RSC Advances

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. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this 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.

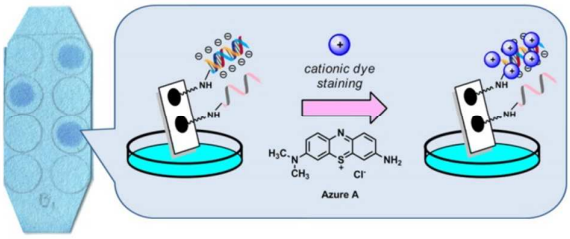
ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Pyrrolidinyl peptide nucleic acids immobilised on cellulose paper as a DNA sensor†

Nuttapon Jirakittiwut, Nattawadee Panyain, Thanesuan Nuanyai,‡ Tirayut Vilaivan and Thanit Praneenararat*

"Immobilisation of pyrrolidinyl peptide nucleic acids on paper resulted in a new DNA sensor with great specificity."



RSC Advances Accepted Manuscript

Journal Name

RSCPublishing

COMMUNICATION

Pyrrolidinyl peptide nucleic acids immobilised on cellulose paper as a DNA sensor†

Cite this: DOI: 10.1039/x0xx00000x

Nuttapon Jirakittiwut, Nattawadee Panyain, Thanesuan Nuanyai,‡ Tirayut Vilaivan and Thanit Praneenararat*

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A new label-free DNA sensor based on pyrrolidinyl peptide nucleic acid on cellulose paper was fabricated. Coupled with straightforward signal detection by cationic dye staining, this sensor has promise as a robust tool for point-of-care DNA detection.

The development of field diagnostics for the developing countries remains a challenge that has gained much interest from researchers around the world. In this regard, paper-based analytical devices are of much interest due to several superior properties of cellulose paper such as low cost and biocompatibility.[1] Pioneered by the Whitesides group in the form of paper-based microfluidic devices,[2-4] several types of paper-based sensors have since fabricated, leading to various applications such as whole cell biosensors,[5] or the sensor capable of multiple determinations of metals.[6, 7] Notably, most of these devices used cellulose merely as a platform for physical adsorption of chemical species, whereas covalent immobilisation could allow detection of species that would otherwise be impossible due to the poor retention of the involving counterparts in cellulose material. A notable example includes a study by Yu *et al.* who demonstrated that divinyl sulfone (DVS) could be used to attach various biomolecules having varying retention on cellulose and utilise them as probes.[8] This paved the way for more economical sensors that can detect various biomolecules including DNA, whose detection can lead to diverse applications such as clinical diagnosis or the detection of pathogens.[9] Note, however, that DNA as a probe for another DNA strand is subject to enzymatic degradation. Therefore, means to improve the stability, as well as specificity and selectivity, of DNA probes are still desired.

Peptide nucleic acid (PNA),[10] a synthetic DNA mimic, has been extensively studied as a probe for DNA detection due to several advantages including higher binding affinity of PNA-DNA over DNA-DNA, and much greater resistance to enzymatic degradation. This has sparked interest from researchers to utilise PNAs, mostly in the original scaffold called aminoethylglycyl PNA (aegPNA), as probes in a number of sensors with great performance in many aspects such as sensitivity, specificity and robustness.[11-14]

During the past few years, our group has developed a new class of pyrrolidinyl PNAs, a conformationally rigid PNA. Our pyrrolidinyl PNA consists of a D-prolyl-2-aminocyclopentane-carboxylic acid (acpc) backbone (Fig. 1).[15, 16] This relatively more rigid structure was shown to exhibit certain desirable properties over aegPNA, while maintaining comparable overall binding affinity and sequence specificity. These advantages include the stronger directional preference for antiparallel binding and the higher affinity towards DNA over RNA. Encouraged by the aforementioned features, we have showcased the utility of this PNA as DNA probes in a number of applications.[17-21]

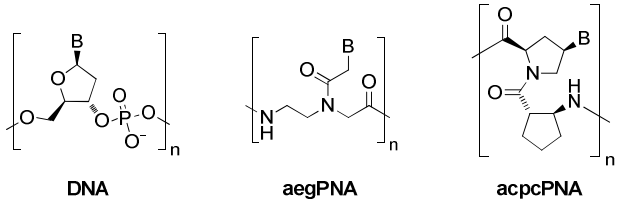


Fig. 1 Chemical structures of DNA, aegPNA, and acpcPNA. B stands for nucleobases (A, T, C, and G).

