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Fluorescent bio-barcode DNA assay for the detection of *Salmonella enterica* serovar Enteritidis

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ABSTRACT

Salmonella enterica serovar Enteritidis is one of the most frequently reported causes of foodborne illness. It is a major threat to the food safety chain and public health. A highly amplified bio-barcode DNA assay for the rapid detection of the insertion element (*iel*) gene of *Salmonella* Enteritidis is reported in this paper. The biosensor transducer is composed of two nanoparticles: gold nanoparticles (Au-NPs) and magnetic nanoparticles (MNPs). The Au-NPs are coated with the target-specific DNA probe which can recognize the target gene, and fluorescein-labeled barcode DNA in a 1:100 probe-to-barcode ratio. The MNPs are coated with the 2nd target-specific DNA probe. After mixing the nanoparticles with the 1st target DNA, the sandwich structure (MNPs-2nd DNA probe/Target DNA/1st DNA probe-Au-NPs-barcode DNA) is formed. A magnetic field is applied to separate the sandwich from the unreacted materials. Then the bio-barcode DNA is released from the Au-NPs. Because the Au-NPs have a large number of barcode DNA per DNA probe binding event, there is substantial amplification. The released barcode DNA is measured by fluorescence. Using this technique, the detection limit of this bio-barcode DNA assay is as low as 2.15×10^{-16} mol (or 1 ng/mL).

1. Introduction

Salmonella species are the most frequently reported causes of foodborne illness. The annual cases of salmonellosis are around 40,000 in the United States from 1995 to 2005 (CDC, 2007). Commercially available detection methods for *Salmonella enterica* serovar Enteritidis include microbiological, immunological and molecular techniques. Although microbiological detection is accurate, it takes several days for incubation and needs special reagents and facilities. Commercially available immunological detection systems are specific but their sensitivity is low. Though PCR-based molecular detection method is very sensitive, PCR is often criticized for its complex, expensive, time-consuming, and labor-intensive procedure and narrow target DNA quantification range. So a rapid detection and valid identification of this foodborne pathogen in food and beverages is vital for food safety and public health.

A biosensor is an analytical device that integrates a biological sensing element with a transducer to quantify a biological event into an electrical output. It is a promising device for rapid detection of pathogenic microorganisms. Use of nanomaterials in biosensors allows the use of many new signal transduction technologies in their manufacture. Many biosensors based on nanotechnology for the detection of foodborne pathogens and bioterrorism agents can be found in the literatures. These detection techniques include conductive polymer nanowire (Pal et al., 2007), nano-porous silicon (Mathew and Alocilja, 2005), gold nanoparticles (Liu et al., 2004) and carbon nanotube (Davis et al., 2003).

The bio-barcoded DNA assay utilizes oligonucleotide-modified gold nanoparticles (Au-NPs) for signal amplification and magnetic nanoparticles (MNPs) for easy separation from the samples. Oligonucleotide-functionalized gold nanoparticles have a demonstrated advantage in terms of sensitivity and selectivity over conventional probes in a variety of biodetection schemes (Cao et al., 2002). Their outstanding performance stems from cooperative binding properties, unique optical properties, catalytic properties, and the existence of robust and versatile surface-functionalization methods (Rosi and Mirkin, 2005). The bio-barcode assay is based on Au-NPs functionalized with a large number of oligonucleotide strands (the barcodes) and a corresponding recognition agent. The large barcode DNA-to-recognition agent ratio provides a means of signal amplification.

At present, considerable attention is being paid to solid-phase extraction as a way to isolate and pre-concentrate desired components from a sample matrix (Tartaj et al., 2003). The MNPs have been widely used as a universal separation tool to purify nucleic acids (Obata et al., 2002), proteins and peptides (Safarik and Safarikova,

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2004), cells (Pamme, 2005) and other biological agents from crude samples. An attractive advantage of these nanoparticles is that it can eliminate nonspecific adsorption of unwanted biological agents by controlling the surface chemistry of the particles (Hsing et al., 2007). The MNPs here provide a fast, convenient and efficient method for the separation and preconcentration of the substance from large volumes of solution.

