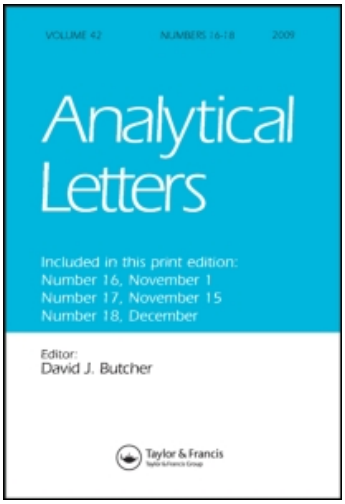

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Enhancing DNA Detection Sensitivity Through a Two-Step Enrichment Method with Magnetic Beads and Droplet Evaporation

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ENHANCING DNA DETECTION SENSITIVITY THROUGH A TWO-STEP ENRICHMENT METHOD WITH MAGNETIC BEADS AND DROPLET EVAPORATION

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A simple and sensitive DNA detection method has been developed through a two-step enrichment process. Trace amount of target DNA in a large volume (1 mL) is selectively separated and condensed with DNA-modified magnetic beads into a small volume (5 μL) by denaturalization. Then, the pre-enriched target DNA solution (1 μL) is transferred onto a smooth hydrophobic surface, where the target DNA is further-enriched by natural sessile droplet evaporation. Using this method, the fluorescence detection sensitivity of the target DNA can be enhanced by 3 orders of magnitude and as low as 3.91 pM of the target DNA can be detected within 2 hours.

Keywords: DNA detection sensitivity; Droplet evaporation; Magnetic beads

INTRODUCTION

Detection of DNA plays a vital role in many research fields such as clinical diagnosis, single-nucleotide polymorphism (SNP), genotyping, environment protection, antiterrorism, and forensic analysis (Heller 2002; Teles and Fonseca 2008). Up to now, the detection methods can be divided into two kinds. One is DNA hybridization technology including southern blotting, dot/slot blotting, several of DNA biosensors, and so on, based on the principle of complementary base pairing. The other is DNA amplification technology such as polymerase chain reaction (PCR), ligase chain reaction (LCR), and rolling circle amplification (RCA), which utilizes the semi-conservative replication of DNA. Despite its high detection sensitivity of DNA with 1∼5 copies (Knemeyer et al. 2000), the amplification technology has some drawbacks such as bulky equipment, skilled manpower, and

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easy contamination for practical application (Johnson 2000; Strömberg et al. 2008). Therefore, it is greatly significant to enhance the DNA detection sensitivity with a simple, low cost method utilizing hybridization technology (Wu et al. 2006; Yu et al. 2008; Mao et al. 2009).

Due to its scientific interest and implication in everyday life, the sessile droplet evaporation has been extensively studied over the past several decades (De Gennes 1985; Deegan et al. 1997; Deegan 2000). Recently, sessile drop evaporation has been extensively used in various self-assembled structure studies (Gratson et al. 2004; Shevchenko et al. 2006; Zhang et al. 2008) and has many applications in biomedical fields (Fang et al. 2006; Small et al. 2006). Droplet evaporation on a smooth hydrophobic surface has an important characteristic. During the solvent evaporating, the droplet shrink and the concentration of solute molecules continue increasing. This effect is favorable to enhance the detection sensitivity of some detection methods associated with concentration (Schuerenbeg et al. 2000; J. Li et al. 2006).

Herein, we report a two-step enrichment method to enhance the DNA detection sensitivity with magnetic beads and droplet evaporation. First, we use DNA-modified magnetic beads to selectively separate and pre-enrich the target DNA. Then, the target DNA is transferred onto a hydrophobic surface to attain further enrichment by sessile droplet evaporation process. By doing this, we obtain a simple, rapid, low cost, selective, and sensitive DNA detection method.

EXPERIMENTAL

The principle of a two-step enrichment method to enhance the DNA detection sensitivity is depicted in Figure 1. First, the NH_2-modified DNA was immobilized onto the carboxylated magnetic beads activated by EDC/NHS. Next, a one-step hybridization reaction was performed by mixing the DNA-modified