In the current study, we aimed to immobilise acpcPNA by covalently attaching the PNA onto cellulose paper, and utilising it as a probe for DNA detection. After DNA incubation by capillary method[22] and washing, we demonstrated that cationic organic dyes could be used to visualise the binding between PNA and DNA by electrostatic interaction between the dye and the bound negatively charged DNA (Fig. 2). By combining the relatively low cost of cellulose paper, and the performance of acpcPNA as a DNA probe, we envisioned that this new DNA sensor could have promise as an economical point-of-care DNA testing device.

RSC Advances Accepted Manuscript

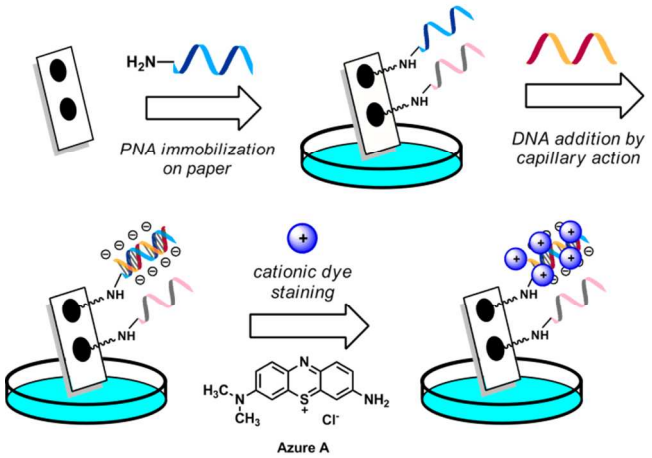
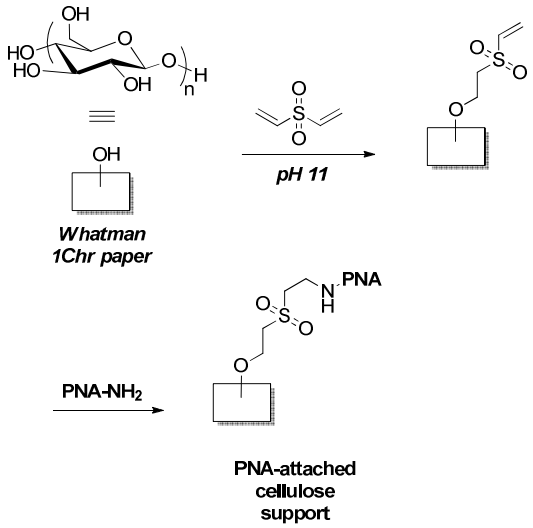


Fig. 2 The fabrication of the new DNA sensor by covalently attaching acpcPNA onto cellulose paper, followed by cationic dye staining.

The fabrication of the sensor commenced with selecting an appropriate chemistry for covalent immobilisation of acpcPNA via an appended lysine residue at the N- or C-termini. The hydroxyl group at C-6 position of the glucose monomer in cellulose is the most common site for functional group interconversion,[23] as it has been used as a "gateway" to attach various chemical scaffolds [8, 18, 22, 24-26] leading to diverse applications ranging from combinatorial synthesis (SPOT and macroarray synthesis)[27] to the immobilisation of biomolecules for sensor construction purposes.[8, 22, 26] For the ultimate goal of naked-eye detection, we found that not all commonly used chemical reactions are equally suitable, as some attachment methods produced false-positive signal from PNA alone (without the presence of DNA – data not shown). Hence, we conducted a preliminary screening and found that divinyl sulfone (DVS) is a suitable attachment method and thus we decided to immobilise acpcPNA by this chemistry (Scheme 1).[8]



Scheme 1. The immobilisation of acpcPNA by DVS-mediated conjugate addition of acpcPNA onto support.

Thereafter, some experiments to prove that the PNA was really covalently attached on the cellulose support were needed. We confirmed the success of attachment by immobilising a fluorescein-labelled PNA onto cellulose (Fig. S1 – see supplementary information). After thorough washing, it was found that the PNA with prior incubation of cellulose with DVS showed significantly higher fluorescence intensity than that without DVS incubation. This indirectly confirmed that the covalent attachment has occurred and is required for PNA retention on the paper. Also, we conducted another preliminary test by immobilising unlabelled PNA onto support, followed by incubation with fluorescently labelled DNA. We found that only the complementary DNA could be retained on support after extensive washing, confirming that the immobilised PNA is still capable of exerting Watson-Crick base pairing with the DNA (Fig. S2). An additional experiment where we varied the concentration (amount) of incubated DNA showed that the fluorescence signals, as quantified by the software ImageJ, had a logarithm relationship with the amount of bound DNA, roughly following the Langmuir isotherm (Fig. S3).

