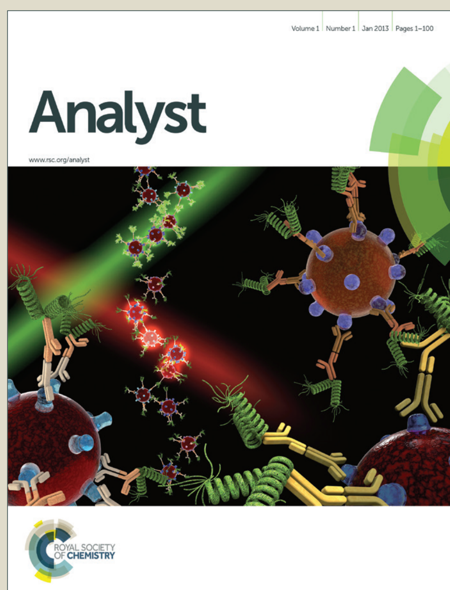
# Analyst

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

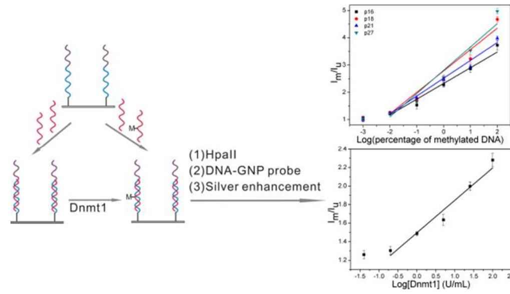
Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

# Table of Contents




A microarray-based resonance light scattering assay has been proposed for sensitively detecting DNA methylation and DNA methyltransferase.

Analyst Accepted Manuscript

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

# Microarray-based resonance light scattering assay for detecting DNA methylation and human DNA methyltransferase simultaneously with high sensitivity

Lan Ma,[a,b] Min Su,[a,b] Tao Li,[a] Zhenxin Wang*[a]

*Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX*
**DOI: 10.1039/b000000x**

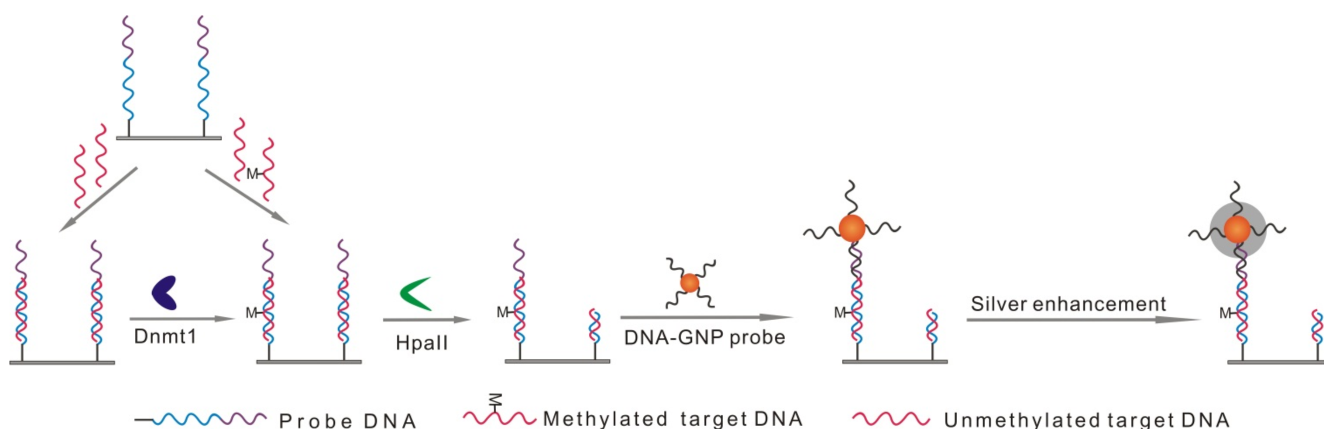
**A microarray-based resonance light scattering assay, with the combination of methylation-sensitive endonuclease and gold nanoparticle (GNP) probe, has been proposed to sensitively distinguish DNA methylation level as low as 0.01% (10 pM methylated DNA in 100 nM total DNA) and detect human DNA methyltransferase 1 (Dnmt1) down to 0.1 U/mL.**

