold sensor chip (Sensor chip Au) (BIAcore AB, Uppsala, Sweden) were used to detect hybridization between probes and extension reaction products. The device has two flow cells which can be used separately or in series to allow for independent detection. The running buffer used in the experiment was hybridization solution (30 mM sodium phosphate, 450 mM NaCl, 3 mM EDTA, 0.25% Triton X100, pH 7.4). All the measurements were performed at a controlled condition (flow rate of 5 μl min^{-1}, 25 □). Hybridization reaction was monitored in real time by measuring refractive index changes over time. The analytical signal，measured in resonance units (RU), was displayed by the difference between values before and after sample injection. All sensorgrams were evaluated by Biaevaluation 4.1 software from Biacore.

2.5. SPR detection

Sensor chip functionalized with DNA probes was used for hybridization reaction. Prior to the detection, the sensor chip surface was rinsed with the running buffer (hybridization solution) for 3 min. The equal volume of hybridization solution was added into 50 μl of the extension reaction products. The final sample was evenly mixed for a few seconds. When the sensorgram reached a steady status in running buffer, the extension products in hybridization buffer were injected into the flow cell over the sensor chip surface at a constant speed of 5 μl min^{-1} with a sample volume of 90 μl. When the injection was completed, the flow cells were washed with running solution to remove unbound material. Each assay was carried out by injecting sample

three times consecutively and the average value was recorded.

After detection, the sensor chip was regenerated with a denaturing solution (10 mM glycine–HCl, pH 3.0) to remove bound analytes from the surface of the gold film for the next measurement. The regeneration process was repeated 30 times to evaluate the regeneration performance and the useful lifetime of the sensor chip.

3. Results and discussion

3.1. Experimental principle

The basic principle of the point mutation detection approach using SPR biosensor in this study is shown in Scheme 1. 3'-thiolated DNA probes with complementary sequences to the target DNA were immobilized to the sensor surface by Au-S binding and MCH was added to block non-specific binding sites. In addition, the employment of MCH was favor to improve the hybridization efficiency by reducing the sterical hindrance [31]. Wild-type target DNA has a specific sequence which is perfectly complementary to the designed primer, in the presence of dNTPs and DNA polymerase, the primer was extended in the 5' to 3' direction to bind target sequence to form double-stranded DNA. On the contrary, when the mutant-type target DNA existed, the primer that ends with one mismatched base at 3'-end to the target sequence was not been elongated due to the property of DNA polymerase for discriminate mismatched configurations. Furthermore, the $Vent_R$ (exo−) DNA polymerase used here without the 3' to 5' proofreading exonuclease activity provided a good selectivity for point mutation detection through extension of primers perfectly matching at their 3'-ends. The products of the mutant-type sequences bearing the

complementary sequence can be captured by the DNA probes immobilized on the sensor chip surface. As a result, significantly higher SPR signals of hybridization reaction were obtained.

3.2. Extension reaction products detection

In order to investigate the ability for discriminating point mutation of the biosensor proposed in this work, hybridization signals with the products of extension reaction mixtures containing wild-type targets and mutant-type targets separately were compared under the same condition. One blank sample using the reaction mixtures without target sequences was employed as control for enzyme adsorption in parallel with samples of extension reaction products. All the three different samples were prepared and then injected into SPR instrument under controlled condition, the response signals were shown in Fig. 1. The response curve increased rapidly within 1 min and showed a trend of steady when the maximum binding capacity was reached during the sample injection. The response signal of mutant sample was higher than the values observed with wild sample and a clear difference was obtained. The injection of blank sample caused no response signal, which was similar to the results obtained from wild sample. It can be deduced that the primers were effectively extended in presence of wild targets and non-specific adsorption of DNA polymerase to the sensor surface was almost negligible.

3.3. Optimization of reaction conditions

Primer extension reaction plays an important role in obtaining high selectivity for the SPR biosensor developed in this study. In order to obtain good optimization for

extension reaction, the effects of Mg^{2+} concentration, reaction temperature and reaction time on assay selectivity were investigated.

The activity of the $vent_R$ (exo^-) DNA polymerase used in this study is dependent on the presence of magnesium ions [32]. Therefore, the effect of Mg^{2+} concentration was first examined. To determine the optimum Mg^{2+} concentration, extension reaction mixtures with a series of concentrations of Mg^{2+} were tested. As shown in Fig. 2a, in the 1 mM to 3 mM concentrations range of Mg^{2+}, the response signal of wild-type sample decreased with the increase of Mg^{2+} concentrations until 2 mM and then increased while the signal of mutant-type sample kept stable. The highest signal difference between wild-type sample and mutant-type sample was obtained at 2 mM Mg^{2+}, so 2 mM Mg^{2+} was selected as the optimal concentration.

Subsequently, the optimum reaction temperature was investigated at a fixed Mg^{2+} concentration of 2 mM. $Vent_R$ (exo^-) DNA polymerase applied for differentiating mutant sequence containing mismatched bases in this work exhibits high thermal stability with half-life of 6.7 h at 95 □. The appropriated reaction temperature for catalytic activity of $Vent_R$ (exo^-) DNA polymerase is about 70 □–80 □. Furthermore, the reaction temperature has an impact upon hybridization efficiency and stability. Considering the above factors, the extension reaction temperature was varied from 60 □ to 80 □. In Fig. 2b, the response signal of wild-type sample firstly decreased with the temperature from 60 □ to 75 □ and began to increase at the temperature higher than 75 □. The optimal hybridization signal was achieved at 75 □. The signal difference of mutant sample under different temperature may be attributed to the

effect of temperature on the hybridization between the targets and primers. Therefore, 75 □ was employed as the best extension reaction temperature in the following experiments.