Aiming to avoid labelling of the DNA samples,[22] a third component that enables visualisation of the PNA-DNA binding event in a label-free and preferably instrument-free fashion is desired.[12, 26] Encouraged by the preliminary results, we screened for methods that allow detection of binding between unlabelled PNA and DNA. We envisaged that the difference of charges between PNA (no charge) and DNA (negatively charged at physiological pH) could provide a unique opportunity for detection – a method that would not be plausible with DNA or other DNA analogues as a probe. Indeed, this feature has been utilised before by Kim *et al.* where they used positively-charged gold nanoparticle with signal enhancement to allow naked-eye detection of aegPNA-DNA binding.[12] Unfortunately, this method did not work well in our condition, likely because of the drastic difference between the surfaces used in each study. We then focused on known, positively-charged, fluorescent dyes for DNA detection including ethidium bromide and SYBR gold, and found that none of them could give appreciable signal. Subtle molecular effects may influence this outcome and we are actively investigating this phenomenon with other cationic fluorescent dyes. Consequently, we turned our attention to optical cationic dyes that can be directly visualised by naked eyes. After some screening, thiazine dyes – a class of molecular biology staining agents including the commonly known methylene blue – was shown to be an appropriate visualising agent. Preliminary tests revealed that Azure A (structure shown in Fig. 2) showed the best staining profile among dyes tested (see details in supplementary information). Hence, we selected this dye for the subsequent studies.

As a preliminary study of the new experimental setup, we studied the effect of the length of incubated DNAs and the orientation of the binding region to better understand the nature of this detection method. Fig. S4 showed the effect of the total length of DNA on signal intensity. It should be noted that longer DNA strands are expected to provide stronger signal since they gave more accessible sites for dye binding. In our case, the shortest DNA among the three in this study did give the lowest signal intensity. On the contrary, the longest DNA strand gave approximately the same signal intensity as the one with the second longest length, likely due to the saturation of signal. We decided to choose the DNA with the second longest length (41 nucleotides) for our subsequent experiments. Furthermore, we studied the effect of the expected binding orientation on the signal intensity, with the hypothesis that the "wrong" orientation where extra bases are forced to clash with the surface should lead to unfavourable interactions with both the probe and the dye, leading to reduced signal intensity. As shown in Fig. S5, the wrong orientation (DNA "C") gave lowest signal intensity as anticipated.

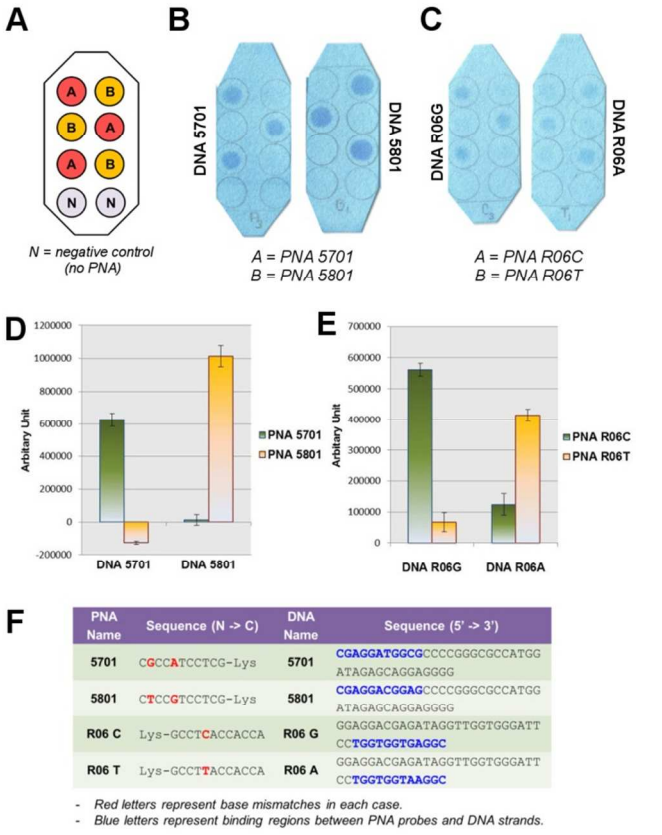
After gaining some basic understanding of the system, we proceeded to showcase the utility of this sensor by applying it to detect DNA sequences that were derived from real biological