DNA methylation involves in many biological processes, such as gene regulation, imprinting, embryonic development and X chromosome inactivation.[1] This heritable epigenetic modification mainly occurs on cytosine residues of the CpG dinucleotides in mammal genes.[2] A variety of studies have demonstrated that the abnormal methylations of genes are related to many severe diseases including cancer and immunodeficiency.[3] The hypermethylation of CpG islands in the promoter region of tumour suppressor genes and the global hypomethylation of the cancer genes are the main DNA methylation signatures in tumorigenesis.[4] Because DNA methylation is catalyzed by DNA methyltransferase (MTase) in the presence of S-adenosylmethionine (SAM), the disordered expression and activity of DNA MTase may result in the abnormal methylation status in many cases.[5] Therefore, the development of sensitive and accurate method for detection of both DNA methylation and DNA MTase can facilitate the study of cancer pathology, early diagnose of cancer and screening of anticancer drugs.

So far, many methods/techniques have been used to separately detect DNA methylation or DNA MTase.[6] For instance, bisulphite sequencing, and methylation-specific PCR (MSP) have been widely employed to analyze DNA methylation in artificial and practical samples.[6b, 6c] Fluorescence resonance energy transfer (FRET)-based method, GNP-based colorimetric method and electrochemistry-based assay have been used to detect bacterial DNA MTase and human DNA MTase (DNMT).[6g-6j, 6l] In particular, DNA microarray-based fluorescent assays have been widely used in the evaluations of methylation status and patterns of genome since DNA microarray allows for the massively parallel detections of a large number of different sequences on a small scale and in an automated fashion.[7] However, few of methods/assays have been developed for detecting DNA methylation and DNA MTase simultaneously.[8]

GNPs have been extensively used as probes for fabricating simple bioassays with high sensitivity because GNPs have unique physical and chemical properties which are strongly dependent on the size, shape and aggregation degree of GNPs.[9] In particular, electroless deposition of silver onto GNPs has been employed for the sensitive detections of biological recognition processes on microarrays.[10]

Herein, we proposed a microarray-based RLS assay which combines with methylation-sensitive endonucleases (HpaII) and DNA-modified gold nanoparticle (DNA-GNP) probes to detect




**Scheme 1** Schematic representation of the DNA microarray-based RLS assay for the analysis of DNA methylation level in target DNA and detection of Dnmt1. The illustration is not drawn to scale.

Analyst Accepted Manuscript

DNA methylation and Dnmt1 activity simultaneously. The schematic diagram of the DNA microarray-based assay is shown in Scheme 1. Briefly, the single strand alkylamine-modified oligonucleotides (probe DNAs) consisting of three fragments (spacer part, complementary part and labelling part) are immobilized on commercial aldehyde-functionalized glass slide through Schiff base linkages. The immobilized probe DNAs are then hybridized with target DNAs (i.e., complementary single-stranded DNAs of probe DNAs or the mixture of complementary single-stranded DNAs of probe DNAs and their methylated variants). For analyzing DNA methylation level in the target DNAs, the newly formed dsDNAs are directly incubated with HpaII. The unmethylated dsDNAs are digested by HpaII while hemimethylated dsDNAs remain on the slide surface since methylated cytosine within the recognition motif could efficiently protect both strands from HpaII digestion.[11] For detecting Dnmt1 activity in solution, the unmethylated dsDNAs are firstly incubated with Dnmt1, then digested by HpaII. After HpaII digestion, DNA microarray is labelled with 13 nm DNA-GNP probes because 13 nm DNA-GNPs have relatively high colloidal stability in probe reaction buffer. Finally, a silver enhancement step is applied to the microarrays for signal amplification since the RLS properties of GNPs by themselves are relatively poor, if the particles are smaller than ca. 40 nm.[12] Using this detecting principle, both DNA methylation level and DNA methyltransferase amount can be detected simultaneously on one microarray slide by RLS signal differences of different subarrays which contain unmethylated dsDNAs or mixed dsDNAs (unmethylated dsDNAs plus hemimethylated dsDNAs), respectively.

