ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2021, **57**, 2073

Received 22nd December 2020,
Accepted 21st January 2021

DOI: 10.1039/d0cc08306b

rsc.li/chemcomm

Integration of single-molecule detection with endonuclease IV-assisted signal amplification for sensitive DNA methylation assay†

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We demonstrate the development of a new fluorescent biosensor for sensitive DNA methylation assay by integrating single-molecule detection with endo IV-assisted signal amplification. This biosensor possesses the characteristics of good selectivity and high sensitivity with a detection limit of 7.3 × 10^{-17} M. It can distinguish as low as 0.01% methylation level, and can analyze genomic DNA methylation even in a single cancer cell.

DNA methylation is one of the most common epigenetic modifications in mammal genomes, and it occurs mainly at the fifth carbon of cytosine residues in cytosine/guanine (CpG) pairs.[1] Through the covalent addition of methyl group to cytosine, DNA methylation can influence the heritable state of gene expression without any change in the DNA sequence.[2] The normal DNA methylation patterns play critical roles in various physiological processes including embryonic development,[3] genomic imprinting,[4] differentiation,[5] cellular memory,[6] and aging.[7] Nevertheless, aberrant DNA methylation may lead to the dysregulation of gene expression and consequently cause various human diseases[8] and cancers including lung, liver, kidney, breast, and cervical cancers.[9] Therefore, accurate and sensitive detection of DNA methylation is of great importance to epigenetic studies and clinical diagnosis.

Though conventional methods such as liquid chromatography,[10] capillary electrophoresis,[11] mass spectrometry,[11] and gel electrophoresis[12] enable direct detection of DNA methylation, they usually suffer from sophisticated instruments, poor specificity, and laborious and time-consuming procedures. Due to their low sensitivity, these methods require large amounts of DNA samples for DNA methylation analysis. Considering the fact that the methylated cytosine accounts only for approximately 1% of total DNA bases,[13] new methods with improved sensitivity are highly required. The polymerase chain reaction (PCR) is now the most popular method for amplified and sensitive detection of DNA methylation,[14] but the requirement of precise control of reaction temperature cycling limits its wide applications. To avoid thermocycling, a series of isothermal nucleic acid amplification strategies have been adapted for DNA methylation assay such as strand displacement amplification,[15] rolling circle amplification,[16] loop-mediated isothermal amplification,[17] ligation chain reaction,[18] and hybridization chain reaction.[19] Despite their enhanced sensitivity, these amplification methods have some obvious drawbacks such as the complicated reaction scheme, diverse enzymes/probes, inherent nonspecific amplification, and relatively high background signal. Therefore, the development of simple, selective, and sensitive methods for DNA methylation assay is still a great challenge.

Herein, we develop a new fluorescent biosensor for DNA methylation assay based on the integration of endonuclease IV (endo IV)-assisted signal amplification with single-molecule detection. Superior to the conventional ensemble fluorescence measurement, single-molecule detection has distinct characteristics of low sample consumption and high signal-to-noise ratio,[20] enabling simple and sensitive detection of DNAs,[21] microRNAs,[22] proteins,[23] enzymes,[24] and even living cells.[25] The proposed biosensor can sensitively detect DNA methylation with a detection limit of 7.3 × 10^{-17} M and distinguish as low as 0.01% methylation level, and it can analyze genomic DNA methylation even in a single cancer cell.

As a proof of concept, we chose the 18 bp DNA fragments derived from the promoter region of the cyclin-dependent kinase inhibitor 2A gene (*p16*) as a model DNA target. The aberrant *p16* promoter methylation is closely associated with

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0cc08306b

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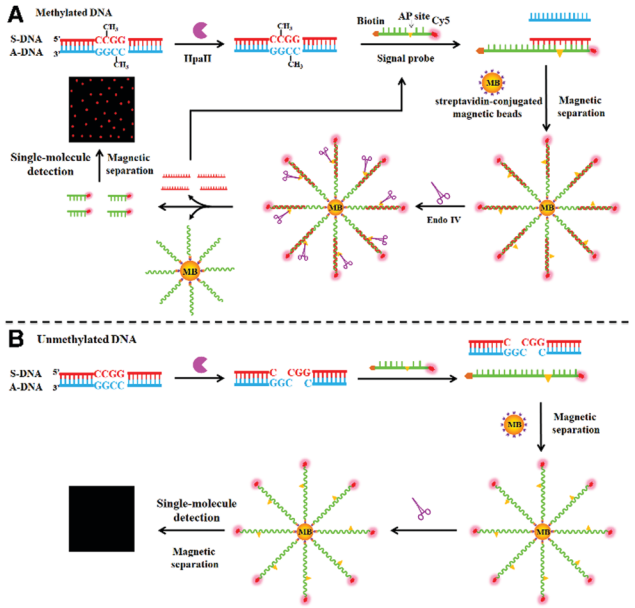