RSC Advances Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

settings. Extra hanging sequences were appended to either the 3' or 5' end of the synthetic DNA targets to mimic the detection of real DNA samples. It should be noted that phenothiazine dyes bind electrostatically to both duplex and single-stranded DNAs,[28] therefore the extra hanging bases should contribute to the dye adsorption.[12] First, we attempted to detect the binding of DNA sequences in human leukocyte antigen (HLA) alleles. HLA alleles such as HLA-B*5701 and HLA-B*5801 used in this study were found to have strong correlation with various forms of cutaneous adverse drug reactions.[29, 30] Therefore, the ability to screen patients for such genes would greatly reduce the risk associated with the prescription of relevant drugs. Another case was about the detection of a single-gene disorder thalassaemia. Codon 26 mutation (R06 G→A) is a cause of *β*-thalassaemia and the ability to detect this single-base mutation could be very useful for prenatal diagnosis and treatment.[31] As shown in Fig. 3B, our detection system could unambiguously distinguish sequences with double mismatches (HLA-B*5701 vs 5801). Notably, since sheets of paper can be scanned by a commercial scanner, images obtained could be used for further processing by imaging software (ImageJ) to produce some quantitative data. Fig. 3D showed a representative graph illustrating that our DNA sensing device could clearly differentiate related DNA sequences, as objectively confirmed by the Student's *t*-test of the obtained numerical data. In the case of single mismatched DNAs (R06C and T), the Student's *t*-test still suggested that values from the two DNA sequences differ with statistical significance. Nevertheless, it was more obvious by naked eyes that the mismatch DNA also gave significant false-positive signal. In order to solve this issue, we found that a more stringent washing (20% acetonitrile in PBS buffer) after the DNA incubation could efficiently remove this non-specific binding, albeit with a slight compromise of signal intensity (Fig. 3C and E). In addition, we also tested the performance of the sensing system in the presence of various interferences. First, the 5701/5801 DNA sensor was tested again with the presence of two non-relevant DNA sequences (R06G and A9). As shown in Fig. S6, nearly the same results as in Fig 3B were obtained, confirming that the sensor is highly specific. Furthermore, we tested the performance of the 5801 system with the presence of nine other DNAs having varying lengths and sequences. The sensor again exhibited extremely good discrimination power by providing positive results only when the complementary sequence was present (Fig. S7). Overall, the system could be applied with closely related DNA sequences with clear differentiation power, even with the presence of multiple DNA interferences. The ability to distinguish between complementary and single mismatch targets at ambient temperature highlights the benefits of the high specificity offered by pyrrolidinyl PNA probes.

Importantly, we also proved that an amino-linked DNA (aminohexyl group attached to 3' end) with the same sequence as the HLA-B*5801 PNA probe used in this work did not provide significantly discernible signal. This is most likely due to the much lower binding affinity of the DNA probe (Fig. S8). In fact, it is common practice to use at least 20-nucleotide DNA as probes. Therefore, the cost of PNA (at the required length and amount) is not considered a significant addition to the overall cost of the sensor although the cost of PNA is about 20-25 times higher than that of DNA oligonucleotides. This further underscores the power of acpcPNA as a DNA probe. For the detection limit, we found the lowest detectable concentration of DNA to be about 200 nM or 3.3 pmol/spot (Fig. S9). This value is comparable to the expected sensitivity of the DNA binding dye (~40 ng based on calculation vs ~40-100 ng for Azure A and related dyes for gel-based detection of DNA)[32] with the advantages of high specificity and being a simple disposable system that requires no complicated instruments for performing the hybridization, detection, and signal analysis. As a comparison to our previous work,[18] the current sensor was found to exhibit greater resistance to signal interference when the sensor was incubated with a mixture of several DNA sequences (see above) with only one limitation of having lower sensitivity. This difference is attributed to the nature of the sensor chemical assembly, which aimed to provide a specific Watson-Crick interaction between PNA and DNA in the very first step, whereas the previous work[18] relied on non-specific electrostatic interaction as the binding site. In addition, an enzymatically generated colorimetric reaction was used as a means for signal amplification,[18] which should naturally lead to a lower detection limit. The results herein should therefore be considered as a promising system that can be further developed into a simple yet highly powerful DNA sensor.