This bio-barcoded assay is particularly promising as it allows the rapid detection of protein targets at low-attomolar concentrations (Chang et al., 2007) and nucleic acids at high-zeptomolar levels under optimized conditions (Nam et al., 2004). In addition, enzymatic amplification of the target sequence prior to detection is not necessary. In this paper, we demonstrate for the first time the feasibility of the bio-barcode assay to detect oligonucleotide targets from one of the most important foodborne pathogens – *Salmonella* Enteritidis, by using two DNA probes associated with the insertion element (*Iel*) gene of the organism.

2. Experimental

2.1. Reagents and materials

Hydrogen tetrochloroaurate (III) trihydrate and sodium citrate dehydrate were used for the synthesis of gold nanoparticles. 1,4-Dithio-DL-threitol (DTT) was used for the cleavage of oxidized thiolated oligonucleotides and release of thiolated barcode DNA from Au-NPs surface. Amine-coated MNPs were used for separation and preconcentration. All the above reagents were purchased from Sigma (St. Louis, MO, USA). Nap-5 column was purchased from GE Healthcare (Piscataway, NJ, USA), which was used to purify the DNA product from DTT solution. Sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Milwaukee, WI, USA) was used as a cross-linker between thiolated DNA probe and amine-coated MNPs. Sulfo-NHS acetate (Pierce, Milwaukee, WI, USA) was used to block unreacted sulfo-SMCC. All solutions were prepared in distilled water.

2.2. Apparatus

For this particular proof-of-concept study, the target DNA was amplified in PCR to generate enough materials for the experiment. The target DNA was amplified by a thermo cycler (Mastercycler Personal, Eppendorf). The PCR product was confirmed by gel electrophoresis (Runone System). After purification of the PCR product, a spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories) was used to measure the concentration of DNA samples, as well as DNA probe and barcode DNA. In the characterization experiments of Au-NPs, a UV–vis–NIR scanning spectrophotometer (UV-3101PC, Shimadzu) was used to determine the absorbance of Au-NPs, and TEM (JEOL100 CXII) was used for characterization of the nanoparticle dimension. All magnetic separation was done using a magnetic separator (FlexiMag, SpheroTech). A centrifuge (Micro12, Fisher Scientific) was used for separation and purification of Au-NPs. An incubator (HS-101, Amerex Instrument Inc.) was used to enrich the bacteria and hybridization reaction. A fluorescent multi-label counter (Victor 3, PerkinElmer) was used for measuring the fluorescent signal.

2.3. Bacteria culture, DNA isolation and PCR

A clinical strain of *S.* Enteritidis (strain S-64) was used in this study. The pathogen was grown in trypticase soy broth (TSB) at 37 °C for 16 h. After enrichment, the cells were enumerated by spiral plating appropriately diluted cultures on brilliant green agar. DNA isolation was performed from 1 mL *S.* Enteritidis culture using the QiaAmp DNA Mini Kit (Qiagen, Valencia, CA). Primers were designed for the detection of *S.* Enteritidis using the insertion element (*Iel*) gene (Wang and Yeh, 2002). The single stranded forward and reverse primers were *Iel*L – 5′-CTAACAGGCGCATACGATCTGACA-3′ (positions 542–565, 24 bases) and *Iel*R – 5′-TACGCATAGCGATCTCCTTCGTTG-3′ (positions 1047–1024, 24 bases). After PCR amplification, the PCR product was purified by PCR purification kits (QIAquick; QIAgen, Valencia, CA) and diluted serially with distilled water. The following thiolated oligonucleotides were used for conjugation with nanoparticles: The first (1st) DNA probe on Au-NPs: 5′-AATATGCTGCCTACTGCCCTACGCTT-thiol-3′ (position: 919–944), The second (2nd) DNA probe on MNPs: 5′-thiol-TTTATGTAGTCCTGTATCTTCGCCGT-3′ (position: 661–686), Barcode DNA on Au-NPs: 5′-TTATTCGTAGCTAAAAAAAAAA-thiol-3′ (Hill and Mirkin, 2006). TEX 613 ($\lambda_{excitation}$ = 596 nm, $\lambda_{emission}$ = 613 nm) was used to label the barcode DNA and 6-Carboxyfluorescein (6-FAM; $\lambda_{excitation}$ = 495 nm, $\lambda_{emission}$ = 520 nm) was used to label the 2nd DNA probe on MNPs. Both TEX 613 and 6-FAM were used to evaluate the conjugation efficiency. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