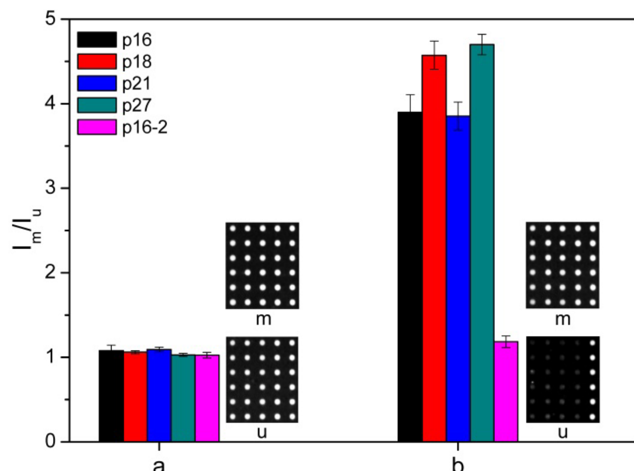



**Fig. 1** The relative RLS intensities ($I_m/I_u$) of five substrates after treatment with 500 U/mL EcoRI (a) and 250 U/mL HpaII (b), respectively. The insets are RLS images of hemimethylated substrates (m) and unmethylated substrates (u), The columns (from left to right) in the RLS images contain p16, p18, p21, p27 and p16-2, respectively.

Four oligonucleotides (see table S1 in ESI† for details of the DNA sequences) containing the fragment of 5'-CCGG-3' derived from promoter CpG islands of p16, p18, p21 and p27 genes are chosen as model systems for evaluating the ability of the assay in analyzing DNA methylation level. P16, p18, p21 and p27 are members of the cyclin-dependent kinase inhibitors (CKIs) which play important roles in regulating the cell cycle.[13] Increasing evidences demonstrate that many diseases have relationship with the abnormal expression of CKIs.[14] The relative RLS intensity ($I_m/I_u$) is used to quantitatively analyze methylation level of target DNAs. The $I_u$ is the RLS intensity generated by 100% unmethylated DNA sample, and $I_m$ is the RLS intensity generated by sample containing methylated DNA. Under the same experimental conditions, high methylation level of target DNA leads to generate high value of $I_m/I_u$. On the basis of our previously reported optimum conditions for detecting restriction endonuclease activity,[15] the concentrations of HpaII and target DNAs are optimized to enhance assay sensitivity. The highest $I_m/I_u$ is obtained under 250 U/mL HpaII in the enzymatic digestion solution and 100 nM target DNA in DNA hybridization solution (as shown in Fig. S1 and S2 in ESI†).

Oligonucleotide p16-2 (nonspecific substrate of HpaII) and EcoRI (nonspecific restriction endonuclease of 5'-CCGG-3' motif) have been chosen to evaluate the selectivity of the assay under optimum conditions. After HpaII digestion, the $I_m/I_u$ of the specific substrates (p16, p18, p21, and p27) are increased more than 3.5 times while the $I_m/I_u$ of p16-2 is not changed (as shown in Fig. 1). In addition, the $I_m/I_u$ of all substrates remain unchanged after treatment with 500 U/mL EcoRI. These results demonstrate that the $I_m/I_u$ difference between methylated DNA and unmethylated DNA is the consequence of HpaII digestion and can be used to discriminate methylated and unmethylated DNAs.

