Electronic Supplementary Information

Highly sensitive detection of CpG methylation in genomic DNA by AuNP-based colorimetric assay with ligase chain reaction

Fengxia Su¹, Limei Wang¹, Yueying Sun¹, Chenghui Liu², Xinrui Duan², Zhengping Li^{1, 2*}

¹Key Laboratory of Medicinal Chemistry and Molecular Diagnosis, Ministry of Education, College of Chemistry and Environmental Science, Hebei University, Baoding 071002, Hebei Province, P. R. China.

² Key Laboratory of Applied Surface and Colloid Chemistry, Ministry of Education, Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710062, Shaanxi Province, P. R. China

List of Contents:

- 1. Materials and apparatus
- 2. Table S1. Sequences of the synthetic oligonucleotides used in the experiments
- 3. Preparation and methylation of genomic DNA
- 4. Preparation and modification of gold nanoparticles
- 5. Ligase chain reaction (LCR)
- **6.** Colorimetric detection
- 7. Optimization of ligation temperature in LCR amplification
- 8. Optimization of the amounts of Salmon sperm DNA in LCR amplification
- 9. Optimization of NaCl concentration for colorimetric detection
- 10. Reproducibility and stability of the LCR-based colorimetric assay
- 11. The LCR-based colorimetric assay performance by using 13 nm AuNPs

Supporting Information

1. Materials and Apparatus

Ampligase thermostable DNA ligase was purchased from Epicenter Technologies (USA). Exonuclease I (Exo I) and Exonuclease III (Exo III) used to digest the LCR products were obtained from Thermo Fisher Scientific Inc. (USA). CpG Methyltransferase (M.SssI) was purchased from New England Biolabs. Salmon sperm DNA was purchased from Invitrogen (USA). HAuCl₄·4H₂O was obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Trisodium citrate dihydrate and HPLC purified DNA oligonucleotides were obtained from Sangon Biotech. (Shanghai, China). The sequences of oligonucleotides used in this study were listed in Table S1. TIANamp Genomic DNA Kit (TIANGEN Biotechnology) was used to extract genomic DNA from human whole blood. EpiTect[®] Bisulfite kit was purchased from Qiagen (Germany). JumpStart Taq DNA polymerase (Sigma-Aldrich, Shanghai, China) and 2.5 mM of dNTPs (TaKaRa, Dalian, China) were used for PCR amplification. All water used in this study was sterilized and deionized water. All other reagents were of analytical reagent grade and used as purchased without further purification. The LCR and PCR reactions were carried out in a 2720 thermal cycler (Applied Biosystems, USA). A TU1901 UV-Vis spectrophotometer (Purkinje General, China) was employed for quantification of genomic DNA. D90 digital camera (Nikon, Japan) was used to take the photographs of the colorimetric assay results, and Epoch Multi-Volume Spectrophotometer system (BioTek, USA) was used for scanning UV-Vis absorption spectra. JEM-2100 transmission electron microscope (TEM) (JEOL, Japan) was employed to characterize the morphology of the gold nanoparticles.

Target M	TTTTTTTTGGAGGGTCGATGAGGTAATG <u>C</u> GGTTTTGTTATTGGTTT
	GAGGGGGGGGT
Target N	TTTTTTTTGGAGGGTTGATGAGGTAATG <u>T</u> GGTTTTGTTATTGGTTT
	GAGGGGGTGGGT
Probe A	phosphate-CATTACCTCATCGACCCT
Probe B	TCAAACCAATAACAAAACCG
Probe A'	AAGGGTCGATGAGGTAATGC

2.	Table S1	. Sequences	of the svi	nthetic oligoni	icleotides used	l in the ex	neriments ((5'-3')
	I HOIC DI	· Dequences	of the syl	nunctic ongoin	icicollucs used	a mi une ca	per miento (00	,

Probe B'	phosphate-GGTTTTGTTATTGGTTTG <u>AGAAA (phosphorothioate)</u>
DNA strand a	CGCATTACCTCATCGACCCTTTTTTTTT-SH C3
DNA strand b	SH C6-TTTTTTTTTTTCTCAAACCAATAACAAAAC
Forward primer	GGGAGTGGGGTTAGGGTGTAGA
Reverse primer	AAACTACTACTCCTCCCAAAATTACC

