Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2015

Target-protected dumbbell molecular probe mediated cascade rolling circle amplification strategy for the sensitive assay of DNA methyltransferase activity

Haiyan Zhao,[a] Lei Wang[b] and Wei Jiang[a]*

[a] Key Laboratory for Colloid and Interface Chemistry of Education Ministry, School of Chemistry and Chemical Engineering, Shandong University, 250100 Jinan, P.R. China

[b] School of Pharmaceutical Sciences, Shandong University, 250012 Jinan, P. R. China

Corresponding author: Tel: 86-531-88363888; fax: 86-531-88564464.

E-mail: wjiang@sdu.edu.cn

1. Experimental

Reagents. Oligonucleotides (Table S1) were synthesized and purified by Sangon Inc. (Shanghai, China). M.SssI, AluI, HaeIII and HhaI DNA methyltransferase (MTase), HpaII and RsaI endonuclease, T4 DNA ligase, Exonuclease I (Exo I), Exonuclease III (Exo III), phi29 DNA polymerase were bought from New England Biolabs (Beijing, China). The 10 × T4 DNA ligase reaction buffer, 10 × Cutsmart reaction buffer, 10 × NEB buffer2 and S-adenosyl-L-methionine (SAM) were also provided by New England Biolabs. Sybr Green I (SG) was obtained from BioTeke Corporation (Beijing, China). 5-azacytidine (5-Aza) and 5-aza-2′-deoxycytidine (5-Aza-dC) were from Sigma-Aldrich. All other chemicals were of analytical grade and used as received. All solutions were prepared using the ultrapure water that was obtained from a Millipore Milli-Q water purification system (> 18.25 MΩ cm^{-1}).

Table S1. Sequences of oligonucleotides used in this study

Name	Sequence (5′→3′)
DP-13bp	p-*CCG G*TT CCT TAG TGC AGA **GTA C**GG AGA TAG GGA GAA AGG GAA *CCG G*GT GTG AAA GAA AGA AAG AAA GCA CAC
DP-16bp	p-*CCG G*TT CCT CTT AGT GCA GA**G TAC** GGA GAT AGG GAG AAA GGA GGA A*CC GG*G TGT GTA AAG AAA GAA AGA AAG ACA CAC
DP-20bp	*p-CCG G*TT CCT CTC TTA GTG CAG A**GT AC**G GAG ATA GGG AGA AAG GAG AGG AA*C CGG* GTG TGT GTA AAG AAA GAA AGA AAG ACA CAC AC
DP-22bp	p-*CCG G*TT CCT CTC TTT AGT GCA GA**G TAC** GGA GAT AGG GAG AAA GAG AGA GGA A*CC GG*G TGT GTG TGA AAG AAA GAA AGA AAG CAC ACA CAC
DP-24bp	p-*CCG G*TT CCT CTC TCT TAG TGC AGA **GTA C**GG AGA TAG GGA GAA AGG AGA GAG GAA *CCG G*GT GTG TGT GTA AAG AAA GAA AGA AAG ACA CAC ACA C
P1	CTC CCT ATC TCC GTA CTC TGC A
P2	TGC AGA **GTA C**GG AGA TAG GGA G

Lower-case letter 'p' indicates phosphate group. Underlines indicate complementary sequences of dumbbell probe. Letters with red and italics (CCGG) indicate the recognition site for MTase and HpaII endonuclease.

The bolded letters (GTAC) is the RsaI recognition sequence.

Apparatus. All fluorescence emission spectrum were record on a Hitachi F-7000 fluorescence spectrometer (Hitachi, Japan). The excitation wavelength was 497 nm, and the spectra were recorded from 510 nm to 610 nm. The slits of both excitation and emission were 5 nm and the photomultiplier tube voltage was 650 V.

Preparation of seal dumbbell probe. Seal dumbbell probe (D-probe) was obtained by self-templated ligation of 5′-phosphorylated dumbbell-shaped DNA sequence by T4 DNA ligase. To obtain the dumbbell structure, the dumbbell probe DNA sequences were first denatured at 95 ºC for 5 min. Then the ligation reaction was performed at 37 ºC for 120 min in 20 μL 1 × T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM Dithiothreitol, 1 mM ATP, pH 7.5) containing 5.0 μM dumbbell probe and 100 U T4 DNA ligase, and then heated at 65 °C for 10 min to terminate the ligation reaction. After that, 20 U Exo I and 50 U Exo III were added to digest the leftover ssDNA and dsDNA to yield closed DNA and denatured by heating at 80 °C for 20 min. These prepared probes were then stored at -20 °C until use.