The effect of extension reaction time on response signal was also explored. Different reaction time from 30 min to 75 min was chosen. In Fig. 2c, when the reaction time increased, the response signal of wild-type sample reduced. This could be attributed to that the increased amount of extended duplex sequences was formed as the extension reaction went on. When the reaction time was more than 60 min, differences between wild-type and mutant-type samples were clearly distinguishable, however, in order to shorten the detection time of the experiment, 60 min was chosen as the ideal reaction time for the identification of mutations.

3.4. Analytical performance of the strategy

Under the optimized experimental conditions, experiments were performed with the samples containing mutant target DNA of different concentrations to illustrate the analytical performance of the biosensor. Each concentration was measured in three replicas on the same chip prepared on the same day, error bars represent the standard deviation among the results obtained from the three measurements. As shown in Fig. 3, with the increase of mutant target DNA concentration in extension reaction volume, the SPR response signal gradually enhanced. The response signal exhibited a good linear relationship with the logarithm of target DNA concentration in the range of 100 pM to 100 nM, the corresponding regression equation $Y(\Delta RU) = 65.02 + 59.30 \times \lg C$ (nM) with the correlation coefficient of 0.9961. In addition, the detection limit of 100

pM can be achieved. Compared to other works relating to point mutation detection by SPR biosensor, the sensitivity of this method is competitive for standard solutions containing synthetic oligonucleotides [31,33].

3.5. Selectivity of the strategy

To evaluate the selectivity of the method proposed in this work, the mixture samples were prepared by mixing 1×10^{-7} M wild target DNA with mutant target DNA at a ratio of 1:10, 1:100, 1:200, 1:500，0:1000 to simulate the heterozygous state that exists in most clinical samples obtained from patients. The control sample, containing all the reagents of the corresponding mixture sample except wild target sequence, was also detected to evaluate the influence of wild target on the discrimination for mutant target in mixture sample. As shown in Fig. 4, each bar represented an average value of three time injections and the error bar indicated the standard deviation. As the ratio of mutant DNA in mixture sample solution decreased, the SPR signal became weaken. The signal of mixture sample was close to the value of control sample. These results demonstrated the proposed method had a high selectivity for distinguishing the low concentration of mutant sequences from heterozygous sample containing abundant wild targets.

3.6. Regeneration performance of sensor surface

Efficient regeneration is important for successful assays, which is good for the performance of the sensor chip for interaction analysis. In this study, 10 mM glycine–HCl (pH 3.0) solution was chosen as regeneration solution to remove the bound analytes from the sensor surface at the end of each measurement. The response

signal decreased <20% (data not shown) after 30 cycles of hybridization/ regeneration steps. The regeneration process can be accomplished within 1 min without significantly loss of analyte binding activity. These results indicated that the sensor surface can be reused for the next analysis cycle by simply washing the chip surface with a denaturing solution.

4. Conclusions

In this work, a label-free and highly sensitive method based on surface plasmon resonance biosensor has been developed for point mutation detection. The method is based on hybridization detection between the products of DNA polymerase-induced extension reaction and the probes immobilized on the sensor surface. The experimental results demonstrated that the SPR biosensor developed in this work had good specificity for point mutation assay via integrating the discrimination property of DNA polymerase for mismatched configurations. Under optimal conditions, our method was able to detect mutant sequences with a detection limit as low as 100 pM. Therefore, the established method could be a potential alternative for point mutation detection.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (21075141), the Science and Technology Plan Project of Yuzhong District of Chongqing (20120212) and Natural Science Foundation Project of CQ (CSTC2012jjAl0008 and CSTC2013jjB10019).

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328-337.

Figure caption

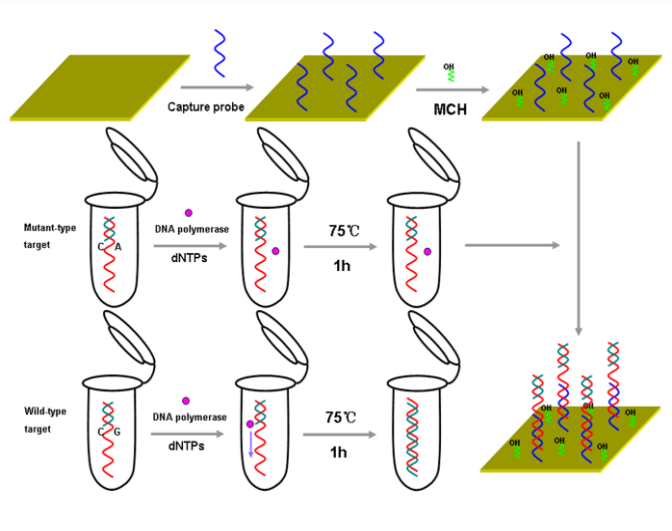
Scheme 1. Schematic representation of point mutation assay using SPR biosensor based on polymerization extension reaction.

Fig. 1. SPR sensorgrams of hybridization with (a) mutant-type sample**,** (b) wild-type sample and (c) sample without target. A volume of 70 μl was injected at a flow rate of 5 μl min^{-1}.

Fig. 2. Response signal after hybridization of DNA probes and extension reaction products. (A) Optimization of Mg^{2+} concentration in the extension reaction volume; (B) Influence of reaction temperature on response signal; (C) Influence of reaction time on response signal.

Fig. 3. (A) SPR sensorgrams for mutant sample with target DNA concentrations of 0.1 nM, 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 250 nM (from a to h). (B) The linear relationship between the response signal and the logarithm of mutant target concentration.

Fig. 4. The response signal of heterozygous solutions containing wild target of 1×10^{-7} M and mutant target of different concentrations from 0 M to 10×10^{-9} M.



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A simple SPR biosensor strategy has been developed for rapid and highly sensitive detection of point mutation.