Note. Target M and target N are synthetic DNA from the promoter of TIMP-3 gene, whose sequences are corresponding to that of methylated and unmethylated DNA after bisulfite-treatment, respectively. The detected sites in the sequences are underlined. Probe A and probe B are complementary to a portion of target M and can adjacently hybridize to target M. Probe A' and probe B' are respectively complementary to probe A and probe B. DNA strand a and b are used respectively to modify gold nanoparticles, which were respectively complementary to probe A' and probe B', and thus can hybridize to the DNA strand A'B' in the LCR products to give the color changes. The forward primer and reverse primer were used to amplify the TIMP-3 gene fragment by PCR for sequencing.

3. Preparation and methylation of genomic DNA

Human genomic DNA was extracted from whole blood of a healthy volunteer in our laboratory by TIANamp Genomic DNA Kit. The extracted genomic DNA was quantified by a UV-Vis spectrophotometer. The genomic DNA was treated with sodium bisulfite and purified according to the manufacturer's protocols. The bisulfite treated genomic DNA was amplified with PCR reaction performed in 50 μ L aqueous solution containing 0.25 mM dNTPs, 5 μ L 10× PCR buffer, 1 μ M each of forward primer and reverse primer (see Table S1) and 2.5 U of JumpStart Taq DNA polymerase in a thermocycler under the following program: 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 1 min, and ended with 72 °C for 7 min. The PCR product was sequenced with an ABI 3730xl DNA analyser (Applied Biosystems) by Lifetech. (Beijing, China). As depicted in Fig. S1, the genomic DNA can be characterized as unmethylated DNA and the sequence was in accordance with the synthetic sequence (Target N) in Table S1.

Fig. S1 The sequencing result of the detection region in bisulfite treated healthy human genomic DNA, in which the CpG sites in the genomic DNA are underlined. All the cytosine in the CpG sites is sequenced as thymine, indicating that the genomic DNA is unmethylated.

The genomic DNA was methylated with the treatment of the M.SssI according to following procedures, while another part was not treated. The methylation mixture, consisting of 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9 @ 25 °C, 0.64 mM S-adenosylmethionine (SAM), 20 U M.SssI, and 2 μ g genomic DNA with a final volume of 20 μ L, was incubated at 37 °C for 5 h. Then 0.8 μ L 16 mM SAM and 20 U M.SssI were added and incubated at 37 °C for 12 h to ensure complete methylation. Afterward, the mixture was heated to 65 °C for 20 min to inactivate the M.SssI. Then two equal quantities of methylated and unmethylated genomic DNA were simultaneously treated with sodium bisulfite. The bisulfite-treated methylated and unmethylated genomic DNA are quantified from the absorption at 260 nm.

4. Preparation and modification of gold nanoparticles

The gold nanoparticles (AuNPs) were synthesized by citrate reduction of HAuCl₄ according to Frens^{S1} and Grabar^{S2} *et al.* with slight modifications. All glassware was steeped in chromate washings (cleansing solution) for 24 h, rinsed with double-distilled water, and oven-dried prior to use. Briefly, after boiling 100 mL of the 0.01% HAuCl₄ solution, 1 mL of the 1.0% trisodium citrate solution was quickly added with vigorous stirring. The color of the solution changed to deep red in a few seconds and the reduction of trisodium citrate to HAuCl₄ was practically completed after 6-8 min of boiling. The solution was allowed to cool to room temperature and then diluted to 100 mL. The average diameter of the prepared gold nanoparticles was about 50 nm as characterized by TEM (see Fig. S2).



Fig. S2 The TEM image of the 50 nm gold nanoparticles.

The gold nanoparticles with an average diameter of 13 nm were prepared in an analogical method with that described above with slight modification. Briefly, after boiling 100 mL of the 0.04% HAuCl₄ solution, 1 mL of the 10% trisodium citrate solution was quickly added with vigorous stirring. The color of the solution changed to deep red in a few seconds and the reduction of trisodium citrate to HAuCl₄ was practically completed after 6-8 min of boiling. The solution was allowed to cool to room temperature and then diluted to 100 mL. The average diameter of the prepared gold nanoparticles was about 13 nm as characterized by TEM (see Fig. S3).



Fig. S3 The TEM image of the 13 nm gold nanoparticles.

