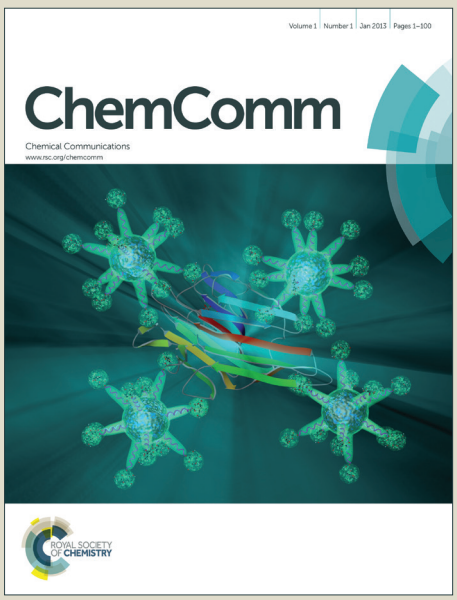
ChemComm

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/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

APTEC: Aptamer-Tethered Enzyme Capture as a Novel Rapid Diagnostic Test for Malaria†

Cite this: DOI: 10.1039/x0xx00000x

Roderick M. Dirkzwager,[a] Andrew B. Kinghorn,[a] Jack S. Richards[b] and Julian A. Tanner*[a]

Received 00th January 2015,
Accepted 00th January 2015

DOI: 10.1039/x0xx00000x

www.rsc.org/

We report the rapid diagnosis of malaria by aptamer-tethered enzyme capture (APTEC) whereby an aptamer captures biomarker *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) then activity is measured colorimetrically. The robust test was sensitive (limit of detection = 4.9 ngmL^{-1}) and could reliably diagnose malaria in clinical blood samples.

Rapid diagnostic tests (RDTs) have had a significant impact on global efforts against malaria over the last two decades. However, there remains a critical need for new RDT technologies that are more sensitive, more robust and more affordable[1] considering that only 64% of the estimated 207 million annual cases of malaria receive a confirmatory diagnostic test before treatment.[2] Further, new technologies suitable for population-based screening programs are required for malaria elimination.[3] All current malaria RDTs rely on the use of monoclonal antibodies for molecular recognition with significant cost, cool-chain transport and stability challenges.[4] Alternative chemistries that are cheap, stable and sensitive are required.

Aptamers are short single-stranded oligonucleotide sequences which specifically bind to various molecular targets and are selected using in vitro evolution techniques.[5] Aptamers are attractive alternatives to antibodies in diagnostic tests because they are more stable, cheaper to synthesize and can be tailored for specificity and sensitivity by altering selection conditions.[6] Colorimetric assays are ideal for developing world RDTs since the readout can be analyzed visually. Aptamer-based colorimetric assays have been widely reported using gold nanoparticles, coupled enzyme or DNAzyme reactions but aptamer-based point-of-care tests are not yet in clinical use.[7]