Figure(s)

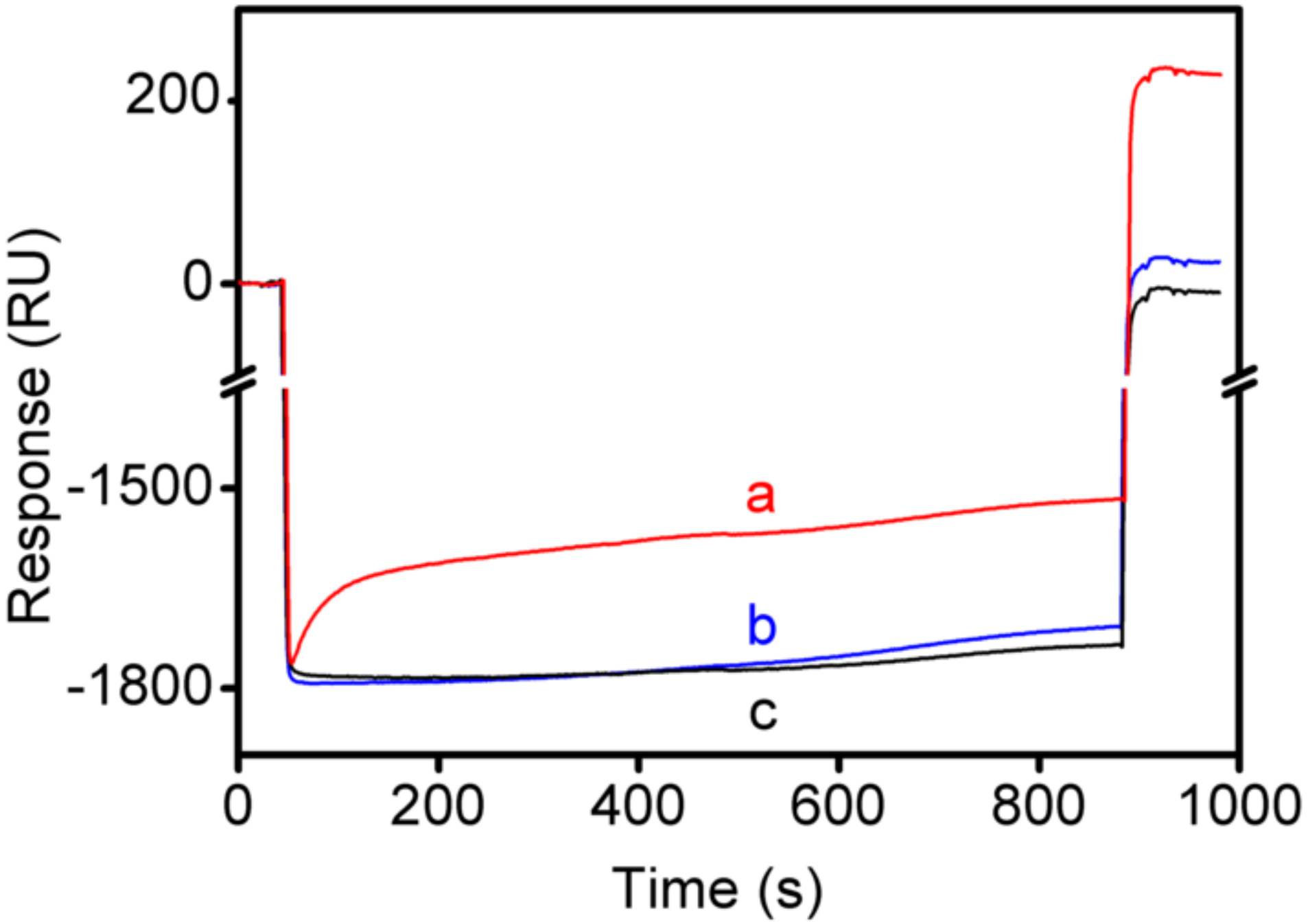
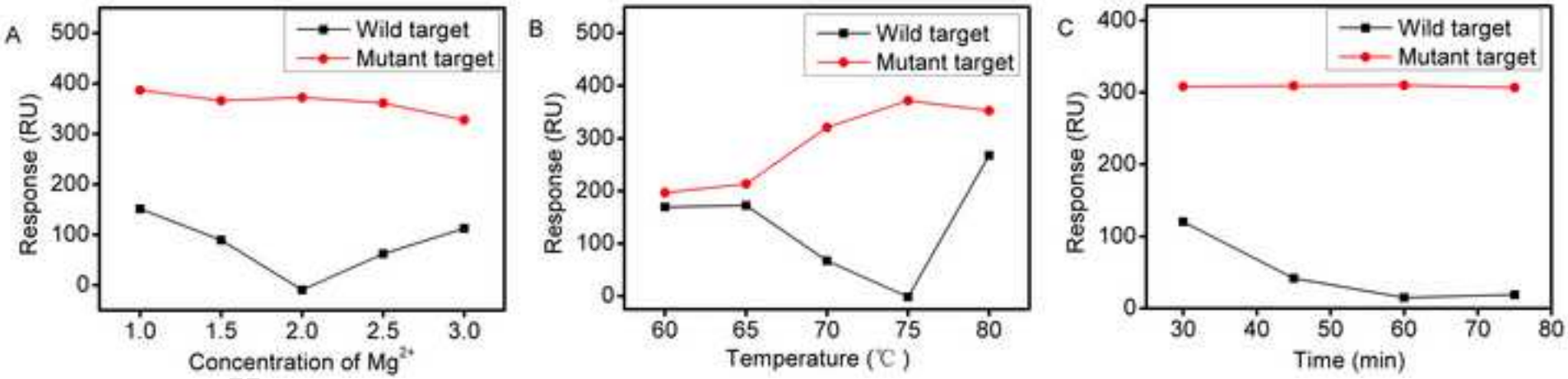


Table 1

Oligonucleotide	Sequence
Probe	5′-ACAAAGCACATCAGATTTTTTTTTTTTTTT -3′-(CH2)6-SH
Wild target	5′-TCTGATGTGCTTTGTTCTGGATTTCGCAGG TCCTCAAGGGCAGAAGAGTCACTTATG-3′
Mutant target	5′-TCTGATGTGCTTTGTTCTGGATTTCACAGG TCCTCAAGGGCAGAAGAGTCACTTATG-3′
Primer	5′-CCCTTGAGGACCTGC-3′

The mutations of the *BRCA*1 chosen for this work were R1443X, which is located in exon 13, codon 1443. Mismatched sites are given in underlined bold.