Methylation and cleavage of dumbbell probe. The methylation experiment was performed at 37 °C for 2.0 h in 10 μL 1 × NEB buffer2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) containing 160 μM SAM, 150 nM D-probe and various concentrations of M.SssI (from 0 to 100 U/mL). Then, HpaII cleavage was performed at 37 °C for 2.5 h in 30 μL 1 × cutsmart buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 μg/ml BSA, pH 7.9) containing 25 U/mL HpaII, followed by inactivation at 80 °C for 20 min.

Cascade rolling circle amplification and fluorescence detection. Firstly, the above methylation products were mixed with 20 μL 1 × cutsmart buffer containing 225 nM primer1,

1.25 mM dNTPs, 1.5 U phi 29 DNA polymerase. Then the 50µL mixture was incubated at 37 °C for 50 min to perform the first round amplification (1-RCA). Subsequently, 10 µL 1 × cutsmart buffer containing 7.5 µM primer2 and 10 U RsaI was added to monomerize the 1-RCA products by incubating at 37 °C for 50 min. After this, the RsaI was inactivated at 65 °C for 20 min and the monomerized products were circularized by adding 100 U T4 DNA ligase with T4 DNA ligase buffer to final volume of 70 µL at 37 °C for 30 min. Next, second round of RCA was performed at 37 °C for 50 min with further addition of 10 µL 1 × cutsmart buffer containing 1.5 U phi 29 DNA polymerase and 4 mM dNTPs and terminated by heating at 65ºC for 10 min. Finally, the CRCA products were mixed with 1 ×SG with a final volume of 100 µL, and record the fluorescence emission spectrum.

Gel electrophoresis. A 15% polyacrylamide gels analysis and 0.7% (w/v) agarose gel electrophoresis analysis were used to confirm the feasibility of methylation process and cascade RCA process, respectively. The gel experiments were both carried out in 1 × TBE (89 mM Tris, 89 mM Boric Acid, 2.0 mM EDTA, pH 8.3) and stained with ethidium bromide for 5 min. The imaging of the gel was performed with UV imaging system (Bio-RAD Laboratories Inc. USA).

Selectivity of the M.SssI MTase activity assay. To investigate the selectivity of the proposed M.SssI assay, three other cyosine MTase including AluI, HaeIII, and HhaI were used as the interference methyltransferases. The selectivity experiments were performed with 50 U/mL interfere enzyme using the same procedure as the M.SssI assay.

MTase activity detection in buffer spiked with cell extracts. In a typical M.SssI activity detection using cell extracts, 10 % (v/v) cell extracts was added in the reaction buffer with all the other conditions the same as the description mentioned above. The cell extracts was

prepared by centrifugation (5 min, 3000 rpm, 4 ºC) of HeLa cells samples to be pelleted and then resuspended in 100 µL of lysis buffer (10 mM Tris-HCl, pH 7.0) on ice using a sonicator (four pulses at 200 W for 30 s with a tapered microtip). The mixture solution was then centrifuged at 12,000 rpm for 30 min at 4 ºC to remove insoluble material (containing nucleus). The resulting supernatant was collected and filtered through a 0.45 µm filter membranes, yielding crude lysate.

Inhibition study of anticancer drugs to M.SssI activity. To study the inhibition effects of the two representative anticancer drugs, 5-Aza and 5-Aza-dC, on M.SssI MTase activity, the influence of drugs on the activity of other involving enzymes were first investigated. The methylation experiment of D-probe was performed at 37 °C for 2.0 h in 1 × NEB buffer2 containing 160 µM SAM, 15 nM D-probe and 100 U/mL M.SssI to ensure the absolute methylation. Then, the HpaII cleavage and CRCA process were carried out as described above, except that 5 µM durg was added into the solution to investigate the influence of drugs on the activity of HpaII, phi29 DNA polymerase, RsaI and T4 DNA ligase.