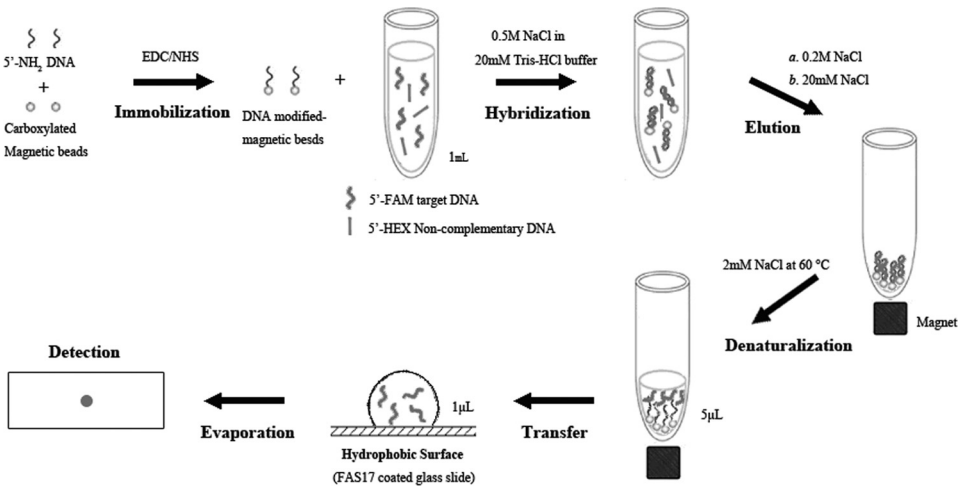

Figure 1. A two-step enrichment method enhances DNA detection sensitivity with magnetic beads and droplet evaporation.

magnetic beads and the target DNA. The volume of the reaction system was about 1 mL with a trace amount of the target DNA in it. After a gradient elution process to achieve selectivity, the pre-enrichment was carried out by segregating the target DNA from the DNA-modified magnetic beads using 5 μL denaturalization solution. Then, 1 μL of the denaturalization solution was transferred onto a smooth hydrophobic glass piece, and the target DNA was further enriched by natural sessile drop evaporation process. Finally, the fluorescence of the target DNA was detected by a fluorescent image analyzer, and the detection sensitivity was obtained by analyzing the images.

Materials and Reagents

The carboxylated magnetic beads (1.0 μm, Dynabeads® Myone™ Carboxylic Acid) were obtained from Invitrogen. 2-(N-morpholino) ethanesulfonic acid (MES), N-hydroxysuccinimide (NHS) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) were purchased from Sigma. 1H, 1H, 2H, 2H-perfluorodecyltrimethoxysilane [FAS17, $CF_3(CF_2)_7(CH_2)_2Si(OCH_3)_3$] was acquired from Nanjing Shuguang Chemical Group Co., Ltd. Standard glass slides (25.4 × 76.2 mm) were bought from Corning Incorporated. All of the oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. and with the following sequences:

Amine-modified DNA sequence:

5′-NH_2-TTTTT ATC CTT ATC AAT ATT TAA CAATAA TCC CTC

Target sequence was modified with 6-FAM at the 5′-end for fluorescence detection:

5′-(6-FAM)-GAG GGA TTA TTG TTA AAT ATT GAT AAG GAT

Non-complementary sequence with HEX fluorescence group was set as a negative control:

5′-HEX-AGT CTA TTA TAA TAA ACT AGT TAT ATA GCA

Apparatus

The fluorescence detection was performed with LAS-4000 mini Fluorescent image analyzer (Fujiflim Life Science) and the images were dealt with Multi Gauge analysis software. Plasma cleaner PDC-32C (Harrick) was applied to clean the standard glass slides. The Milli-Q water was prepared by Milli-Q Ultrapure Water Purification Systems (Millipore).

Preparation of Hydrophobic Surface

A standard glass slide was cleaned by a Plasma cleaner and employed as the substance, and fluoroalkylsilane, 1H, 1H, 2H, 2H-perfluorodecyl-trimethoxysilane [FAS17, $CF_3(CF_2)_7(CH_2)_2Si(OCH_3)_3$] was introduced as the hydrophobic agent.

Generally, the cleaned glass slide was carefully cut into about ten small pieces and placed in a culture dish. Then three lids of micro-centrifugal tubes with 100 μL FAS17 each were put into the culture dish. Finally, the culture dish was sealed by a piece of preservative film and laid in a fume hood overnight at 37°C. By doing this, the glass pieces were covalently bound a layer of FAS17 with a contact angle about 113° for water. (H. Sugimura 2002; Sugimura et al. 2002).