Figure(s)



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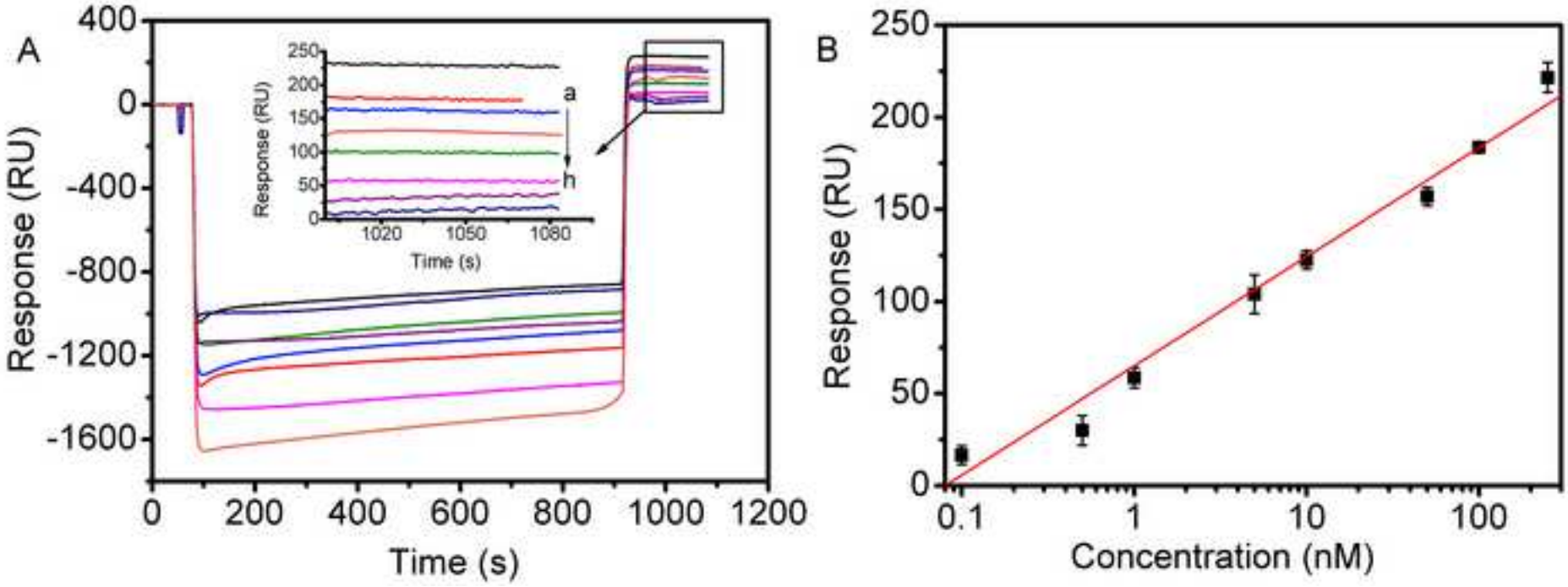