Subsequently, the inhibition effects of the two anticancer drugs on M.SssI MTase activity were evaluated. All the inhibition experiments were performed in conditions similar to those of M.SssI MTase activity assay, except for that various concentrations of drugs were added into the solution before the addition of 100 U/mL M.SssI MTase in the methylation step. Then after the HpaII cleavage and CRCA process, the fluorescence signal was measured.

2. Results and discussion

Verification of circularized dumbbell probe. The ligation products were analyzed by 15% polyacrylamide gels. As shown in Fig. S1, compared lane 4 to lane 5, only the band of sealed

probe remained after digested by Exo I and Exo III, indicating that pure closed seal probe was obtained. Moreover, compared lane 2 to lane 3 and 4, we can found that the sealed probe had faster migration rate than nonsealed probe. The phenomenon was consistent with the prior report, [1] which may induced by the change of DNA secondary structure.

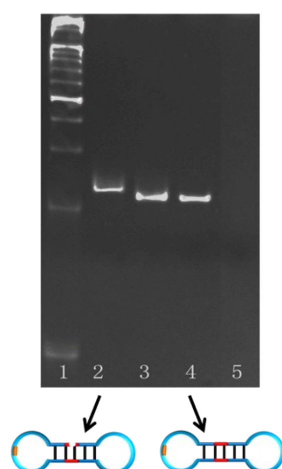

Fig. S1 Nondenaturating PAGE analysis of sealed D-probe. Lane 1, DNA ladder marker; Lane 2, Sealed probe precursor; Lane 3, Sealed D-probe by ligation; Lane 4, Sealed D-probe by ligation and subsequent digestion by Exo I and Exo III; Lane 5, Sealed probe precursor digested by Exo I and Exo III.

Optimization of experimental conditions. Given that D-probe, which serves together as DNA MTase and endonuclease recognition probe, CRCA template, and signal probe, is the key design of the M.SssI MTase activity assay strategy. And its stem length may be related to both amplification rate and binding capacity to SG, thus the effect of D-probe stem length on analytical performance was first investigated. We designed five different D-probes with 13, 16, 20, 22, 24 base pairs (bp) in the stem, respectively. We first investigated the effects of reaction time on 1-RCA of the five D-probes. As shown in Fig. S1B, the stem length with 13-bp reached a plateau firstly, but the signal of plateau is low. These results verified the fact that short stem length would give a high amplification rate due to its small conformational constraint, but short stem length

gives low fluorescence signal due to weak binding capacity for SG.[2] To fulfil rapid assay, 50 min was selected for each round amplification in our assay. As shown in Fig. S1A, the negative system exhibited a gradually signal increased, owing to longer stem length has higher binding capacity for SG. Naturally, much enhanced signal was observed in the positive system. But interestingly, an increased stem length of D-probes from 13-bp to 20-bp resulted in an increased signal, but further increased stem length from 20-bp to 24-bp reduced to a decreased signal intensity. This may because that binding capacity for SG was the determinant in the stem length region from 13-bp to 20-bp, while in further increased stem length region the amplification rate was the determinant. Finally, the D-probe with 20-bp stem length, which had the maximum net signal, was chosen for the M.SssI activity sensing system.

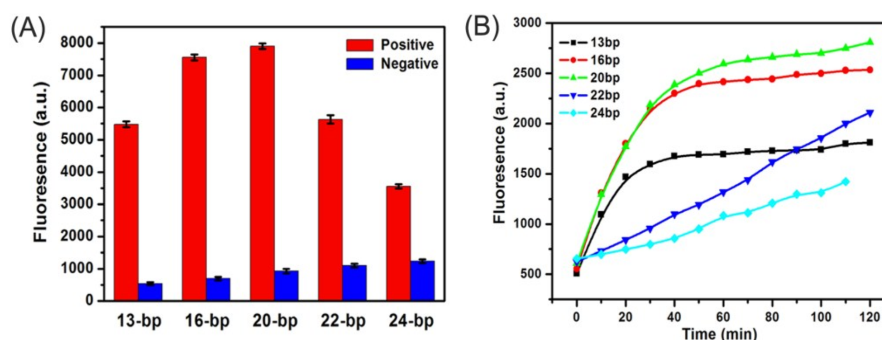

Fig. S2 (A) Effects of D-probe stem length on the fluorescence intensity of the M.SssI acitivity sensing system. The concentration of M.SssI MTase in positive system and negative system is 16 U/mL and 0 U/mL, respectively. (B) Effects of reaction time on the fluorescence intensity of 1-RCA with the five designed different D-probes.