Scheme 1 Construction of a fluorescent biosensor for DNA methylation assay based on the integration of single-molecule detection with endo IV-assisted signal amplification.

various human cancers such as lung,[26] liver,[27] and colorectal cancers.[28] The proposed biosensor involves four essential elements including endonuclease HpaII, a signal probe, endo IV, and a magnetic bead. The HpaII is a methylation-sensitive restriction endonuclease and it can cleave the unmethylated DNA containing symmetrical HpaII recognition sequence 5′-CCGG-3′, but it cannot cleave the methylated DNA 5′-C^{m}CGG-3′.[29] The single-stranded signal probe contains an apurinic/apyrimidinic (AP) site in the middle, and is labeled with biotin at the 5′ end and Cy5 at the 3′ end, respectively. Endo IV can catalyze the cleavage of AP site within double-stranded DNA (dsDNA), but it exhibits no activity toward single-stranded DNA (ssDNA).[30] The surface of a magnetic bead is modified with streptavidin. In the presence of methylated DNA target (Scheme 1A), both the sense (S) and antisense (A) DNA strands are methylated, leading to their resistance to HpaII cleavage due to the CpG methylation of HpaII recognition site 5′-C^{m}CGG-3′. The S-DNA strand of the remaining methylated DNA can subsequently anneal with a signal probe to form the S-DNA/signal probe duplexes, which can be captured by streptavidin-conjugated magnetic beads through streptavidin–biotin interaction. After the removal of the unbound signal probes through magnetic separation, endo IV is added to cleave the AP site in the S-DNA/signal probe duplex, resulting in the release of the Cy5-labeled cleaved signal probe and S-DNA. The free S-DNA can hybridize with the new signal probe to form a new S-DNA/signal probe duplex, initiating the next round of endo IV-induced cleavage of signal probes and releasing numerous Cy5 fluorophores, which can be simply quantified *via* total internal reflection fluorescence (TIRF)-based single-molecule imaging. In contrast, the unmethylated DNAs will be completely digested by HpaII (Scheme 1B). Because endo IV has no activity toward the AP site in ssDNA,[30] no signal probe can be cleaved due to the lack of complementary S-DNA, and consequently no Cy5 signal is detected.

We employed 20% non-denaturating polyacrylamide gel electrophoresis (PAGE) to monitor the cleavage of signal probes by direct excitation of Cy5 (Fig. 1A). In the presence of unmethylated DNA, a distinct band of intact signal probe is observed (Fig. 1A, lane 2), which is confirmed by the band of signal probe (Fig. 1A, lane 3), indicating that the signal probe remains intact and uncleaved when unmethylated DNA is present. In contrast, in the presence of methylated DNA, the band of the signal probe disappears, and a band of cleaved signal probe with faster migration is observed (Fig. 1A, lane 1), indicating the cleavage of signal probes. Moreover, the presence of methylated DNA induces a significantly enhanced fluorescence signal with a characteristic emission peak of 665 nm (Fig. 1B, red line), while no distinct fluorescence signal is detected in the presence of unmethylated DNA (Fig. 1B, black line). These results suggest that the DNA methylation can induce the cleavage of signal probes and the subsequent recovery of Cy5 fluorescence.

Prior to single-molecule imaging, the reaction products are diluted 10 000-fold, followed by spreading on a glass coverslip. The Cy5 signal is excited by a 640 nm laser and filtered by a Cy5-emission filter. As shown in Fig. 1C, distinct Cy5 spots are observed in the presence of methylated DNA, but no Cy5 spot is detected when unmethylated DNA is present. The Cy5 counts in response to methylated DNA are 25.6-fold higher than those in response to unmethylated DNA (Fig. 1D). These results demonstrate that the single-molecule detection of Cy5 signals can be used for DNA methylation assay.