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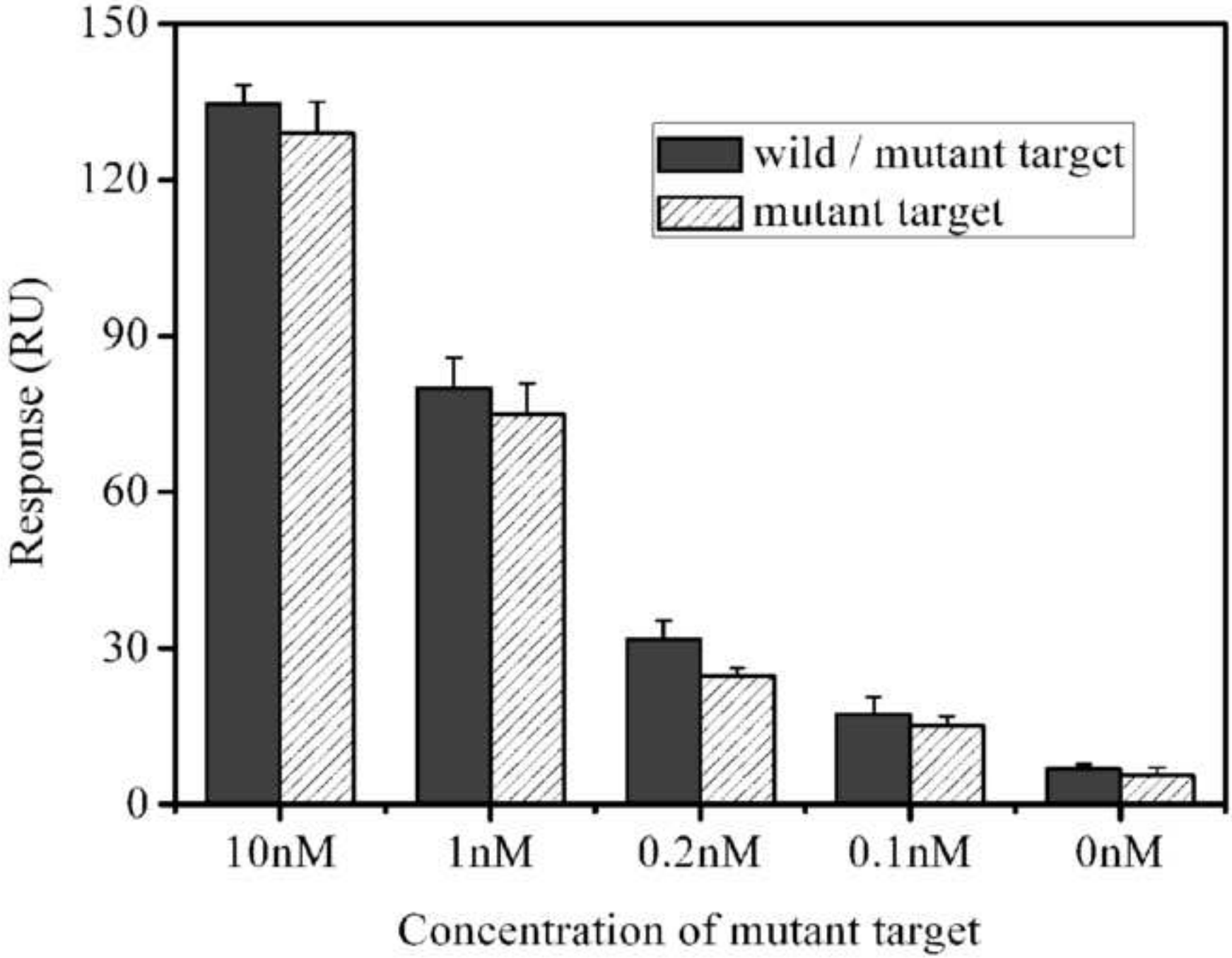
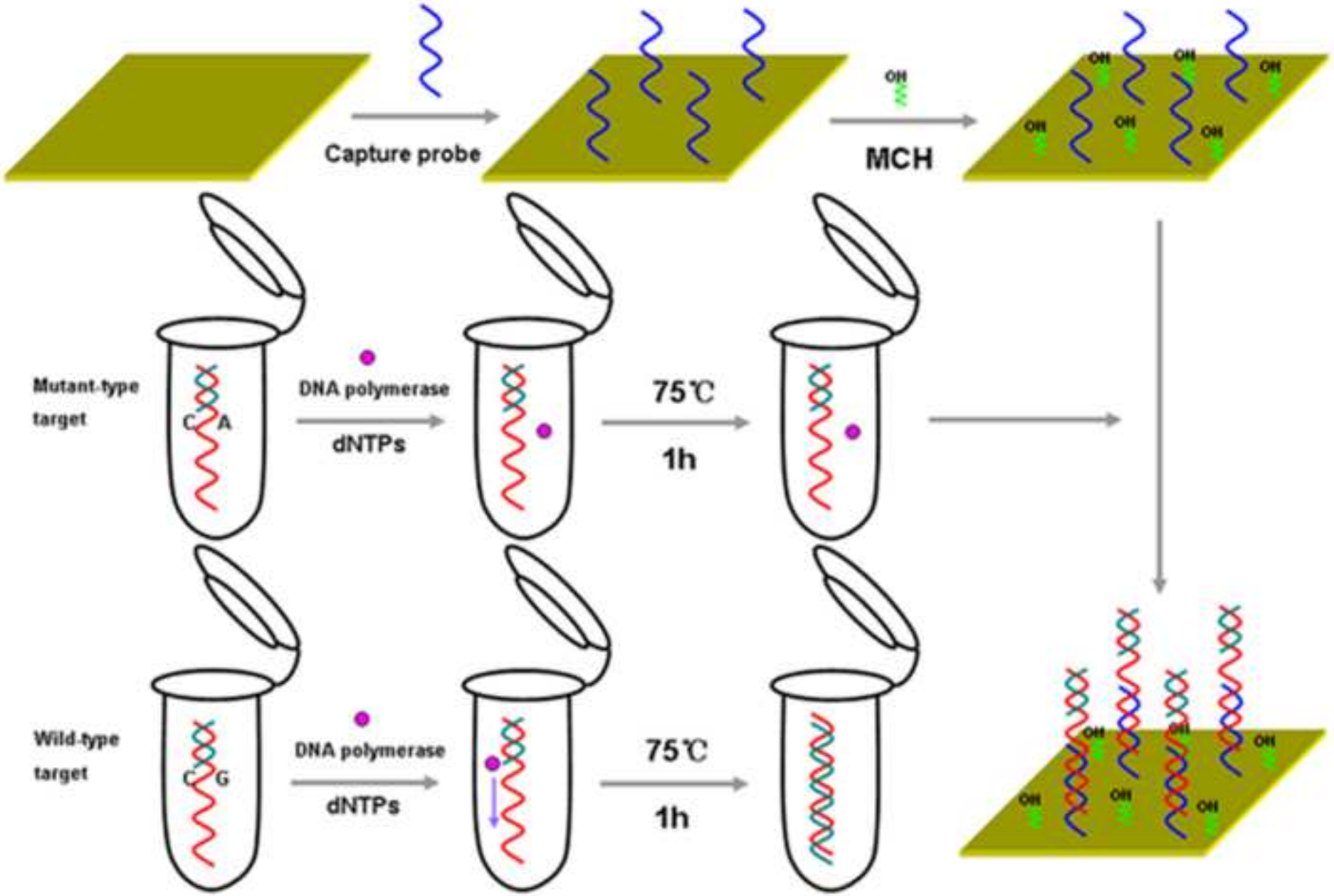
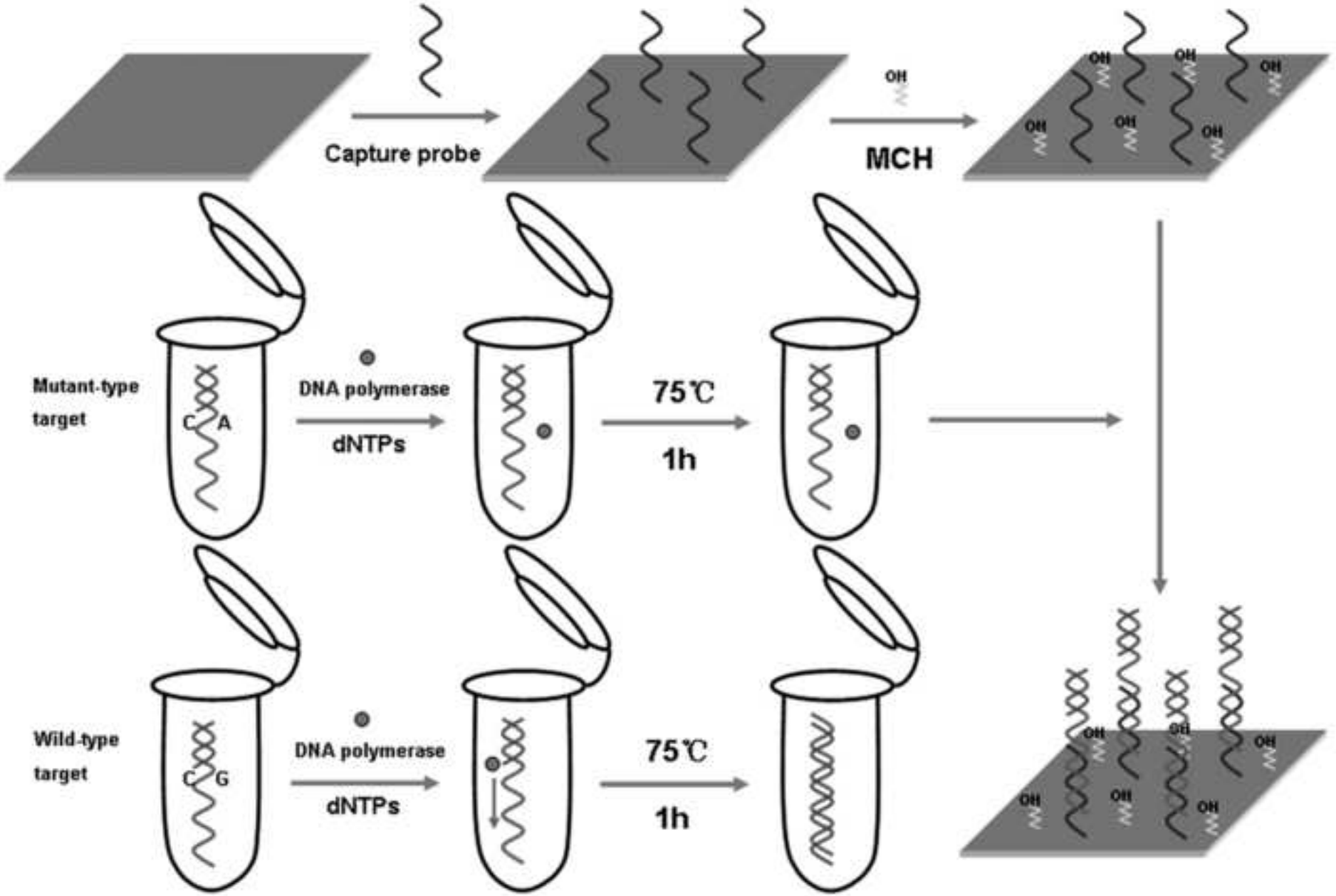