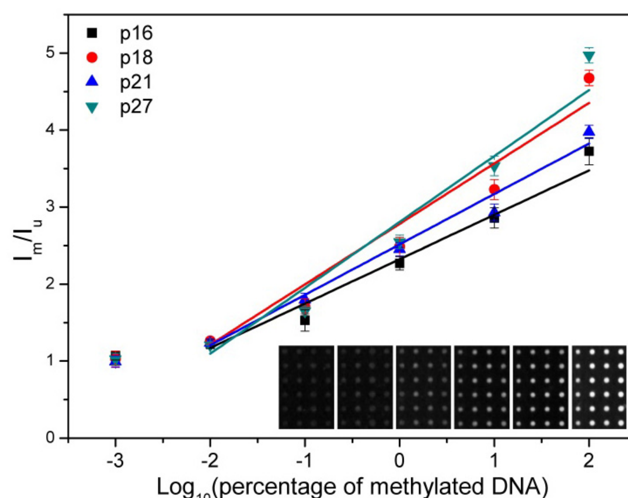



**Fig. 2** The relative RLS intensities ($I_m/I_u$) as a function of the logarithm of percentage of methylated DNAs in DNA mixture. The columns (from left to right) in the RLS images (insets) contain p16, p18, p21 and p27, respectively. The sample containing 100% unmethylated target DNA is used as a control sample.

To investigate the ability of proposed assay in multiple methylated DNAs detection, target DNAs (100 nM, 25 μL) containing various percentages of methylated DNAs were hybridized with the probe DNAs on microarray. As shown in Fig. 2, the $I_m/I_u$ values are increased linearly as a function of the logarithm of percentages of methylated DNAs from 0.01% to 100%, indicating a dynamic range of 4 orders of magnitude. The detection limit (the $I_m/I_u$ reaches $I_m/I_u$ of control experiment plus 3 times of its standard deviation) is calculated to be 0.01% methylated DNA in target DNA, i.e. 10 pM methylated DNA in 100 nM target DNA. The sensitivity of our assay is much better

Analyst Accepted Manuscript

 This journal is © The Royal Society of Chemistry [year]

or comparable to those of previously reported methods (e.g., cationic conjugated polyelectrolyte-based approach, ligation-mediated HRCA method and MALDI mass spectrometry method).[6a, 6d, 6e] Although only 4 methylated DNAs have been detected in the proof of principle experiment, the assay can be employed to simultaneously analyze DNA methylation in large scale because of the inherent nature of microarray technique.

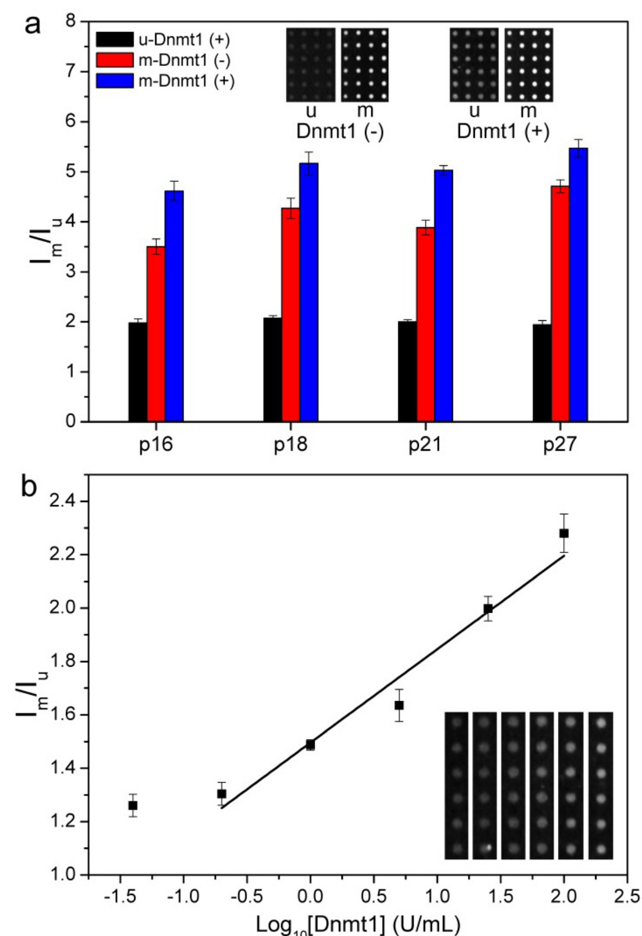



**Fig. 3** (a) The relative RLS intensities ($I_m/I_u$) of unmethylated (u) and hemimethylated (m) substrates in the presence or absence of 25 U/mL Dnmt1. The columns (from left to right) in the RLS images (insets) contain p16, p18, p21 and p27, respectively. (b) Relative RLS intensity ($I_m/I_u$) as a function of the logarithm of Dnmt1 concentration. The insets are the corresponding RLS images of unmethylated p21 after treatment by Dnmt1.