2.4. Synthesis of nanoparticles

Gold nanoparticles were synthesized by a chemical reduction method (Hill and Mirkin, 2006). Hydrogen tetrochloroaurate (III) trihydrate aqueous solution (1 mM, 50 mL) was prepared in a flask. The gold solution was heated while stirring on a hotplate. Once it refluxed vigorously, the solution was slowly titrated with 5 mL of 38.8 mM sodium citrate. The solution turned from yellow to clear, to black, to purple and finally deep red. The UV–vis absorption spectrum of the Au-NPs solution was measured by a UV–vis–NIR scanning spectrophotometer (UV-3101PC, Shimadzu Inc.).

2.5. Functionalization of nanoparticles

To ensure full reactivity, thiol-modified oligonucleotides should be reduced immediately before use. Otherwise, the thiol group on the oligonucleotides could not form a self-assembled monolayer on the surface of Au-NPs due to loss of active thiol group. DTT solution (0.1 M) was prepared in the disulfide cleavage buffer (170 mM PBS buffer, pH 8.0). The thiolated oligonucleotide was suspended in 100 μL DTT solution and the solution was allowed to stand at room temperature for 2 h. Nap-5 columns were then used for purifying the reduced thiolated oligonucleotides. The procedure was followed according to the manufacture's manual. After purification, a UV–vis spectrophotometer was used to determine the DNA concentration. The Au-NPs synthesized previously (1 mL), the purified thiolated barcode DNA (5 nmol), and the purified thiolated 1st DNA probe (0.05 nmol) were then mixed together. Thiolated DNA would form a self-assembled monolayer on the surface of Au-NPs. Fig. 1A shows a schematic of the Au-NPs functionalization. After a serial salt addition (Hill and Mirkin, 2006), the particles were stabilized for long-time storage at room temperature.

For the MNPs, the polyamine-functionalized iron oxide particles (1 mg) were reacted with 300 μg of sulfo-SMCC bifunctional linker for 2 h in 1 mL coupling buffer (0.1 M PBS buffer, 0.2 M NaCl, pH 7.2). The supernatant was removed after magnetic separation and the MNP-cross-linker conjugate was rinsed with the coupling buffer three times. The reduced thiolated 2nd DNA probe (1 nmol) was added into 1 mL coupling buffer containing sulfo-SMCC-modified MNPs and reacted for 8 h. Fig. 1B shows the schematic of the MNP–DNA probe conjugation. After conjugation, the DNA probe-immobilized MNPs were rinsed with the coupling buffer three