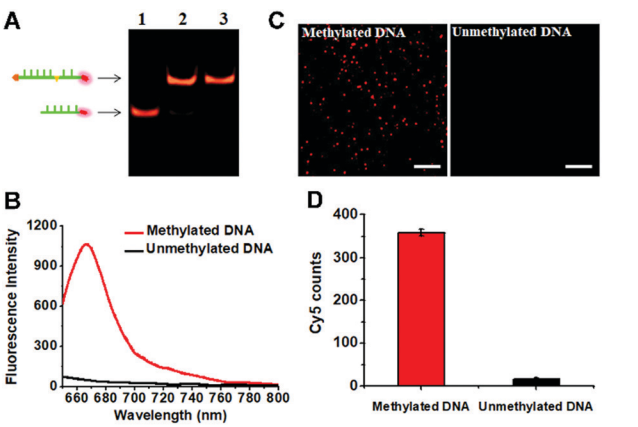

Fig. 1 (A) Non-denaturating PAGE analysis of signal probe cleavage in the presence of methylated DNA (lane 1), unmethylated DNA (lane 2), and signal probe (lane 3), respectively. (B) Measurement of Cy5 fluorescence emission spectra in the presence of methylated DNA (red line) and unmethylated DNA (black line), respectively. (C) Single-molecule imaging of Cy5 in the presence of methylated DNA and unmethylated DNA, respectively. The scale bar is 5 μm. (D) Measurement of Cy5 counts in response to methylated DNA (red column) and unmethylated DNA (black column), respectively. The 100 nM methylated DNA and 100 nM unmethylated DNA were used in the experiments. Error bars show the standard deviation of three experiments.

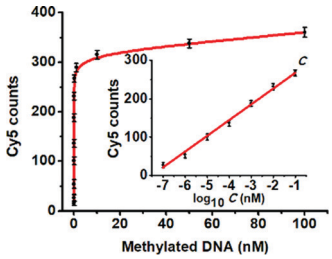

Fig. 2 (A) Variance of the Cy5 counts with different-concentration methylated DNA. Inset shows the linear relationship between Cy5 counts and the logarithm of methylated DNA concentration in the range of 100 aM – 100 pM. Error bars show the standard deviation of three experiments.

Under the optimal experimental conditions (see ESI,† Fig. S1, *i.e.*, 300 nM signal probe, 8 U of HpaII, HpaII reaction time of 80 min, 5 U of endo IV, and endo IV reaction time of 60 min), we investigated the detection sensitivity by measuring Cy5 counts in response to different concentrations of methylated DNA. As shown in Fig. 2, the Cy5 counts enhance with the increasing concentration of methylated DNA from 0 to 100 nM. In the logarithmic scale, the Cy5 counts exhibit a good linear correlation with the concentration of methylated DNA over a large range of 6 orders. The regression equation is $N = 308.74 + 41.14 \log_{10} C$ with a correlation coefficient (R^2) of 0.9953, where N represents the Cy5 counts and C represents the concentration of methylated DNA. The limit of detection (LOD), the limit of quantitation (LOQ, defined as the lowest concentration measured in the calibration) and the linear range are determined to be 73 aM, 100 aM, and 100 aM – 100 pM, respectively. The sensitivity of the proposed biosensor is superior to reported fluorescence methods for DNA methylation assay, with 4 orders of magnitude higher than that of a two-stage isothermal amplification method (0.78 pM),[15] 10.9-fold higher than that of a hyperbranched rolling circle amplification method (0.8 fM),[16] and 4.2-fold higher than that of Fe@Au nanoparticle-based nanosensors (310 aM).[31] The improved sensitivity can be ascribed to (1) the high amplification efficiency of the endo IV-induced cyclic cleavage reaction,[30] (2) the low background resulting from magnetic separation, and (3) inherent high sensitivity of single-molecule detection.[20]

To evaluate the selectivity of the proposed method, two random DNA fragments including randoms 1 and 2 (see ESI,† Table S1) are used as the interferences. As shown in Fig. 3A, a significantly enhanced Cy5 signal is detected in response to methylated DNA (Fig. 3A, red column), while no obvious Cy5 signal is observed when random 1 (Fig. 3A, blue column) and random 2 (Fig. 3A, cyan column) DNA fragments are present, with a similar signal response to unmethylated DNA (Fig. 3A, green column). These results demonstrate good selectivity of the proposed biosensor toward methylated DNA. We further investigate the specificity of the proposed biosensor by measuring the methylated DNA in a mixture containing both unmethylated and methylated DNAs. The measured methylation level is calculated on the basis of eqn (1):