Preparation of DNA-Modified Magnetic Beads

The DNA-modified magnetic beads were prepared by conjugating the carboxylated magnetic beads with the 5′-NH_2 modified DNA. First, 600 μL of 10 mg/mL carboxylated magnetic beads were pipetted into a micro-centrifuge tube and washed twice with the equal volume of MES buffer (25 mM, pH 6.0). Then, 120 μL of 10 μM 5′-NH_2 modified DNA in MES buffer was added to the washed magnetic beads and incubated for 30 min at room temperature with gentle shaking. Afterwards, 60 μL of 1 M EDC and 20 μL of 1 M NHS fresh solution in MES buffer were injected into the micro-centrifuge tube and mixed well, and then the suspension reacted for 2 hours at room temperature with gentle shaking. Finally, the coated magnetic beads were incubated with 50 mM Tris buffer (pH 7.4) for 15 min at room temperature to quench the unreacted activated carboxylic acid groups. The coated magnetic beads were washed three times with 200 μL hybridization buffer (20 mM Tris, pH 8.0, 0.5 M NaCl) and then resuspended in 200 μL hybridization buffer and stored at 4 □ (Greg 1996; H. Li and He 2009).

In this process, we increased the yield of DNA-modified magnetic beads dramatically by adding NHS to the EDC reactions, for a NHS ester intermediate is less hydrolysis and easier reaction with amine group than EDC ester intermediate in aqueous solutions (Greg 1996).

Hybridization and Denaturalization Procedure

The DNA-modified magnetic beads, the target DNA, and the Non-complementary DNA were mixed together to perform the hybridization reaction in one-step. In a typical experiment, 50 μL of 30 mg/mL coated magnetic beads were pipetted into a micro-centrifuge tube, and then 1 mL of target DNA and non-complementary DNA mixed solution in hybridization buffer were added and incubated for 1 hour at room temperature with gentle shaking. The hybrid-conjugated beads were washed once with 100 μL elution buffer A (0.2 M NaCl, 0.1% SDS) and once with 100 μL elution buffer B (20 mM NaCl) to remove the non-complementary DNA.

In order to detect the fluorescence of the target DNA, which was modified with 6-FAM fluorescent group at the 5′-end, the target DNA had to segregate from the DNA-modified magnetic beads by a denaturalization process. In this process, the hybrid-conjugated beads were suspended in 5 μL of 2 mM NaCl at 60°C for 10 min with gentle shaking. Then the tube was placed on a magnet for 4 min. Finally, 1 μL of the supernatant fluid was cautiously pipetted out of the tube and transferred onto a hydrophobic glass piece.

Droplet Evaporation and Fluorescence Detection

The droplet of 1 μL supernatant containing target DNA was concentrated by natural evaporation process for the sake of higher detection sensitivity than without concentrated. During the evaporating, the droplet would shrink on the smooth hydrophobic glass piece. The evaporation process usually took about 15 min at room temperature. After evaporation, the fluorescence detection was performed on the LAS-4000 mini fluorescent image analyzer with Fluorescence Method of SYBR Green (Light: Blue; Filter: Y515-Di; Iris: F0.85) and the exposure time was chosen as 5 sec. The images were analyzed with Multi Gauge analysis software.

RESULT AND DISCUSSION

Effect of the Hybridization Time

The hybridization time influenced the target DNA conjugating with the DNA-modified magnetic beads. The study showed that the hybridization efficiency increased saliently during the initial 60 min and decreased slightly with an extension of time (Figure 2). The phenomenon accounted for a maximum of hybridization after reaction of about 60 min, and the fluorescence intensity was slightly quenched during the reaction. Therefore, 60 min was chosen as an optimal hybridization time for the following experiments.

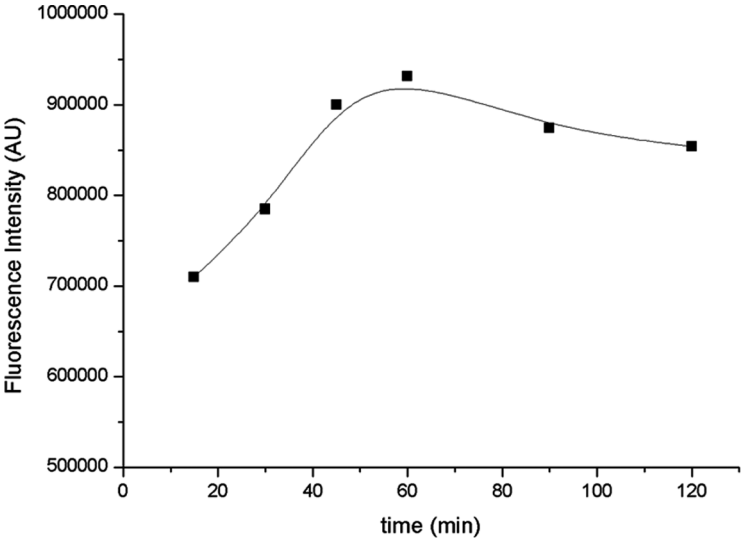

Figure 2. Fluorescence intensity related to the hybridization time. 50 μL of 30 mg/mL coated magnetic beads and 1 mL of 0.5 nM target DNA for each reaction, n = 6. The fluorescence detections were taken by 1 μL denaturalization solution without droplet evaporation. AU is an arbitrary unit of the fluorescence intensity.