Methylation time, HpaII cleavage time and HpaII concentration play key roles in methylation and subsequent cleavage process of D-probe. Thus, the effects of methylation time, HpaII cleavage time and HpaII concentration on the ΔF of M.SssI acitivity sensing system were investigated, respectively. Moreover, primer1 concentration, RsaI cleavage time, dNTPs concentration, primer2 concentration and RsaI concentration have important effects on the

amplification efficiency of CRCA, thus the effects of these factors were also investigated, respectively. As shown in Fig. S3, the optimized methylation time, HpaII cleavage time and RsaI cleavage time were 2.0 h, 2.5 h, and 50 min, respectively (Fig. S3A, B and C). The optimized concentrations of P1, HpaII endonuclease, dNTPs, P2 and RsaI were 45 nM, 25 U/mL, 500 μM, 750 nM and 100 U/mL, respectively (Fig. S3D–H).

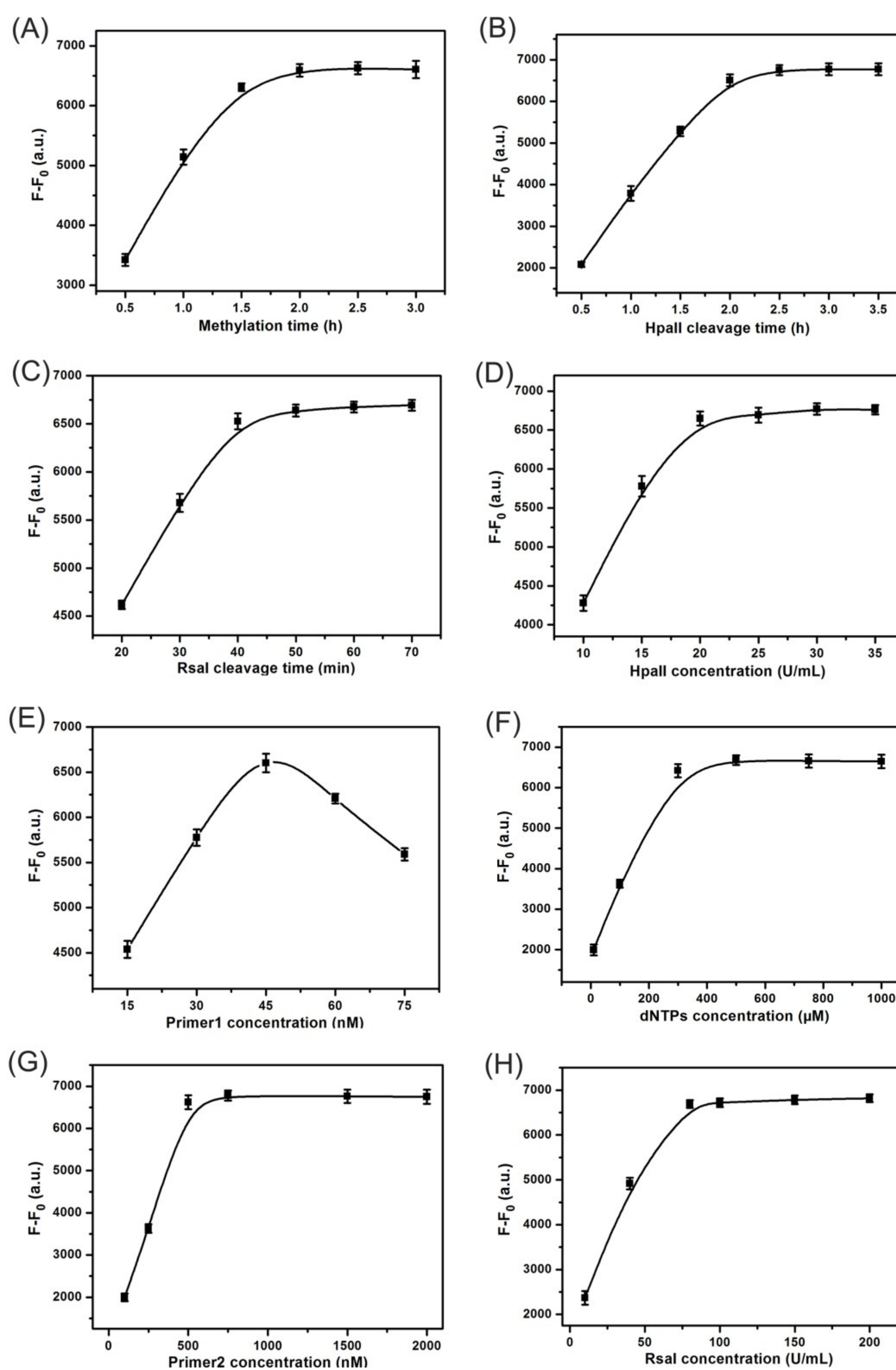

Fig. S3 (A) Effect of methylation time on the ΔF of M.SssI activity sensing system. (B) Effect of HpaII cleavage time on the ΔF of M.SssI activity sensing system. (C) Effect of RsaI cleavage time on the ΔF of M.SssI activity sensing system. (D) Effect of HpaII concentration on the ΔF of M.SssI activity sensing system. (E) Effect of primer1 