$$\text{Methylation level (\%)} = M/(M + U) \times 100\% \qquad (1)$$

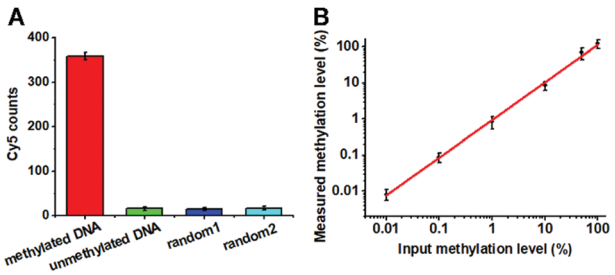

Fig. 3 (A) Measurement of Cy5 counts in response to methylated DNA (red column), unmethylated DNA (green column), random 1 DNA (blue column), and random 2 DNA (cyan column). The concentration of each tested DNA is 100 nM. (B) Correlation between the measured and input methylation levels. The total concentration of methylated and unmethylated DNA is 100 pM. Error bars show the standard deviation of three experiments.

where M is the content of methylated DNA measured and U is the content of unmethylated DNA. As shown in Fig. 3B, a good linear relationship is obtained between the measured methylation level and the input methylation level in the range of 0.01% – 100%. The regression equation is $Y = 1.04\ X - 0.02$ with $R^2 = 0.9984$, where Y is the measured methylation level (%) and X is the input methylation level (%). This result suggests that the proposed biosensor can be applied for accurate detection of DNA methylation in the complicated mixture.

To verify the feasibility of the proposed biosensor for real sample analysis, we measured the *p16* DNA methylation extracted from genomic DNA of both cancer cells and normal cells including the human hepatoma cell line (Hep G2 cells), human breast cancer cell line (MDA-MB231 cells), human breast cancer cell line (MCF-7 cells), human cervical carcinoma cell line (HeLa cells), human lung adenocarcinoma cell line (A549 cells), and normal human epithelial mammary cell line (MCF-10A cells). As shown in Fig. 4A, a low Cy5 signal is observed in normal MCF-10A cells, while a significantly enhanced Cy5 signal is produced in cancer cell lines including Hep G2, MDA-MB231, MCF-7, HeLa and A549 cells due to the increased DNA methylation level in the promotion region of the *p16* gene in cancer cells.[32] Moreover, the Cy5 counts enhance

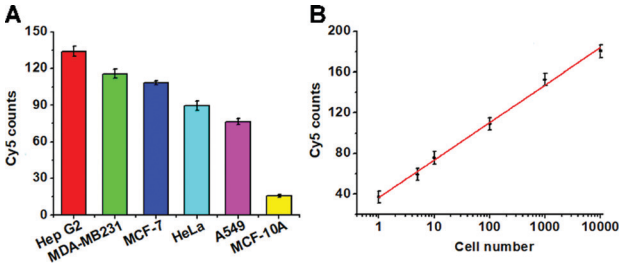

Fig. 4 (A) Measurement of Cy5 counts in response to genomic DNA extracted from Hep G2 (red column), MDA-MB231 (green column), MCF-7 (blue column), HeLa (cyan column), A549 (purple column), and MCF-10A (yellow column) cells, respectively. 500 cells are used for genomic DNA extraction for each cell line. (B) Linear relationship between Cy5 counts and the logarithm of Hep G2 cell number. Error bars represent the standard deviation of three experiments.

with the increasing number of Hep G2 cells in the range from 1 to 10 000 cells (Fig. 4B). The regression equation is $N = 36.46 + 36.94 \log_{10} X$ ($R^2 = 0.9954$), where N represents the Cy5 counts and X represents the number of Hep G2 cells. These results indicate that the proposed biosensor can be used for accurate and sensitive quantification of genomic DNA methylation in human cells with single-cell sensitivity.