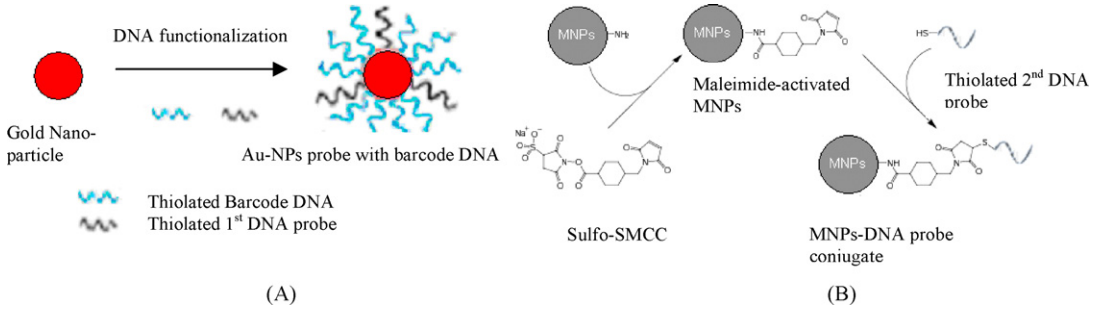

Fig. 1. Schematic of the functionalization of nanoparticles: (A) Au-NPs; (B) MNPs.

times. The unreacted DNA probe in the supernatant was collected for the evaluation of conjugation efficiency. The functionalized MNPs were then suspended in 35 mL of 10 mM sulfo-NHS acetate. The solution was incubated and shaken at room temperature to block the unreacted sulfo-SMCC on the surface of MNPs. After passivation, the particles were centrifuged at 4000 rpm for 1 min and washed with passivation buffer (0.2 M Tris, pH 8.5) and then with a storage buffer (10 mM PBS buffer, 0.2 M NaCl, pH 7.4).

2.6. Evaluation of conjugation efficiency

Fluorescein-labeled 2nd DNA probe and barcode DNA were used to evaluate the conjugation efficiency. To separate the conjugates from unreacted oligonucleotides, different separation procedures were taken. After centrifuging at 13,000 rpm for 20 min, the fluorescence signal of the supernatant solution of 1st DNA probe/Au-NPs/barcode DNA-TEX 613 was measured. The supernatant solution of MNPs/sulfo-SMCC/2nd DNA probe-6-FAM was measured after magnetic separation. The same separation procedure was applied to their respective controls.

2.7. Target DNA detection

A solution containing target DNA in PCR tube was put in the thermocycler at 95 °C for 10 min to separate the dsDNA into ssDNA. Serially diluted DNA samples (40 μL) were mixed with 0.8 mg MNPs in 200 μL assay buffer (10 mM PBS buffer, 0.15 M NaCl, 0.1%SDS, pH 7.4). The hybridization reaction was maintained at a temperature of 45 °C for 45 min in an incubator. After hybridization, the MNPs with DNA target were washed twice with the assay buffer, then were resuspended in 200 μL assay buffer. The functionalized Au-NPs (100 μL) were centrifuged at 13,000 rpm for 20 min and the unreacted thiolated oligonucleotides in the supernatant were removed. Finally the purified Au-NPs complex was resuspended in 500 μL assay buffer. The Au-NPs complex (40 μL) was then added into 200 μL solution containing MNPs with DNA target. The hybridization was incubated at 45 °C for 2 h with shaking.

After the sandwich structure (MNPs-2nd DNA probe/Target DNA/1st DNA probe-Au-NPs-barcode DNA) was formed, the assay was put on the magnetic separator for 3 min and then the supernatant was removed. Unreacted solution components (DNA and Au-NPs) were washed away five times with 500 μL assay buffer in order to effectively remove Au-NPs that were not specifically bound to the MNPs through hybridization.

The MNP-target-Au-NP complex was resuspended in 200 μL of 0.5 M DTT solution. To release the barcode DNA from the surface of Au-NPs, the complex was incubated at 50 °C for 15 min, and then 45 min at 25 °C under vortex. After magnetic separation and centrifugation, the released barcode DNA was ready for fluorescent measurement.

3. Results and discussion

3.1. Characterization of gold nanoparticles

The Au-NP dimension and spectroscopic property were characterized by using a transmission electron microscope (TEM) and a UV–vis–NIR scanning spectrophotometer, respectively.