concentration on the ΔF of M.SssI activity sensing system. (F) Effect of dNTPs concentration on the ΔF of M.SssI activity sensing system. (G) Effect of primer2 concentration on the ΔF of M.SssI activity sensing system. (H) Effect of RsaI concentration on the ΔF of M.SssI activity sensing system. $\Delta F = F - F_0$, where F and F_0 were fluorescence intensities of the system with and without M.SssI MTase, respectively. $C_{M.SssI}$ = 16 U/mL.

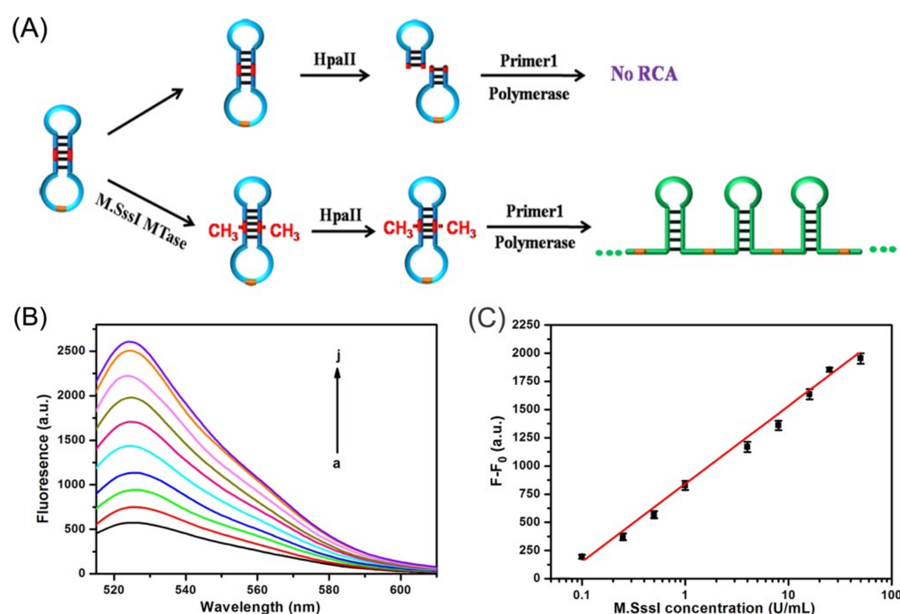

Fig. S4 (A) Schematic illustration of M.SssI MTase activity assay based on target-protected dumbbell molecular probe mediated linear rolling circle amplification strategy. (B) Fluorescence emission spectra of linear rolling circle amplification strategy towards to different concentrations of the M.SssI MTase. From a to j were 0, 0.10, 0.25, 1, 4, 8, 16, 25, 50, 100 U/mL, respectively. (C) Linear relationship of linear rolling circle amplification strategy between ΔF and M.SssI MTase concentration. The linear relationship could be described as $\Delta F = 825.7 + 693.2 \lg C$ with a correlation coefficient of R = 0.997.

Table S2 Comparison of RCA assays for MTase activity detection.

Method	Strategy	Linear Range (U/mL)	Detection Limit (U/mL)	Ref.