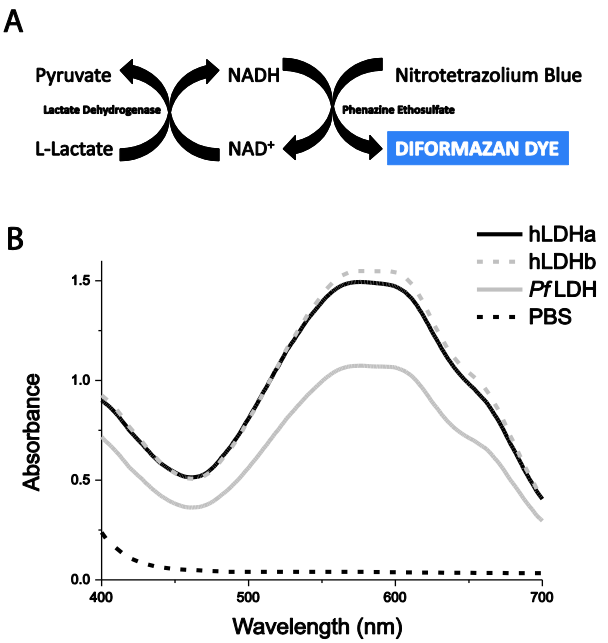


Fig. 1 (a) The reaction scheme which couples LDH activity to the reduction of NTB through the shuttling of NAD$^+$/NADH to produce a blue diformazan dye product. (b) Absorbance scan results when human LDH isoforms (hLDHa and hLDHb) and *Pf*LDH samples (20 ng) are diluted in phosphate buffered saline (PBS) buffer and incubated with the L-lactate/NTB solution after 45 min.

[a] *Department of Biochemistry, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong S.A.R.*
E-mail: jatanner@hku.hk
[b] *The Burnet Institute of Medical Research and Public Health, 85 Commercial Road, Melbourne, Victoria 3004, Australia*
† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/c000000x/

ChemComm Accepted Manuscript

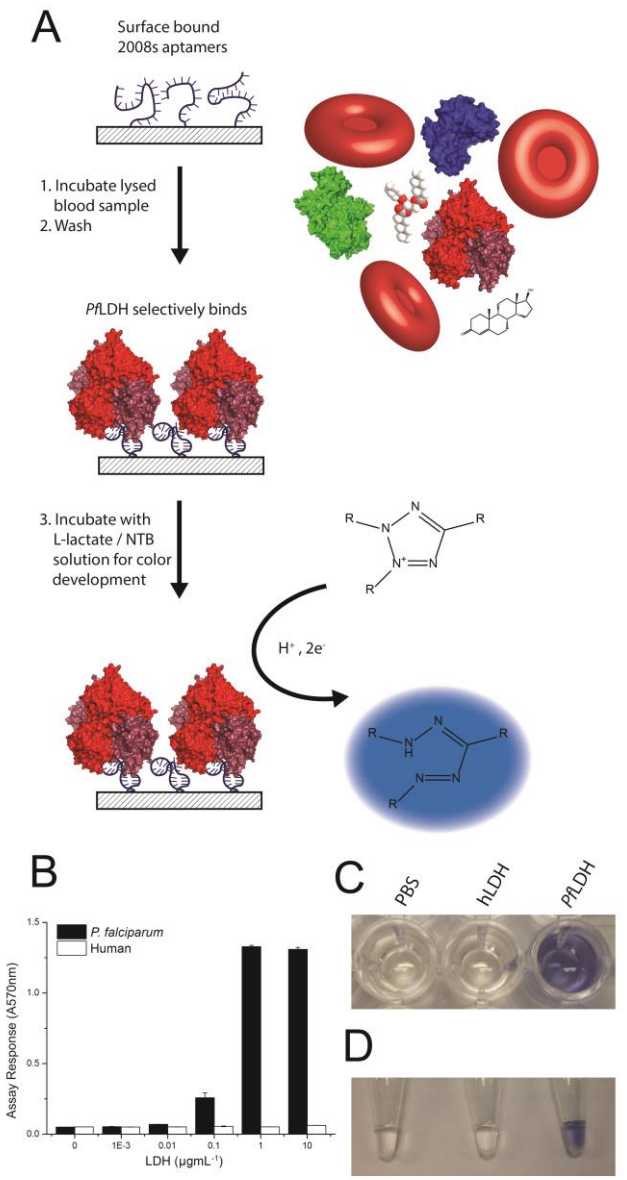


Fig. 2 APTEC assay for *Pf*LDH detection (a) APTEC combines an aptamer tethering step with enzymatic color production (PDB: 3ZH2). (b) Assay response using purified recombinant *Pf*LDH versus native human LDH (hLDHb) in 2 x PBS buffer. (c) Image of the resulting signal for the assay when PBS, hLDHb and *Pf*LDH (100 ngmL^{-1}) are compared. (d) Example of aptamer-decorated magnetic beads being used for APTEC instead of the 96-well plate format. Only 10 µL of LDH sample (100 ngmL^{-1}) was required for this colorimetric response.