The gold nanoparticles were modified by alkanethiol-capped oligonucleotides according to previous method^{S3} with minor modifications. DNA strand a or DNA strand b (see Table S1) (2.7 nmoles) was added into 1 mL AuNP solution and incubated at 45°C for 24 h. Then the solution was adjusted to pH value of 7.0 and ionic strength of 10 mM phosphate buffer, and allowed to stand at 45°C for 12h. Afterwards, aqueous 3 M NaCl was added to the mixture to adjust the concentration of NaCl to 0.1 M. Then, 10% (w/w) sodium dodecyl sulfonate (SDS) was added to the solution to a final concentration of 0.01%. After standing for another 12 h at 45 °C, the concentration of NaCl in the solution was increased to 0.2 M. This procedure was repeated to

adjust the NaCl concentration to 0.3 M. After an additional salt aging process for 24 h at 45 °C, the nanoparticles were isolated by centrifugation for 15 min with 12000 r.p.m. and the supernatant was discarded. The precipitate was resuspended by 1 mL buffer (0.3 M NaCl, 10 mM phosphate and 0.01% SDS, pH 7.0). Then, the steps of centrifugation and resuspension were repeated three times. Finally, the oligonucleotide-modified AuNPs were resuspended by 400 μ L resuspension buffer.

The 13 nm AuNPs were diluted 3-fold and then modified with alkanethiol-capped oligonucleotides. DNA strand a or DNA strand b (1.3 nmoles) was added into 1 mL diluted AuNP solution and incubated at 45°C for 24 h. Then the solution was adjusted to pH value of 7.0 and ionic strength of 10 mM phosphate buffer, and allowed to stand at 45°C for 12h. Afterwards, aqueous 3 M NaCl was added to the mixture to adjust the concentration of NaCl to 0.1 M. After an additional incubating process for 40 h at 45 °C, the nanoparticles were isolated by centrifugation for 30 min with 12000 r.p.m. and the supernatant was discarded. The precipitate was resuspended by 1 mL buffer (0.3 M NaCl, 10 mM phosphate and 0.01% SDS, pH 7.0). Then, the steps of centrifugation and resuspension were repeated three times. Finally, the oligonucleotide-modified AuNPs were resuspended by 1 mL resuspension buffer.

5. Ligase chain reaction (LCR)

Probe A, probe B (with final concentration of 30 nM each) and 0.5 μg Salmon sperm DNA, which is usually employed to improve the specificity of LCR, were mixed with an appropriate amount of target DNA firstly. The mixture was heated at 95 °C for 5 min. Then a mixture of the ligase buffer (20 mM Tris-HCl, 25 mM KCl, 10 mM MgCl, 5 mM NAD, and 0.1 % Triton X-100, pH 8.3) and 2 U Ampligase was added to the hot mixture at 80 °C. Then ten thermal cycles of 95 °C for 30 s and 61 °C for 1 min were performed. After adding probe A' and probe B' (with final concentration of 30 nM each) at 80 °C, the LCR reaction with a final volume of 20 μL was carried out with following 30 thermal cycles of 95 °C for 30 s and 61 °C for 1 min.

6. Colorimetric detection

Exo I (20 U) and Exo III (100 U) were added into the LCR product to digest the unreacted probes and the unphosphorothioate-labeled products by incubation at 37 °C for 1 h. The

degradation reaction was terminated by heating the mixture at 80 °C for 20 min. The digested LCR product (5 μ L) was then mixed with 20 μ L solution of AuNPs modified with DNA strand a and 20 μ L solution of AuNP modified with DNA strand b in supplementary buffer (0.5 M NaCl, 10 mM phosphate, and 0.01% SDS, pH 7.0) with the final volume of 50 μ L. The mixture was put on ice for 5 min and then incubated at 25 °C for 10 min to perform the hybridization. The photographs of the final solutions were taken by D90 digital camera and the absorption spectra were aquired with Epoch Multi-Volume Spectrophotometer system.