Chemiluminescence	HCR-based branched RCA triggered by methylation-responsive double-stranded DNA	1 to 10	0.52	3
Chemiluminescence	Primer generation exponential RCA triggered by methylation-responsive hairpin probe	0.025 to 2.5	0.00013	4
Fluorescence	RCA triggered by methylation-responsive hairpin probe	0.5 to 30	0.18	5
Fluorescence	Linear RCA triggered by methylation-protected dumbbell probe	0.10 to 50	0.08	This work[s]
Fluorescence	Cascade RCA triggered by methylation-protected dumbbell probe	0.01 to 50	0.0024	This work

[s]Supporting information

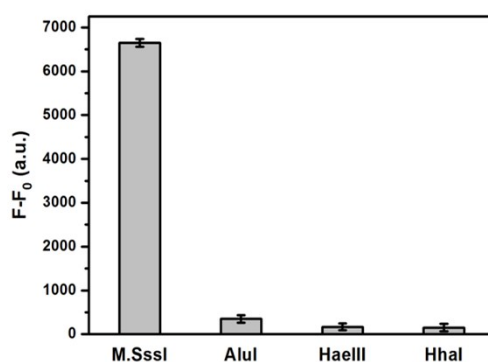

Fig. S5 Comparison of the ΔF in the presence of M.SssI, AluI, HaeIII and HhaI MTase. The concentration of M.SssI was 16 U/mL, and the concentration of AluI, HaeIII and Hhal were 50 U/mL.

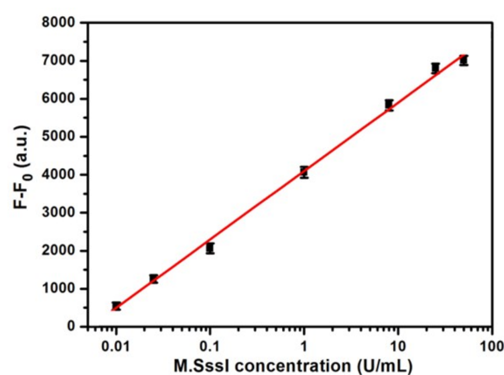

Fig. S6 Linear relationship between ΔF and M.SssI MTase concentration in reaction buffer spiking with cell extracts. The linear relationship can be described as ΔF = 4269.9 + 1665.7 lgC with a correlation coefficient of R = 0.995.

Table S3 Recovery of MTase activity detection in reaction buffer spiking with cell extracts.

Sample	Added (U/mL)	Detected (U/mL)[a]	Recovery (%)	RSD (%)
1	0.025	0.023 ± 0.001	92%	6.4%
2	1.0	0.91± 0.03	91%	3.9%
3	25	24.5 ± 0.6	98%	2.3%

[a]The average value for three measurement results ± standard deviation.

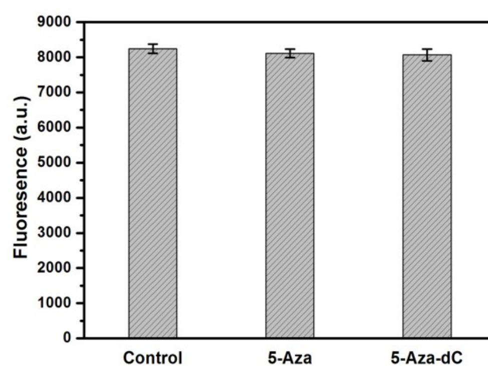

Fig. S7 Effects of anticancer drugs on other involved enzymes activity. The concentration of each drug was 5.0 µM.

3. References

1. Y. Long, X. M. Zhou and D. Xing, *Biosens. Bioelectron.*, 2013, **46**, 102.

2. Y. T. Zhou, Q. Huang, J. M. Gao, J. X. Lu, X. Z. Shen and C. H. Fan, *Nucleic Acids Res*., 2010, **15**, e156.

3. S. Bi, T. T. Zhao, B. Y. Luo and J. J. Zhu, *Chem. Commun*., 2013, **49**, 6906.

4. Y. P. Zeng, J. Hu, Y. Long and C. Y. Zhang, *Anal. Chem.*, 2013, **85**, 6143.

5. P. Liu, X. H. Yang, Q. Wang, J. Huang, J. B. Liu, Y. Zhu, L. L. He and K. M. Wang, *Chinese Chem. Lett.,* 2014, **25**, 1047.