Fig. 2A shows a transmission electron microscopy image of our synthesized Au-NPs with an average diameter of 15 nm. The dimension of Au-NPs is homogenous. Fig. 2B shows the absorption peak of the Au-NPs at 519 nm wavelength. After one month storage in the room temperature, the Au-NPs did not aggregate and their spectroscopic absorbance property was stable.

3.2. Functionalization of nanoparticles

The 6-FAM labeled 2nd DNA probe was used for confirming the functionalization of MNPs. Two controls were taken for evaluation of the conjugation efficiency. One was only the 6-FAM DNA probe,

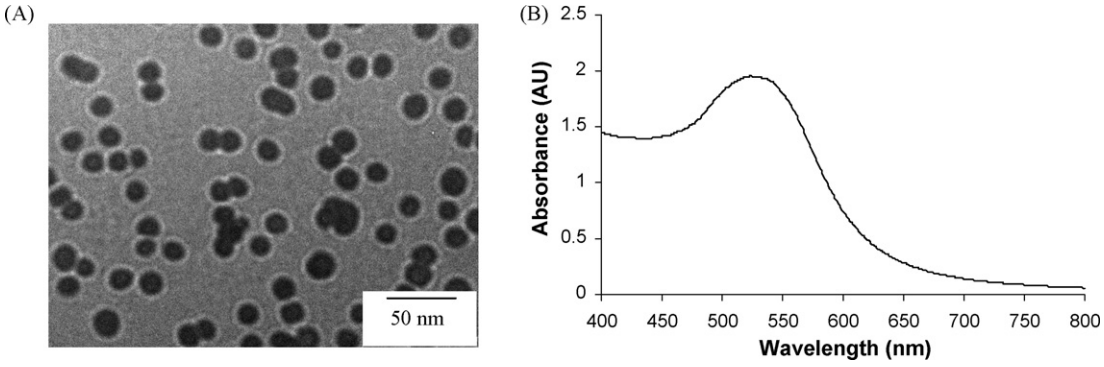

Fig. 2. Characterization of Au-NPs: (A) TEM image of Au-NPs, showing an average of 15 nm in diameter; (B) absorbance spectrum of Au-NPs at 519 nm.

Table 1
Fluorescence signal of supernatant solution for evaluation of conjugation efficiency between MNPs and 2nd DNA probe

	MNPs + Sulfo-SMCC + 6-FAM DNA	MNPs + 6-FAM DNA (control 2)	6-FAM DNA (control 1)
Fluorescence (0.1 s) (counts)	72,735	293,003	368,146
Standard deviation	248.7	821.9	1942.1

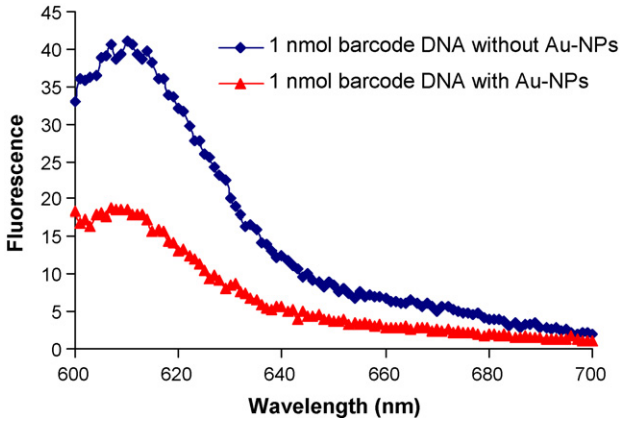

Fig. 3. Fluorescence signal of supernatant solution for evaluation of conjugation efficiency between Au-NPs and barcode DNA.