Previously, we reported the crystal structure and function of a 35 base DNA aptamer (2008s) specific for the malaria biomarker Plasmodium falciparum lactate dehydrogenase (PfLDH) with a dissociation constant (Kd) of 42 nM.8 When bound to 2008s, PfLDH retains enzyme activity. PfLDH catalyses the interconversion of L-lactate to pyruvate and can be coupled to the reduction of a tetrazolium dye for a colorimetric response.9 Our novel APTEC assay captures and assays PfLDH directly, and unlike common enzyme-linked immunosorbent assays (ELISAs), does not require additional enzymes or antibodies to facilitate detection.

Table 1. LODs for the APTEC assay with various samples

Sample	Limit of Detection (LOD)
Recombinant *Pf*LDH	4.9 ngmL^{-1} ± 2 ngmL^{-1}
3D7 asynchronous parasites	0.024% ± 0.01% parasitemia
3D7 ring stage parasites	0.14% ± 0.06% parasitemia

Table 2. Results of the APTEC and OptiMAL-IT when testing *P. falciparum* malaria patient blood samples and negative controls

P. falciparum Parasitemia (%)	No. Patient Samples	APTEC Positives	OptiMAL-IT Positives
0	12	0	-
0 ≤ 0.1	6	3	3
0.1 ≤ 1	6	6	6
> 1	2	2	2

The colorimetric response relies on the production of a diformazan dye. Conversion of L-lactate to pyruvate produces the cofactor NADH which reduces nitrotetrazolium blue chloride (NTB) into a diformazan dye product, facilitated by the electron transporter phenazine ethosulfate (PES) as illustrated in Fig. 1(a).[8] The scanning absorbance graph in Fig. 1 (b) shows a large shift in colorimetric properties in the presence of various LDH isoforms under these conditions. Incubation time for color development was optimized using a response time assay shown in Fig. S1. Different dynamic ranges could be achieved by varying incubation time.

In APTEC, the coupled colorimetric reaction is combined with an initial aptamer-capture step that can specifically tether *Pf*LDH from complex solutions. The 2008s aptamer shows highly specific discriminatory recognition of *Pf*LDH versus human LDH, and when bound, *Pf*LDH activity is not inhibited.[8] The assay follows a simple 3-step protocol illustrated in Fig. 2(a). First, lysed blood samples are incubated in 2008s decorated wells where *Pf*LDH binds to the aptamers. Second, the wells are washed. Third, the wells are incubated with the L-lactate/NTB solution to produce the colorimetric response quantifiable by absorbance at 570 nm. Like the 2008s aptamer itself, APTEC is highly specific for recombinant *Pf*LDH over human LDH (hLDHb) as illustrated in Fig. 2 (b-c). Detection systems using aptamer mediated enzyme capture have been previously reported for thrombin, activated protein C and neutrophil elastase but not for malaria diagnosis as described here.[10]

The APTEC assay successfully detects *Pf*LDH in whole blood samples, initially shown using whole rat blood spiked with recombinant *Pf*LDH (see Fig. S2) and later confirmed by experiments with patient blood samples. Non-specific hydrophobic interaction was avoided thanks to the plates coming preblocked (SuperBlock). For purified *Pf*LDH solutions, non-specific electrostatic interaction with the DNA aptamers was observed but could be avoided by adjusting binding buffer conditions (Fig. S3).