F

PNA Name	Sequence (N -> C)	DNA Name	Sequence (5' -> 3')
5701	CGCCATCCTCG-Lys	5701	CGAGGATGGCGCCCCGGGCGCCATGG ATAGAGCAGGAGGGG
5801	CTCCGTCCTCG-Lys	5801	CGAGGACGGAGCCCCGGGCGCCATGG ATAGAGCAGGAGGGG
R06 C	Lys-GCCTCACCACCA	R06 G	GGAGGACGAGATAGGTTGGTGGGATT CCTGGTGGTGAGGC
R06 T	Lys-GCCTTACCACCA	R06 A	GGAGGACGAGATAGGTTGGTGGGATT CCTGGTGGTAAGGC

- *Red letters represent base mismatches in each case.*
- *Blue letters represent binding regions between PNA probes and DNA strands.*

Fig. 3 A) Layout of the paper-based DNA sensor; B) and C) The scanned images of DNA sensors for HLA-B*5701/5801, and R06 C/T, respectively. Each sheet was subject to DNA incubation of one sequence while each spot contained one PNA sequence as outlined in the figure; D) and E) Signal intensities (HLA-B*5701/5801 and R06 C/T, respectively) derived from scanned images via the ImageJ image processing software. F) The table showing all PNA and DNA sequences used in this study. All experiments were performed in triplicates of triplicates at the DNA concentration of 6 µM (100 pmol/spot).

Conclusions

We report herein the fabrication of a new DNA sensing device. This cellulose-based DNA sensor offers an economical way to detect DNA with great specificity. With low-cost fabrication and straightforward visual detection without requiring labelled samples nor strict temperature control, we envision that this new DNA sensor could become a robust tool for DNA detection in point-of-care testing. Further optimisation is being investigated in our laboratory to improve the sensitivity and

RSC Advances Accepted Manuscript

allow label-free detection of double-stranded long DNA targets – this will be reported in due course.

Acknowledgements

This work is fully supported by the Thailand Research Fund (TRF – DBG5580007 and partially from DPG5780002) and the Ratchadapiseksompot Fund (Chulalongkorn University). The authors also acknowledge the Department of Chemistry, Faculty of Science, Chulalongkorn University for instruments funding.

Notes and references

[a] Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd., Pathumwan, Bangkok, Thailand, 10330.

† Electronic Supplementary Information (ESI) available: Experimental methods and relevant preliminary studies. See DOI: 10.1039/c000000x/

‡ Present address: Faculty of Liberal Arts, Rajamangala University of Technology Rattanakosin, Wang Klai Kangwon Campus, Huanhin, Prachuap Khiri Khan, Thailand, 77110.