In summary, we have developed a new fluorescent biosensor for selective and sensitive detection of DNA methylation at the single-molecule level. The methylation state in target DNA can be distinguished by methylation-sensitive HpaII, and the methylated DNA can subsequently trigger endo IV-assisted cyclical cleavage of signal probes, greatly amplifying the signal of methylated DNA, which can be easily visualized and quantified by single-molecule detection. This biosensor has some distinct advantages: (1) in comparison with other nucleic acid amplification-based DNA methylation assays,[15–19] the proposed biosensor exploits endo IV for signal amplification, efficiently avoiding the complicated thermocycling and the non-specific amplification. (2) The introduction of magnetic separation strategy can minimize the interference from complex real samples, greatly reducing the background signal. (3) The single-molecule counting enables simple and sensitive detection of DNA methylation with low sample consumption and high signal-to-noise ratio. (4) Due to the high efficiency of endo IV-assisted signal amplification, low background resulting from magnetic separation, and high sensitivity of single-molecule detection, the proposed biosensor can achieve a detection limit of as low as 7.3×10^{-17} M and it can distinguish as low as 0.01% methylation level, superior to the reported methods for DNA methylation assay.[15,16,31] Moreover, this biosensor can be applied for accurate detection of DNA methylation even in a single cell, holding great promise for DNA methylation-related epigenetic research and clinical diagnosis.