150
120
90
60
30
0
Response (RU)
wild / mutant target
mutant target
10nM
1nM
0.2nM
0.1nM
0nM
Concentration of mutant target

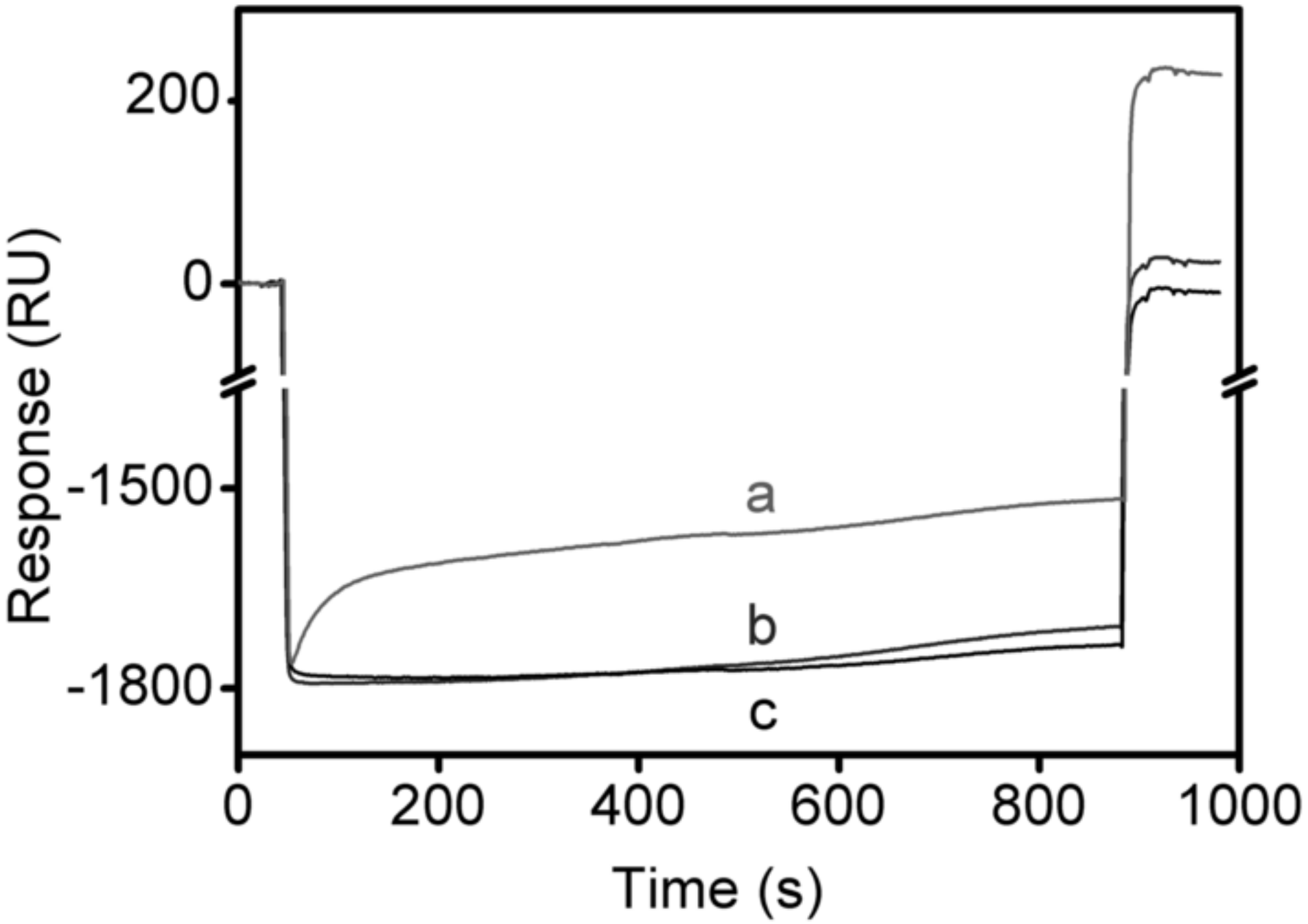
Figure(s)