To determine a limit of detection (LOD) for APTEC with recombinant *Pf*LDH, samples were tested in 5% BSA with background hLDHb to mimic the protein density of blood. A LOD of 4.9 ngmL^{-1} ± 2 ngmL^{-1} *Pf*LDH was calculated, see Table 1 and Fig. S4 (a). *Plasmodium* LDH levels in malaria patients are typically found at µgmL^{-1} concentrations therefore the assay is well within clinical range.[11] In moles, the LOD is 14 fmole ± 6 fmole of *Pf*LDH which compares well to the LOD for free *Pf*LDH in solution at 8.8 fmole ± 0.7 fmole, see Fig. S4 (b). This similarity implies the limiting factor of the test is the enzyme-

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2015

coupled signal amplification step rather than the inherent binding affinity of the aptamer.

The LOD for APTEC was also calculated on malaria parasite cultures. As an integral metabolic enzyme, *Pf*LDH is a suitable biomarker for malaria since it is present during all life stages of the parasite.[12] The *P. falciparum* laboratory line 3D7 was used to infect human red blood cells (RBCs) at various parasitemias. For asynchronous parasite culture LOD = 0.024% ± 0.01% parasitemia (600 ± 250 parasites/ µL), and for ring stage parasites LOD = 0.14% ± 0.06% (3500 ± 250 parasites/ µL), see Table 1 and Fig. S4 (c-d).[13] Other studies which use gold nanoparticles and other DNA aptamers to detect *Pf*LDH have similarly reported detection limits in the clinical range and so support the future potential of aptamers in malaria diagnosis.[7b, 14]

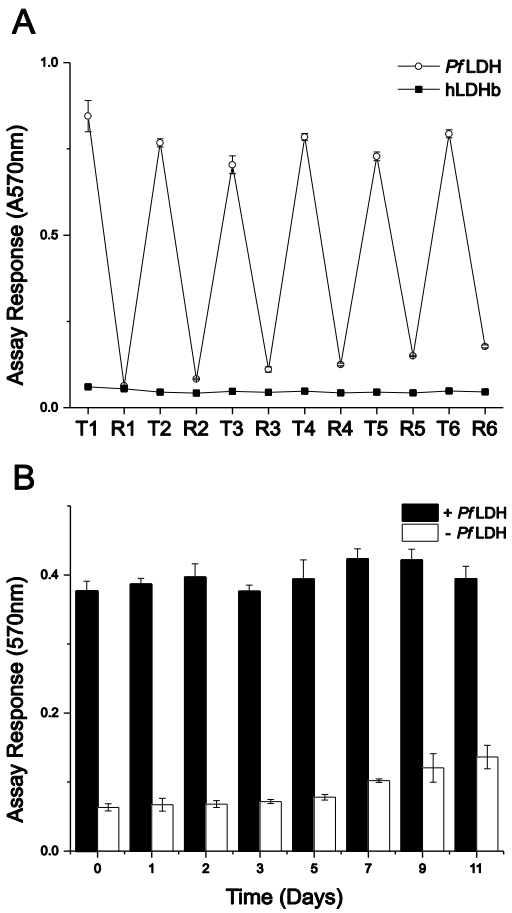


Fig. 3 (a) Regeneration of the aptamers in the APTEC assay using a shortened protocol. Six consecutive incubations with LDH samples (1 µgmL^{-1}) were performed by washing with a urea-based elution buffer between tests. L-lactate/ NTB substrate solution was added for 20 min for each test (T) and after each regeneration (R) to confirm successful *Pf*LDH elution and compared to a hLDHb control. (b) Stability of the L-lactate/NTB solution when stored protected from light at room temperature for 11 days and tested with *Pf*LDH in solution (7.5 ng).

Table 3. Semi-quantitative APTEC assay results. By varying aptamer density within assay wells, visual semi-quantification of *Pf*LDH concentration was possible: negative (− − −), low (+ − −), medium (+ + −), or high (+ + +).