Optimization of Elution Process

In order to choose an ideal elution condition, 4 groups of trials were set with or without target DNA and/or non-complementary DNA, respectively. In detail, 50 μL of 30 mg/mL coated magnetic beads were mixed with 10 μL of target DNA and/or non-complementary, and appropriate hybridization buffer (20 mM Tris, pH 8.0, 0.5 M NaCl) was added as necessary for final volume up to 70 μL. After hybridization for 1 hr at room temperature, the beads were first washed with 5 μL elution solution and then 95 μL for both the elution buffer A and B. During the washing, 1 μL supernatant fluid of the 5 μL elution solution was dropped on the hydrophobic surface for fluorescence detection. After the washing procedure, 5 μL of 2 mM NaCl was used for denaturalization and the detection was taken with 1 μL denaturalization solution without evaporation as mentioned previously. By comparing the results, we concluded that, by this gradient elution process, all of the non-complementary DNA could be washed away without losing the target DNA.

Effect of the Denaturalization Temperature

Five parallel trials of denaturalization were carried out under different temperature between 30°C and 70°C to choose an optimal denaturalization temperature. The efficiency of denaturalization was calculated by comparing each trial with a standard, and results showed that it increased quickly in the range 30–60□ and remained unchanged even when the temperature was higher (Figure 3). Thus, 60°C was selected for the denaturalization.

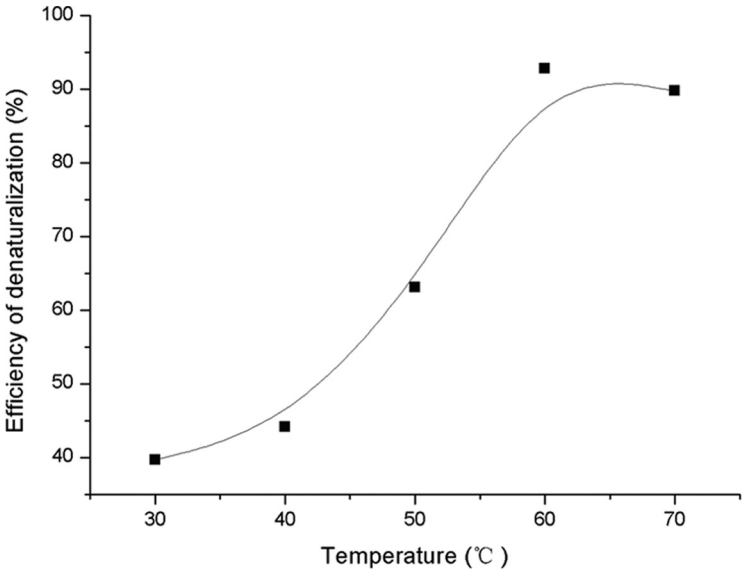

Figure 3. Denaturalization efficiency under different temperature. 50 μL of 30 mg/mL coated magnetic beads and 1 mL of 0.5 nM target DNA for each reaction, n = 5. The fluorescence detections were taken by 1 μL denaturalization solution without droplet evaporation, and 1 μL of 100 nM target DNA was set as a standard.

Efficiency of the Magnetic Enrichment

Six Groups were set with 50 μL of 30 mg/mL coated magnetic beads and 1 mL of 0.5 nM of both target DNA and non-complementary DNA for hybridization. After the elution and denaturalization were carried out as mentioned previously, the fluorescence was detected with 1 μL denaturalization solution without evaporation. The 1 μL of 100 nM target DNA was set as the standard. Results showed that the efficiency of the magnetic enrichment was about $79.6 \pm 1.7\%$.

DNA Detection Sensitivity

The DNA detection sensitivity was compared with three procedures: before the magnetic enrichment, after the magnetic enrichment, and the ultimate detection. For each procedure, the relationship between the fluorescence intensity and the concentration of the target DNA and the DNA detection sensitivity were investigated. Before the enrichment, the fluorescence intensity was linearly proportional to the concentration of target DNA in the range 3.91 nM~1 μM, and the correlation equation was log AU = 0.899log C (nM) + 4.541 ($R^2 = 0.9977$), where AU is the fluorescence intensity with an arbitrary unit and C is the concentration of the target DNA. After the magnetic beads enrichment, the correlation equation was log AU = 0.903log C (pM) + 3.439 ($R^2 = 0.9969$) in the range 19.5 pM~5 nM, and the equation of the ultimate detection was log AU = 0.922log C (pM) + 4.741 ($R^2 = 0.9922$) in the range 3.91 pM~250 pM (Figure 4). The detection sensitivity was estimated by average signal to background noise ratio opting for 2 (S/N = 2)