Capture probe
MCH
OH
Mutant-type target
C A
DNA polymerase
dNTPs
75℃
1h
Wild-type target
C G
DNA polymerase
dNTPs
75℃
1h

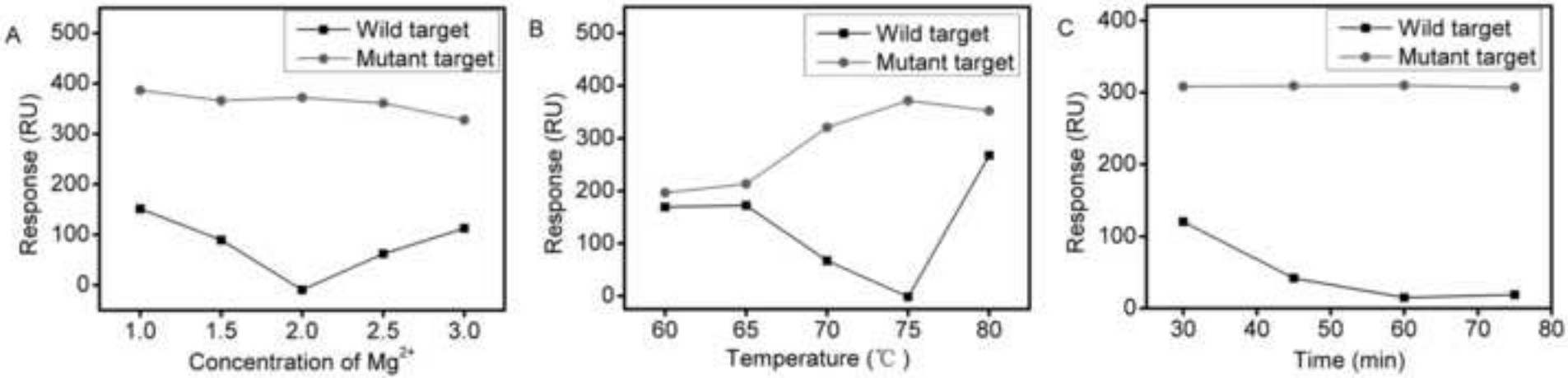
Figure(s)


Capture probe
OH
MCH
Mutant-type target
C A
DNA polymerase
dNTPs
75℃
1h
Wild-type target
C G
DNA polymerase
dNTPs
75℃
1h

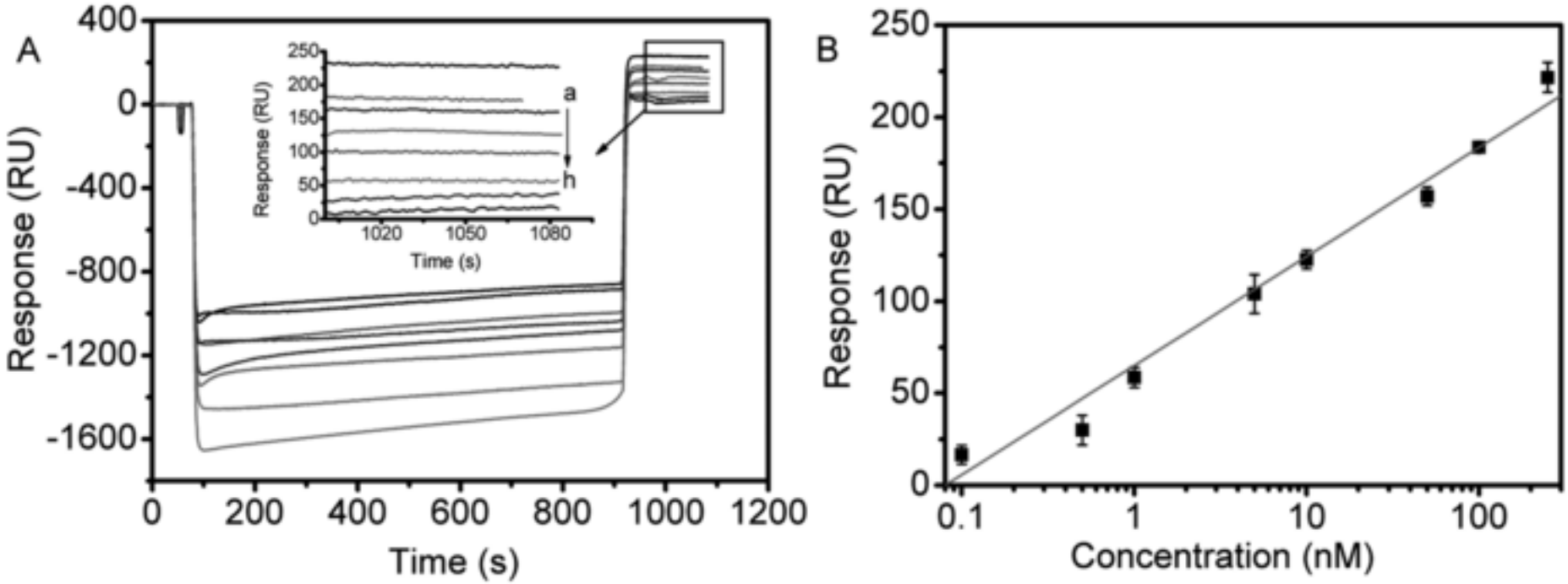
Figure(s)