*Pf*LDH (µgmL^{-1})	Aptamer Density High	Medium	Low	Test Result
0	−	−	−	Negative
0.05	+	−	−	Low
0.1	+	−	−	Low
0.5	+	+	−	Medium
1	+	+	−	Medium
5	+	+	+	High
10	+	+	+	High

Clinical whole blood samples from patients infected with *P. falciparum* malaria were tested using our APTEC system and compared with results from the antibody-based OptiMAL-IT RDT.[15] This demonstrated that 12/15 light microscopy confirmed *P. falciparum* infections were correctly diagnosed by both the APTEC assay and the OptiMAL-IT dipsticks (Table 2). Parasitemia as low as 0.01% was detected and the 3 false negative results observed were from low parasitemia samples (≤0.06).

The ability for aptamers to be easily regenerated suggests the potential for aptamer-based RDT reuse, with benefits of decreased cost and population-level disease screening.[16] We showed the aptamer decorated wells could be reused six times for the APTEC assay using purified *Pf*LDH samples with no significant reduction in response by using a urea elution step as shown in Fig. 3(a). A slight increase in baseline response over time (R1-6) was attributed to deposition of NTB dye product. After urea denaturing, the aptamers were found to regain their active conformation without the need for a refolding steps.

*Pf*LDH has been shown to closely follow parasitemia levels in patients.[17] This makes it is a suitable biomarker to monitor parasite clearance rates in identifying anti-malarial drug resistance however antibody-based RDTs can usually only provide qualitative diagnosis.[18] By varying the aptamer density across 3 test wells, APTEC samples could be categorized as having negative (---),low (+--), medium (++-) or high (+++) concentrations of *Pf*LDH by visual assessment without the need for an absorbance reader. The results and aptamer density experiment are shown in Table 3 and Fig. S5.

The potential adaptation of the APTEC assay into an alternative format was assessed using 2008s functionalised magnetic beads to capture *Pf*LDH. Magnetic beads can be easily separated, functionalized with biomolecules, and incorporated into fluidic systems for RDTs.[19] Sample volumes of 10 µL, attainable using finger-prick sampling in the field, gave a clear color response similar to that of the 96-well plate assay as shown in Fig. 2 (d). The stability of the L-lactate/NTB solution was also assessed and good activity was still observable after storage for 11 days at room temperature shielded from light. Expiration of the L-lactate/NTB solution began to show after one week with a gradual increase in signal for *Pf*LDH negative samples. Future work on desiccating the reagents and adding stabilizing agents will aim to enhance long term stability.[20]

In summary, we have described the APTEC assay which tethers *Pf*LDH from blood samples using the *Pf*LDH specific aptamer 2008s and then harnesses *Pf*LDH enzyme activity to produce a colorimetric response which is sensitive, specific and quantitative. Unlike most examples of aptamer-based colorimetric assays, the APTEC system

ChemComm Accepted Manuscript

requires no additional enzymes or nanoparticles for its response and functions well, even in complex whole blood samples. Compared to other examples of aptamer mediated enzyme capture, intense color development can be observed in less than 45 minutes and can confirm diagnosis without the need for laboratory machinery. With patient blood samples, the APTEC test performed favourably compared to a commercially available *Pf*LDH RDT confirming its clinical viability. The regenerability of the APTEC test and its ability to be semi-quantitative has important implications for monitoring slow parasite clearance associated with anti-malarial drug resistance. Future work will seek to adapt the assay to a more user friendly magnetic bead-based test or lateral flow dipstick system for point-of-care implementation. APTEC technology has the potential to confront many of the issues associated with antibody-based point-of-care diagnostics in various applications.

This work was supported by the Hong Kong University Grants Council under General Research Fund Grant HKU778813M and the Burnet Institute is supported by the Australian National Health and Medical Research Council Infrastructure for Research Institutes Support Scheme and a Victorian State Government Operational Infrastructure Support grant. We acknowledge the HKU emerging Strategic Research Theme in Integrative Biology.

Notes and references

1 (a) R. M. Fairhurst, G. M. Nayyar, J. G. Breman, R. Hallett, J. L. Vennerstrom, S. Duong, P. Ringwald, T. E. Wellems, C. V. Plowe and A. M. Dondorp, *Am. J. Trop. Med. Hyg.*, 2012, **87**, 231; (b) C. Sansom, *Lancet Infect. Dis.*, 2009, **9**, 596.