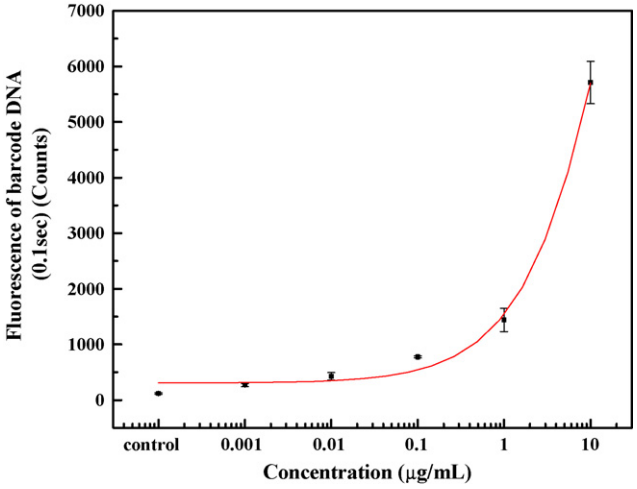

Fig. 5. The fluorescence signal of released barcode DNA with different concentrations of target DNA.

and the other was 6-FAM DNA probe and MNPs without sulfo-SMCC cross-linker.

If the conjugation is efficient, less unconjugated 6-FAM labeled DNA probes will be left in the supernatant solution after magnetic separation.

Table 1 shows that the 6-FAM labeled barcode DNA has the highest fluorescence signal because there is no MNPs in the solution. Magnetic separation has no effect on it. Without sulfo-SMCC as a cross-linker in solution, the DNA probe is not conjugated to the MNPs, hence the magnetic separation will not remove the 6-FAM labeled DNA probe. However, some of the DNA probe in solution could be washed out during magnetic separation, hence a decrease in fluorescence signal is observed. After adding sulfo-SMCC into the conjugation reaction, the conjugation efficiency improved greatly and the fluorescence signal deceased markedly. The result shows sulfo-SMCC worked as an efficient cross-linker to conjugate the thiolated oligonucleotides and amine-coated MNPs. Because the synthesized Au-NPs have a peak absorbance at 519 nm, in order to avoid its interference with the excitation wavelength (520 nm) of 6-FAM, another fluorescent dye (TEX 613) was used to confirm the functionalization of Au-NPs. The unconjugated TEX 613 labeled barcode DNA was left in the supernatant solution after centrifuga-