Dnmt1 detecting experiments were designed to further demonstrate the extended utility of our assay. Dnmt1 is a maintenance MTase and responsible for copying the existing methylation DNA pattern in vivo.[16] Previous studies demonstrate that both hemimethylated and unmethylated CpG sites can be methylated by Dnmt1.[17] In the presence of Dnmt1, $I_m/I_u$ values of both hemimethylated substrate and unmethylated substrate are increased (as shown in the Fig. 3a). Generally, the $I_m/I_u$ enhancement capability (ca. 2 times) of unmethylated substrate is stronger than that of hemimethylated substrate (ca. 1.2 times). Therefore, unmethylated p21 was arbitrarily used as typical DNA substrate to detect Dnmt1 activity. As shown in Fig. 3b, the $I_m/I_u$ values are increased linearly with the logarithm of Dnmt1 concentration in the range of 0.2 U/mL to 100 U/mL, indicating a dynamic range of nearly 3 orders of magnitude. The detection limit is estimated to be 0.1 U/mL, which is much lower than those reported methods including GNP probe-based colorimetric assay and methylation-sensitive endonuclease-based electrochemical method.[6j, 6k]

In conclusion, combining methylation-sensitive restriction endonuclease and GNP probe, a microarray-based RLS assay has been proposed for detecting DNA methylation level and DNA MTase with wide dynamic ranges and low detection limits. Moreover, the microarray-based RLS assay can be read by a white-light source scanner. It provides a low-cost high throughput alternative for early diagnose of cancer and screening of anticancer drugs (e.g., DNA methyltransferase inhibitors).

The authors would like to thank the NSFC (Grant No. 21127010) for financial supports.

## Notes and references

[a] *State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, P. R. China. Fax/Tel: (+86) 431-85262243; E-mail: wangzx@ciac.ac.cn.*
[b] *University of Chinese Academy of Sciences, No.19A Yuquan Road, Beijing, 100049, P. R. China.*
† Electronic Supplementary Information available. See DOI: 10.1039/b000000x/