1 R. Pelton, *Trac-Trends Anal. Chem.*, 2009, **28**, 925-942.
2 A. W. Martinez, S. T. Phillips, M. J. Butte and G. M. Whitesides, *Angew. Chem. Int. Ed.*, 2007, **46**, 1318-1320.
3 A. W. Martinez, S. T. Phillips, G. M. Whitesides and E. Carrilho, *Anal. Chem.*, 2010, **82**, 3-10.
4 A. K. Yetisen, M. S. Akram and C. R. Lowe, *Lab Chip*, 2013, **13**, 2210-2251.
5 A. Struss, P. Pasini, C. M. Ensor, N. Raut and S. Daunert, *Anal. Chem.*, 2010, **82**, 4457-4463.
6 P. Rattanarat, W. Dungchai, D. M. Cate, W. Siangproh, J. Volckens, O. Chailapakul and C. S. Henry, *Anal. Chim. Acta*, 2013, **800**, 50-55.
7 P. Rattanarat, W. Dungchai, D. Cate, J. Volckens, O. Chailapakul and C. S. Henry, *Anal. Chem.*, 2014, **86**, 3555-3562.
8 A. Yu, J. Shang, F. Cheng, B. A. Paik, J. M. Kaplan, R. B. Andrade and D. M. Ratner, *Langmuir*, 2012, **28**, 11265-11273.
9 A. Sassolas, B. D. Leca-Bouvier and L. J. Blum, *Chem. Rev.*, 2008, **108**, 109-139.
10 P. Wittung, P. E. Nielsen, O. Buchardt, M. Egholm and B. Norden, *Nature*, 1994, **368**, 561-563.
11 O. Brandt, J. Feldner, A. Stephan, M. Schröder, M. Schnölzer, H. F. Arlinghaus, J. D. Hoheisel and A. Jacob, *Nucleic Acids Res.*, 2003, **31**, e119.
12 S. K. Kim, H. Cho, J. Jeong, J. N. Kwon, Y. Jung and B. H. Chung, *Chem. Commun.*, 2010, **46**, 3315-3317.
13 O. Brandt and J. D. Hoheisel, *Trends Biotechnol.*, 2004, **22**, 617-622.
14 N. Zhang and D. H. Appella, *J. Am. Chem. Soc.*, 2007, **129**, 8424-8425.
15 C. Suparpprom, C. Srisuwannaket, P. Sangvanich and T. Vilaivan, *Tetrahedron Lett.*, 2005, **46**, 2833-2837.
16 T. Vilaivan and C. Srisuwannaket, *Org. Lett.*, 2006, **8**, 1897-1900.
17 B. Boontha, J. Nakkuntod, N. Hirankarn, P. Chaumpluk and T. Vilaivan, *Anal. Chem.*, 2008, **80**, 8178-8186.
18 P. S. Laopa, T. Vilaivan and V. P. Hoven, *Analyst*, 2013, **138**, 269-277.
19 S. Sankoh, S. Samanman, O. Thipmanee, A. Numnuam, W. Limbut, P. Kanatharana, T. Vilaivan and P. Thavarungkul, *Sens. Actuators B - Chem.*, 2013, **177**, 543-554.
20 T. Theppaleak, M. Rutnakornpituk, U. Wichai, T. Vilaivan and B. Rutnakornpituk, *J. Nanopart. Res.*, 2013, **15**, 2106.
21 S. Jampasa, W. Wonsawat, N. Rodthongkum, W. Siangproh, P. Yanatatsaneejit, T. Vilaivan and O. Chailapakul, *Biosens. Bioelectron.*, 2014, **54**, 428-434.
22 A. C. Araujo, Y. J. Song, J. Lundeberg, P. L. Stahl and H. Brumer, *Anal. Chem.*, 2012, **84**, 3311-3317.
23 S. C. Fox, B. Li, D. Q. Xu and K. J. Edgar, *Biomacromolecules*, 2011, **12**, 1956-1972.
24 D. Stollner, F. W. Scheller and A. Warsinke, *Anal. Biochem.*, 2002, **304**, 157-165.
25 M. Y. He and Z. H. Liu, *Anal. Chem.*, 2013, **85**, 11691-11694.
26 Y. J. Song, P. Gyarmati, A. C. Araujo, J. Lundeberg, H. Brumer and P. L. Stahl, *Anal. Chem.*, 2014, **86**, 1575-1582.
27 H. E. Blackwell, *Curr. Opin. Chem. Biol.*, 2006, **10**, 203-212.
28 M. Ortiz, A. Fragoso, P. J. Ortiz and C. K. O'Sullivan, *J. Photochem. Photobiol. A: Chem.*, 2011, **218**, 26-32.
29 S. Mallal, D. Nolan, C. Witt, G. Masel, A. M. Martin, C. Moore, D. Sayer, A. Castley, C. Mamotte, D. Maxwell, I. James and F. T. Christiansen, *The Lancet*, 2002, **359**, 727-732.
30 S. I. Hung, W. H. Chung, L. B. Liou, C. C. Chu, M. Lin, H. P. Huang, Y. L. Lin, J. L. Lan, L. C. Yang, H. S. Hong, M. J. Chen, P. C. Lai, M. S. Wu, C. Y. Chu, K. H. Wang, C. H. Chen, C. S. J. Fann, J. Y. Wu and Y. T. Chen, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 4134-4139.
31 P. Winichagoon, V. Saechan, R. Sripanich, C. Nopparatana, S. Kanokpongsakdi, A. Maggio and S. Fucharoen, *Prenatal Diagn.*, 1999, **19**, 428-435.
32 M. R. Green and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, 4th edn., Cold Spring Harbor Laboratory Press, 2012.

RSC Advances Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012