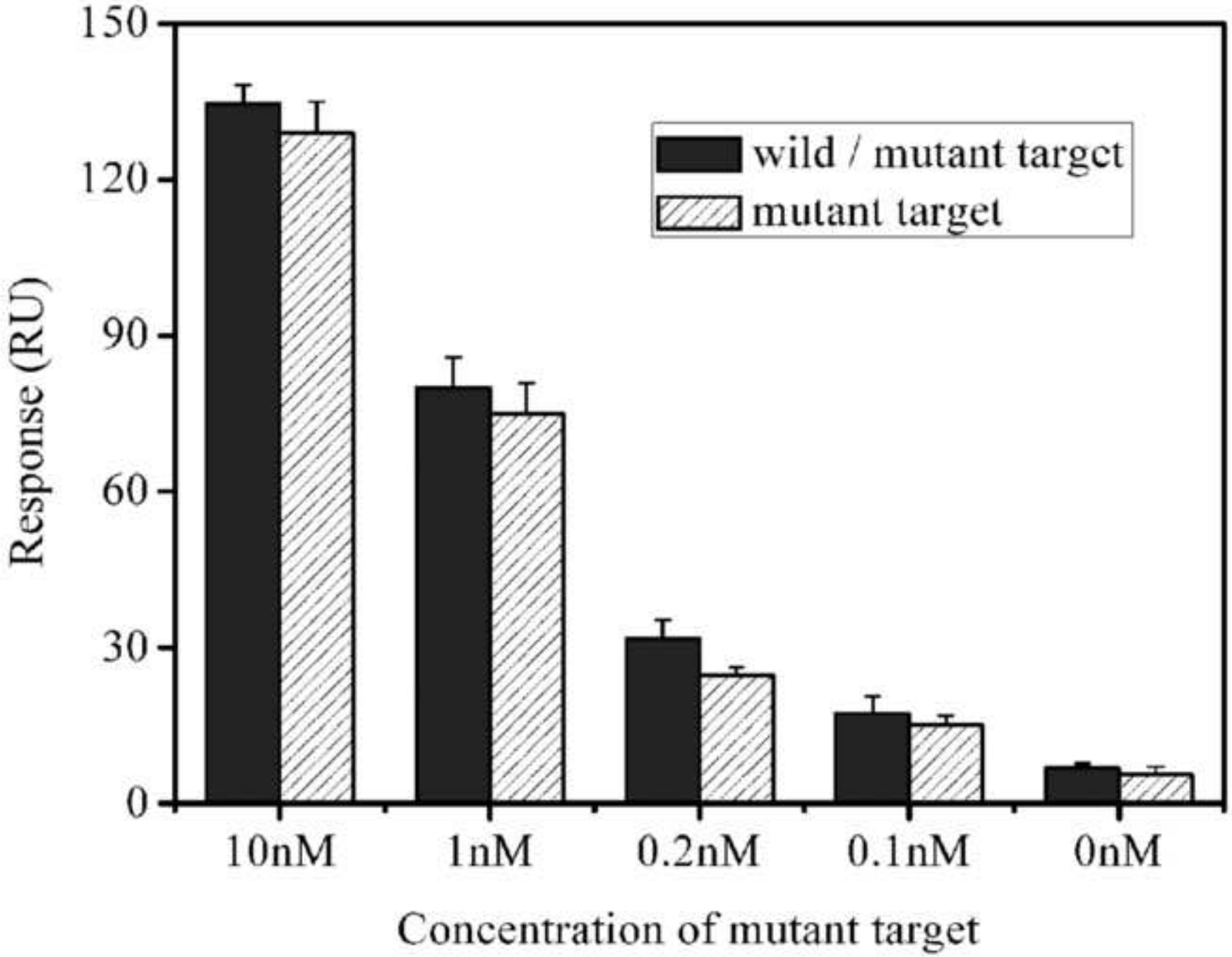
Figure(s)



Figure(s)


A
Response (RU)
400
0
-400
-800
-1200
-1600
0
200
400
600
800
1000
1200
Time (s)
Response (RU)
250
200
150
100
50
0
a
h
1020
1050
1080
Time (s)
B
Response (RU)
250
200
150
100
50
0
0.1
1
10
100
Concentration (nM)

Figure(s)


150
120
90
60
30
0
Response (RU)
wild / mutant target
mutant target
10nM
1nM
0.2nM
0.1nM
0nM
Concentration of mutant target