7. Optimization of ligation temperature in LCR amplification

In LCR amplification, ligation temperature is a crucial factor for achieving high specificity to discriminate between methylated and unmethylated DNA. Therefore, the effect of ligation temperature on the specificity of the LCR-based colorimetric assay was investigated by simultaneously detecting the blank, 0.1 fM target M, and 0.1 fM target N at different ligation temperature. As depicted in Fig. S4 (a-b), when the ligation was performed at 59 °C and 61 °C, the color arised by target M and target N can be clearly discriminated. However, at 59 °C of ligation temperature, the slight color change produced by target N can be observed compared to the blank. No color change can be observed between target N and the blank at 61 °C of ligation temperature. When the ligation temperature was increased to 63 °C, the target M cannot produce color change compared to the blank and target N, indicating that no ligation occurs at 63 °C. Therefore, 61 °C is selected to be optimized ligation temperature in this work.



Fig. S4 Effect of ligation temperature on the specificity of the LCR-based colorimetric assay. The blank (1), 0.1 fM target M (2), and 0.1 fM target N (3) were simultaneously detected with the assay. The ligation temperature in LCR amplification is (a) 59°C, (b) 61°C, and (3) 63°C, respectively. The assay was performed according to the procedures as described in the section of LCR and colorimetric detection except the ligation temperature in LCR.

8. Optimization of the amounts of Salmon sperm DNA in LCR amplification

Generally, template-independent ligation in LCR is a major factor to produce non-specific

amplification, which will limit the sensitivity and selectivity of LCR-based assay. The addition of Salmon sperm DNA as the steric hindrance can efficiently block the non-specific amplification in LCR. Thus, the effect of the amounts of Salmon sperm DNA on detection of DNA methylation was studied. The blank, 0.1 fM target M, and 0.1 fM target N were simultaneously detected with the LCR-based colorimetric assay in the presence of different amounts of Salmon sperm DNA. As demonstrated in Fig. S5 (a), target M and target N can not be discriminated in the absence of Salmon sperm DNA, indicating that the non-specific amplification in LCR occurs. In the presence of 0.5 µg Salmon sperm DNA, as shown in Fig. S5 (b), target M can be well detected with the color change. Moreover, target N can not produce any color changes compared to the blank. Therefore, the LCR-based assay shows high specificity for detection of DNA methylation in the presence of 0.5 µg Salmon sperm DNA. However, when the amount of Salmon sperm DNA was increased to 1 µg (Fig. S5 (c)), target M can not be detected with the color change. Therefore, 0.5 µg Salmon sperm DNA is chosen to be optimal amount for the LCR-based colorimetric assay.



Fig. S5 Effect of the amounts of Salmon sperm DNA on the LCR-based colorimetric assay. The blank (1), 0.1 fM target M (2), and 0.1 fM target N (3) were simultaneously detected with the assay in the presence of (a) 0, (b) 0.5 μ g, and (c) 1 μ g Salmon sperm DNA. The assay was performed according to the procedure as described in the section of LCR and colorimetric detection except the amounts of Salmon sperm DNA.

9. Optimization of NaCl concentration for colorimetric detection

The colorimetric detection is based on the sandwich hybridization among DNA strand A'B' (produced by LCR) and DNA strand a and b (respectively immobilized on different AuNPs), which induces the aggregation of AuNPs and thus, gives the readout of color changes. NaCl contributes to reducing the electrostatic repulsion of DNA phosphate backbones, which can stabilize the sandwich hybridization. So NaCl concentration has important effect on the sensitivity and dynamic range of the colorimetric detection. The synthetic DNA stand A'B' was used for investigation of the effect of NaCl concentration on the colorimetric detection. As shown in Fig.

S6 (a-b), when NaCl concentration is less than 0.15 M, 1 nM and 5 nM DNA strand A'B' can not produce detectable color changes compared to the blank. When NaCl concentration is greater than 0.7 M (Fig. S6 (e-f)), the DNA stand A'B' can arise obvious red-to-blue color changes compared to the blank. However, 1 nM and 5 nM A'B' are difficult to be discriminated. When NaCl concentration is 0.5 M, the best discrimination by color changes can be achieved among the blank, 1 nM, and 5 nM DNA strand A'B' (Fig. S6d). By consideration of both sensitivity and dynamic range of the colorimetric detection, 0.5 M NaCl was chosen as to be the optimal concentration for the colorimetric detection.