This work was supported by the National Natural Science Foundation of China (Grant No. 21735003, 21527811 and 21705096) and the Award for Team Leader Program of Taishan Scholars of Shandong Province, China.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 (*a*) C. Luo, P. Hajkova and J. R. Ecker, *Science*, 2018, **361**, 1336–1340; (*b*) X. S. Liu, H. Wu, X. Ji, Y. Stelzer, X. Wu, S. Czauderna, J. Shu, D. Dadon, R. A. Young and R. Jaenisch, *Cell*, 2016, **167**(233-247), e217.
2 (*a*) P. A. Jones and D. Takai, *Science*, 2001, **293**, 1068–1070; (*b*) F. Ma, Q. Zhang and C.-Y. Zhang, *J. Mater. Chem. B*, 2020, **8**, 3488–3501.
3 H. Guo, P. Zhu, L. Yan, R. Li, B. Hu, Y. Lian, J. Yan, X. Ren, S. Lin, J. Li, X. Jin, X. Shi, P. Liu, X. Wang, W. Wang, Y. Wei, X. Li, F. Guo, X. Wu, X. Fan, J. Yong, L. Wen, S. X. Xie, F. Tang and J. Qiao, *Nature*, 2014, **511**, 606–610.
4 E. Li, C. Beard and R. Jaenisch, *Nature*, 1993, **366**, 362–365.
5 F. Izzo, S. C. Lee, A. Poran, R. Chaligne, F. Gaiti, B. Gross, R. R. Murali, S. D. Deochand, C. Ang, P. W. Jones, A. S. Nam, K.-T. Kim, S. Kothen-Hill, R. C. Schulman, M. Ki, P. Lhoumaud, J. A. Skok, A. D. Viny, R. L. Levine, E. Kenigsberg, O. Abdel-Wahab and D. A. Landau, *Nat. Genet.*, 2020, **52**, 378–387.
6 M. Kim and J. Costello, *Exp. Mol. Med.*, 2017, **49**, e322.
7 A. E. Field, N. A. Robertson, T. Wang, A. Havas, T. Ideker and P. D. Adams, *Mol. Cell*, 2018, **71**, 882–895.
8 (*a*) C. Davegårdh, S. García-Calzón, K. Bacos and C. Ling, *Mol. Metab.*, 2018, **14**, 12–25; (*b*) K. D. Robertson, *Nat. Rev. Genet.*, 2005, **6**, 597–610.
9 (*a*) P. W. Laird, *Nat. Rev. Cancer*, 2003, **3**, 253; (*b*) M. Esteller, P. G. Corn, S. B. Baylin and J. G. Herman, *Cancer Res.*, 2001, **61**, 3225–3229; (*c*) D. K. Vanaja, M. Ehrich, D. Van den Boom, J. C. Cheville, R. J. Karnes, D. J. Tindall, C. R. Cantor and C. Y. Young, *Cancer Invest.*, 2009, **27**, 549–560; (*d*) C. Boltze, S. Zack, C. Quednow, S. Bettge, A. Roessner and R. Schneider-Stock, *Pathol., Res. Pract.*, 2003, **199**, 399–404; (*e*) K. Miyamoto, T. Fukutomi, S. Akashi-Tanaka, T. Hasegawa, T. Asahara, T. Sugimura and T. Ushijima, *Int. J. Cancer*, 2005, **116**, 407–414; (*f*) P. M. Das and R. Singal, *J. Clin. Oncol.*, 2004, **22**, 4632–4642.
10 K. C. Kuo, R. A. McCune, C. W. Gehrke, R. Midgett and M. Ehrlich, *Nucleic Acids Res.*, 1980, **8**, 4763–4776.
11 D. Huang, Q. Yang, S. Jin, Q. Deng and P. Zhou, *Anal. Bioanal. Chem.*, 2014, **406**, 2771–2777.
12 C. A. Eads, K. D. Danenberg, K. Kawakami, L. B. Saltz, C. Blake, D. Shibata, P. V. Danenberg and P. W. Laird, *Nucleic Acids Res.*, 2000, **28**, e32.
13 Q. Zhang, Y. Wu, Q. Xu, F. Ma and C.-Y. Zhang, *Biosens. Bioelectron.*, 2021, **171**, 112712.
14 (*a*) J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin and S. B. Baylin, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 9821–9826; (*b*) M. Li, L. Zhang, G. Chen, J. Zhou, Y. Yuan, J. Zou, M. Yuan, R. Chen, F. Du, X. Cui, X. Huang, J. Dong and Z. Tang, *Chem. Commun.*, 2018, **54**, 1710–1713.
15 G. Zhu, K. Yang and C.-Y. Zhang, *Biosens. Bioelectron.*, 2013, **49**, 170–175.
16 A. P. Cao and C.-Y. Zhang, *Anal. Chem.*, 2012, **84**, 6199–6205.
17 F. Zerilli, C. Bonanno, E. Shehi, G. Amicarelli, D. Adlerstein and G. M. Makrigiorgos, *Clin. Chem.*, 2010, **56**, 1287–1296.
18 (*a*) Z.-y. Wang, L.-J. Wang, Q. Zhang, B. Tang and C.-Y. Zhang, *Chem. Sci.*, 2018, **9**, 1330–1338; (*b*) F. Su, L. Wang, Y. Sun, C. Liu, X. Duan and Z. Li, *Chem. Commun.*, 2015, **51**, 3371–3374.
19 S. Bi, T. Zhao, B. Luo and J.-J. Zhu, *Chem. Commun.*, 2013, **49**, 6906–6908.
20 F. Ma, Y. Li, B. Tang and C. Y. Zhang, *Acc. Chem. Res.*, 2016, **49**, 1722–1730.
21 C. Y. Zhang and J. Hu, *Anal. Chem.*, 2010, **82**, 1921–1927.
22 F. Ma, Q. Zhang and C.-Y. Zhang, *Nano Lett.*, 2019, **19**, 6370–6376.
23 D. M. Rissin, C. W. Kan, T. G. Campbell, S. C. Howes, D. R. Fournier, L. Song, T. Piech, P. P. Patel, L. Chang, A. J. Rivnak, E. P. Ferrell, J. D. Randall, G. K. Provuncher, D. R. Walt and D. C. Duffy, *Nat. Biotechnol.*, 2010, **28**, 595–599.
24 L. J. Wang, F. Ma, B. Tang and C. Y. Zhang, *Anal. Chem.*, 2016, **88**, 7523–7529.
25 F. Ma, S.-H. Wei and C.-Y. Zhang, *Anal. Chem.*, 2019, **91**, 7505–7509.
26 S. Jarmalaite, A. Kannio, S. Anttila, J. R. Lazutka and K. Husgafvel-Pursiainen, *Int. J. Cancer*, 2003, **106**, 913–918.
27 I. H. N. Wong, Y. M. Dennis Lo, J. Zhang, C.-T. Liew, M. H. L. Ng, N. Wong, P. B. S. Lai, W. Y. Lau, N. M. Hjelm and P. J. Johnson, *Cancer Res.*, 1999, **59**, 71–73.
28 H.-Z. Zou, B.-M. Yu, Z.-W. Wang, J.-Y. Sun, H. Cang, F. Gao, D. H. Li, R. Zhao, G.-G. Feng and J. Yi, *Clin. Cancer Res.*, 2002, **8**, 188–191.
29 A. Quint and H. Cedar, *Nucleic Acids Res.*, 1981, **9**, 633–646.
30 X. Xiao, C. Song, C. Zhang, X. Su and M. Zhao, *Chem. Commun.*, 2012, **48**, 1964–1966.
31 M. Dadmehr, M. Hosseini, S. Hosseinkhani, M. R. Ganjali, M. Khoobi, H. Behzadi, M. Hamedani and R. Sheikhnejad, *Biosens. Bioelectron.*, 2014, **60**, 35–44.
32 J. G. Herman, A. Merlo, L. Mao, R. G. Lapidus, J. P. Issa, N. E. Davidson, D. Sidransky and S. B. Baylin, *Cancer Res.*, 1995, **55**, 4525–4530.