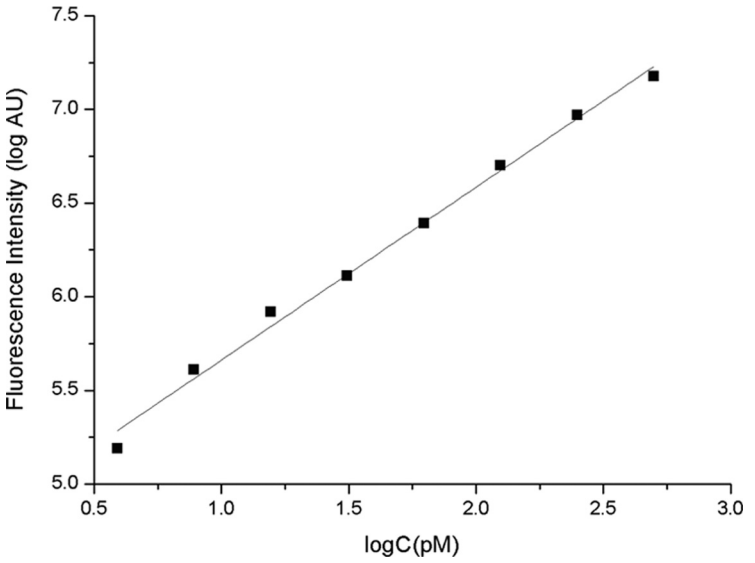

Figure 4. Correlation between Fluorescence Intensity and concentration of the target DNA after the two-step enrichments. 50 μL of 30 mg/mL coated magnetic beads, 1 mL of target DNA with gradient concentration and 0.5 nM non-complementary DNA were mixed for hybridization. The fluorescence detections were taken by 1 μL denaturalization solution after the droplet evaporation.

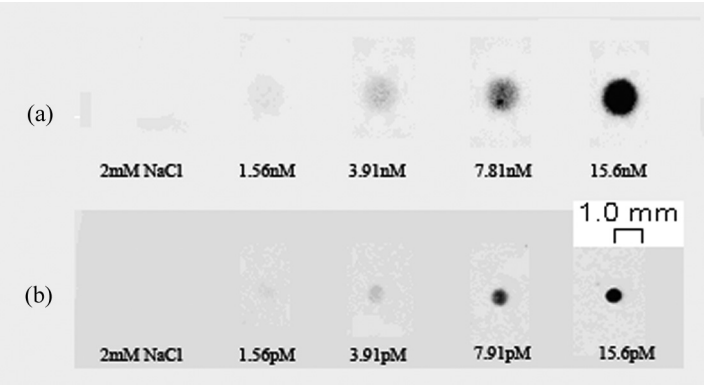

Figure 5. Comparison of the fluorescence images between before and after the two-step enrichments: (a) before the enrichments and (b) after the enrichments (ultimate detection).

when the target DNA could be clearly distinguished from the blank sample (Figure 5). The results indicated that the detection sensitivity was 3.91 nM, 19.5 pM and 3.91 pM for the three procedures, respectively. Therefore, the detection sensitivity was enhanced 3 orders of magnitude by the two-step enrichments.

In this method, the DNA-modified magnetic beads can be reused because the covalent bond between the carboxylated magnetic beads and 5′-NH_2-DNA was strong enough. However, for doing this, the DNA-modified magnetic beads must be thoroughly cleaned by denaturalization. Study showed that after reused ten times, the DNA-modified magnetic beads had no difference with the freshly prepared. In addition, the DNA detection could be accomplished within 2 hours for both the hydrophobic surface, and a substantial amount of DNA-modified magnetic beads can be prepared once and stored at 4°C for several months.

CONCLUSIONS

In conclusion, a two-step enrichment method to enhance the DNA detection sensitivity with magnetic beads and droplet evaporation was described. The method takes advantage of the facility for selectively separating and enriching DNA with magnetic beads and the simple increase of DNA concentration by a droplet evaporation process. Moreover, the method can be expanded for the similar detection of bio-molecule reaction such as RNA-DNA hybridization, RNA-RNA hybridization, Antigen-antibody immune response, and aptamer-protein reaction. Therefore, this simple, rapid, and sensitive analysis method is expected to be very useful for biotechnology and biomedical applications.

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