Fig. S6 Effect of NaCl concentration on the colorimetric detection. (1) 0, (2) 1 nM, and (3) 5 nM synthetic DNA strand A'B' was detected with AuNPs respectively modified with DNA strand a and b according to procedures as described in the section of colorimetric detection except NaCl concentration, which was (a) 0.1 M, (b) 0.15 M, (c) 0.3 M, (d) 0.5 M, (e) 0.7 M, and (f) 1.0 M, respectively.

10. Reproducibility and stability of the LCR-based colorimetric assay

To validate the reproducibility of the LCR-based colorimetric assay, 0, 0.01 fM, 0.1 fM and 1 fM of methylated target (target M) were detected with the LCR-based colorimetric assay at different days. As shown in Fig. S7, the color is red in absence of target M, and changes from purple to blue when the concentration of target M increases from 0.01 fM to 0.1 fM. The three different experiments show the same color change with the increase of target M concentration, which demonstrates the good run-to-run reproducibility of the LCR-based colorimetric assay.



Fig. S7 Visual detection of three repeated experiments by using the LCR-based colorimetric assay, of which the result is (a), (b) and (c), respectively. The concentration of target M is (1) 0, (2) 0.01 fM, (3) 0.1 fM, and (4) 1 fM, respectively. The assay was performed at different days according to the procedures as described in the section of LCR and colorimetric detection.

To validate the stability of the LCR-based colorimetric assay, 0, 0.01 fM, 0.1 fM and 1 fM of methylated target M were detected with the LCR-based colorimetric assay and the color of AuNP solution was observed in room temperature over a period of 90 min. As shown in Figure S8, the color of AuNP solution produced by different amounts of methylated DNA can be stable with different time. When the methylated DNA at low concentration (0.01 fM), the color can stable for 90 min. The color produced by 0.1 fM methylated DNA can be stable for 40 min, then the color becomes light. When the concentration of methylated DNA is 1.0 fM, the color continuously becomes light until colorless after 75 min. The color changes are arisen from the hybridization between LCR products and the oligonucleotides immobilized on the AuNPs, which induces the aggregation of AuNPs with a polymeric network structure. The methylated DNA at low concentration produces smaller amount of LCR products, which form smaller AuNP aggregates that do not precipitate over 90 min. The greater the concentration of methylated DNA, the bigger the AuNPs aggregates, which result in faster precipitation and thus the color of AuNP becomes light. However, the AuNP solutions can keep different colors for detection of different amounts of methylated DNA over the course of the experiment (90 min). Therefore, the LCR-based colorimetric assay is practical to detect methylated DNA by the naked eye.



Fig. S8 The stability of the LCR-based assay in a period of 90 min. The concentration of target M is (1) 0, (2) 0.01 fM, (3) 0.1 fM, and (4) 1 fM, respectively. The standing time of the resulting solution is (a) 5 min, (b) 20 min, (c) 40 min, (d) 60 min, (e) 75 min, and (f) 90 min, respectively. The assay was performed according to the procedures as described in the section of LCR and colorimetric detection.

11. The performance of LCR-based colorimetric assay by using 13 nm AuNPs

Initially, 13 nm AuNPs are used for detection of DNA targets, where the detection limit is typically in nanomolar range in the homogeneous solution. We try to detect the methylated target M with the proposed LCR-based colorimetric assay by using oligonucleotide-modified 13 nm AuNPs. As shown in Fig. S9, the 13 nm AuNPs cannot produce detectable color changes for detection of $0\sim1$ fM methylated target M. In the LCR amplification, the DNA probes are used at 30 nM. After LCR, 5 µL of LCR products are added into 45 µL AuNPs solution. Therefore, even if all the DNA probes are ligated in the LCR, the maximum concentration of the LCR products is 3 nM, which still cannot be detectable by using 13 nm AuNP. Therefore, the results are reasonable.



Fig. S9 Visual detection results by using 13 nm AuNPs. (a) 0, (b) 0.01 fM, (c) 0.1 fM, and (d) 1 fM of target M were detected simultaneously with the LCR-based colorimetric assay. The assay was performed according to the procedures as described in the section of LCR and colorimetric detection.

References:

S1. G. Frens, Nat. Phys. Sci., 1973, 241, 20.

S2. K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, Anal. Chem., 1995, 67, 735.

S3. R. A. Reynolds, C. A. Mirkin and R. L. Letsinger, J. Am. Chem. Soc., 2000, 122, 3795.