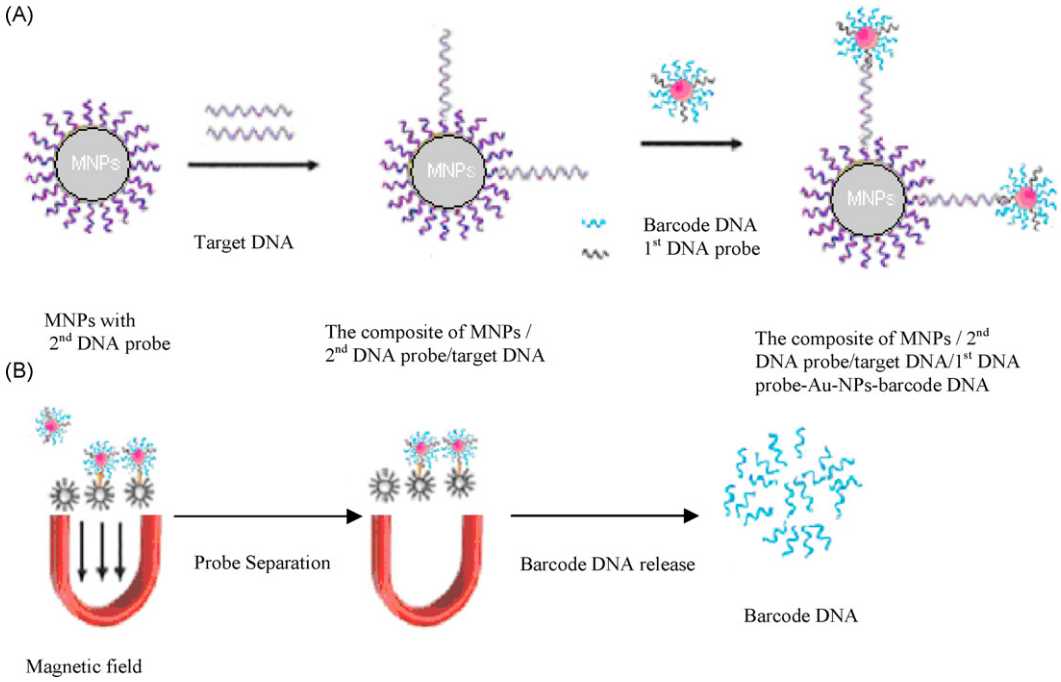

Fig. 4. Schematic of the bio-barcode assay: (A) formation of MNP-2nd DNA probe/target DNA/1st DNA probe-Au-NPs-barcode DNA; (B) barcode DNA separation and release.

Table 2
The relationship between target DNA and the fluorescence signal of released barcode DNA

Sample concentration (μg/mL)	10	1	0.1	0.01	0.001	0
Fluorescence reading (counts)	5711	1440	777	426	270	117
3 × Standard deviation (±)	380.88	209.16	18.52	67.26	28.84	12.01

tion at 13,000 rpm for 20 min. The control was the TEX 613 labeled barcode DNA in the same buffer solution without Au-NPs.

Fig. 3 shows that almost half of the thiolated barcode DNA has been immobilized on the surface of Au-NPs. The bio-barcoded Au-NPs were found to be stable in solution in terms of non-aggregation. This phenomenon is probably due to the negatively charged barcode DNA repelling to each other, hence no aggregation occurs even at long storage time. The solution of bio-barcoded Au-NPs retained its deep red color under room temperature for over a month, demonstrating stability of conjugation. On the contrary, the Au-NPs in the solution without DNA probe aggregated to larger particles and precipitated finally under the same buffer condition.

3.3. Bio-barcode DNA assay amplification and detection

Fig. 4 is a schematic showing the bio-barcode assay. After immobilization of oligonucleotides on the surface of nanoparticles, both nanoparticles can bind with the target DNA to form a sandwich structure, due to the specificity of DNA probes. Fig. 4A shows the target DNA sample hybridizing with the DNA probe on the MNPs, forming a MNP-2nd DNA probe/target DNA complex (middle picture). Then bio-barcoded Au-NPs are added to form a sandwich structure consisting of MNP-2nd DNA probe/target DNA/1st DNA probe-Au-NPs-barcode DNA (picture on the right). After hybridization is complete, the barcode DNA is released from the surface of Au-NPs and the fluorescence signal is measured by a fluorescence counter (Fig. 4B).

Fig. 5 shows that the fluorescence signal of released barcode DNA has an exponential growth relationship with different concentrations of target DNA. At a concentration of 10 μg/mL target DNA, the fluorescence signal has an average count of 5711. The signal exponentially increases with increasing concentration of target DNA.

Table 2 shows the mean and variance of the signal. The results show that the assay can detect as low as 1 ng/mL target DNA (or 2.15×10^{-16} mol).

4. Conclusion

A bio-barcoded DNA assay for the detection of the insertion element (*Iel*) gene of *S.* Enteritidis has been described in this paper. The system is based on two nanoparticles: gold nanoparticles and magnetic nanoparticles. The Au-NPs work as transducer and signal amplifier and the MNPs act as separator and pre-concentrator. Gold nanoparticles were synthesized by a chemical reduction method. The conjugation reaction between the two nanoparticles and thiolated oligonucleotides was efficient. After mixing the nanoparticles with the target DNA, the sandwich structure (MNPs-2nd DNA probe/Target DNA/1st DNA probe-Au-NPs-barcode DNA) was formed. The fluorescence signal of released barcode DNA had an exponential relationship with target DNA concentration. The results show that the detection limit of this bio-barcoded DNA assay is 1 ng/mL (or 2.15×10^{-16} mol).

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