1. R. Goyal, R. Reinhardt and A. Jeltsch, *Nucleic Acids Res.*, 2006, **34**, 1182-1188.
2. T. H.-M. Huang, M. R. Perry and D. E. Laux, *Hum. Mol. Genet.*, 1999, **8**, 459-470.
3. P. A. Jones and D. Takai, *Science*, 2001, **293**, 1068-1070.
4. J. Sandoval, H. Heyn, S. Moran, J. Serra-Musach, M. A. Pujana, M. Bibikova and M. Esteller, *Epigenetics*, 2011, **6**, 692-702.
5. (a) V. Shukla, X. Coumoul, T. Lahusen, R. H. Wang, X. Xu, A. Vassilopoulos, C. Xiao, M. H. Lee, Y. G. Man, M. Ouchi, T. Ouchi and C. X. Deng, *Cell Res.*, 2010, **20**, 1201-1215; (b) J. D. Roll, A. G. Rivenbark, W. D. Jones and W. B. Coleman, *Mol. Cancer*, 2008, **7**, 15; (c) K. D. Robertson, E. Uzvolgyi, G. Liang, C. Talmadge, J. Sumegi, F. A. Gonzales and P. A. Jones, *Nucleic Acids Res.*, 1999, **27**, 2291-2298.
6. (a) F. Feng, H. Wang, L. Han and S. Wang, *J. Am. Chem. Soc.*, 2008, **130**, 11338-11343; (b) J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin and S. B. Baylin, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 9821-9826; (c) K. H. Taylor, R. S. Kramer, J. W. Davis, J. Guo, D. J. Duff, D. Xu, C. W. Caldwell and H. Shi, *Cancer Res.*, 2007, **67**, 8511-8518; (d) A. Cao and C. Y. Zhang, *Anal. Chem.*, 2012, **84**, 6199-6205; (e) J. Tost, P. Schatz, M. Schuster, K. Berlin and I. G. Gut, *Nucleic Acids Res.*, 2003, **31**, e50; (f) M. Wang, Z. Xu, L. Chen, H. Yin and S. Ai, *Anal. Chem.*, 2012, **84**, 9072-9078; (g) X. J. Zheng, J. D. Qiu, L. Zhang, Z. X. Wang and R. P. Liang, *Chem. Commun.*, 2013, **49**, 3546-3548; (h) S. Bi, T. Zhao, B. Luo and J. J. Zhu, *Chem. Commun.*, 2013, **49**, 6906-6908; (i) T. Liu, J. Zhao, D. Zhang and G. Li, *Anal. Chem.*, 2010, **82**, 229-233; (j) N. B. Muren and J. K. Barton, *J. Am. Chem. Soc.*, 2013, **135**, 16632-16640; (k) Z. Wu, Z. K. Wu, H. Tang, L. J. Tang and J. H. Jiang, *Anal. Chem.*, 2013, **85**, 4376-4383; (l) X. Ouyang, J. Liu, J. Li and R. Yang, *Chem. Commun.*, 2012, **48**, 88-90.
7. (a) A. Schumacher, P. Kapranov, Z. Kaminsky, J. Flanagan, A. Assadzadeh, P. Yau, C. Virtanen, N. Winegarden, J. Cheng, T. Gingeras and A. Petronis, *Nucleic Acids Res.*, 2006, **34**, 528-542; (b) H. S. Raad S. Gitan, Chuan-Mu Chen, Pearlly S. Yan, and Tim Hui-Ming Huang, *Genome Res.*, 2002, **12**, 158-164.
8. (a) Y. Li, X. Luo, Z. Yan, J. Zheng and H. Qi, *Chem. Commun.*, 2013, **49**, 3869-3871; (b) W. Li, P. Wu, H. Zhang and C. Cai, *Anal. Chem.*, 2012, **84**, 7583-7590.

Analyst Accepted Manuscript

9. (a) Y. Li, C. Jing, L. Zhang and Y. T. Long, *Chem. Soc. Rev.*, 2012, **41**, 632-642; (b) L. Zhang, Y. Li, D. W. Li, C. Jing, X. Chen, M. Lv, Q. Huang, Y. T. Long and I. Willner, *Angew. Chem. Int. Ed.*, 2011, **50**, 6789-6792; (c) Z. Wang and L. Ma, *Coordin. Chem. Rev.*, 2009, **253**, 1607-1618.
10. (a) Z. Wang, J. Lee, A. R. Cossins and M. Brust, *Anal. Chem.*, 2005, **77**, 5770-5774; (b) X. Cao, Y. Ye and S. Liu, *Anal. Biochem.*, 2011, **417**, 1-16; (c) J.-M. Nam, C. S. Thaxton and C. A. Mirkin, *Science*, 2003, **301**, 1884-1886.
11. S. Tardy-Planechaud, J. Fujimoto, S. S. Lin and L. C. Sowers, *Nucleic Acids Res.*, 1997, **25**, 553-558.
12. L. Sun, D. Liu and Z. Wang, *Anal. Chem.*, 2007, **79**, 773-777.
13. M. F. Roussel, *Oncogene*, 1999, **18**, 5311-5317.
14. (a) J. Hayslip and A. Montero, *Mol. Cancer*, 2006, **5**, 44; (b) J. Yu, H. Zhang, J. Gu, S. Lin, J. Li, W. Lu, Y. Wang and J. Zhu, *BMC Cancer*, 2004, **4**, 65.
15. L. Ma, Z. Zhu, T. Li and Z. Wang, *Biosens. Bioelectron.*, 2014, **52**, 118-123.
16. G. Vilkaitis, I. Suetake, S. Klimasauskas and S. Tajima, *J. Biol. Chem.*, 2005, **280**, 64-72.
17. A. Hermann, R. Goyal and A. Jeltsch, *J. Biol. Chem.*, 2004, **279**, 48350-48359.

Analyst Accepted Manuscript


This journal is © The Royal Society of Chemistry [year]