2 World Health Organization, Malaria Rapid Diagnostic Test Performance - Results of the WHO Product Testing of Malaria RDTs: Round 4, 2012

3 L. von Seidlein, *PLoS Med.*, 2014, **11**, e1001595.

4 (a) R. E. Coleman, N. Maneechai, N. Rachaphaew, C. Kumpitak, R. S. Miller, V. Soyseng, K. Thimasarn and J. Sattabongkot, *Am. J. Trop. Med. Hyg.*, 2002, **67**, 141; (b) C. Wongsrichanalai, M. J. Barcus, S. Muth, A. Sutamihardja and W. H. Wernsdorfer, *Am. J. Trop. Med. Hyg.*, 2007, **77**, 119; (c) P. L. Chiodini, K. Bowers, P. Jorgensen, J. W. Barnwell, K. K. Grady, J. Luchavez, A. H. Moody, A. Cenizal and D. Bell, *Trans. R. Soc. Trop. Med. Hyg.*, 2007, **101**, 331.

5 (a) C. Tuerk and L. Gold, *Science*, 1990, **249**, 505; (b) D. H. J. Bunka and P. G. Stockley, *Nat. Rev. Microbiol.*, 2006, **4**, 588; (c) M. Famulok and G. Mayer, *Chem. Biol.*, 2014, **21**, 1055.

6 (a) A. E. G. Cass and Y. Zhang, *Faraday Discuss.*, 2011, **149**, 49; (b) M. Famulok and G. Mayer, *Acc. Chem. Res.*, 2011, **44**, 1349.

7 (a) J. W. Liu and Y. Lu, *Angew. Chem., Int. Ed.*, 2006, **45**, 90; (b) S. Lee, D. H. Manjunatha, W. Jeon and C. Ban, *PLoS One*, 2014, **9**, e100847; (c) L. Zhang, J. Zhu, T. Li and E. Wang, *Anal. Chem.*, 2011, **83**, 8871.

8 Y. W. Cheung, J. Kwok, A. W. Law, R. M. Watt, M. Kotaka and J. A. Tanner, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 15967.

9 (a) M. T. Makler and D. J. Hinrichs, *Am. J. Trop. Med. Hyg.*, 1993, **48**, 205; (b) A. L. Babson and G. E. Phillips, *Clin. Chim. Acta*, 1965, **12**, 210.

10 (a) L. Cheng and Q. Zhao, *Talanta*, 2013, **106**, 315; (b) Q. Zhao, X.-F. Li and X. C. Le, *Anal. Chem.*, 2011, **83**, 9234; (c) Q. Zhao and X. Wang, *Biosens. Bioelectron.*, 2012, **34**, 232; (d) J. Müller, M. Friedrich, T. Becher, J. Braunstein, T. Kupper, P. Berdel, S. Gravius, F. Rohrbach, J. Oldenburg, G. Mayer and B. Pötzsch, *Thromb. Haemostasis*, 2012, **10**, 390.

11 J. Jang, C. Cho, E. Han, S. S. An and C. Lim, *Malar. J.*, 2013, **12**, 181.

12 A. M. Talman, L. Duval, E. Legrand, V. Hubert, S. Yen, D. Bell, J. Le Bras, F. Ariey and S. Houze, *Malar. J.*, 2007, **6**, 140.

13 L. H. Miller, D. I. Baruch, K. Marsh and O. K. Doumbo, *Nature*, 2002, **415**, 673.

14 (a) W. Jeon, S. Lee, M. Dh and C. Ban, *Anal. Biochem.*, 2013, **439**, 11; (b) S. Lee, K. M. Song, W. Jeon, H. Jo, Y. B. Shim and C. Ban, *Biosens. Bioelectron.*, 2012, **35**, 291.

15 A. H. Moody and P. L. Chiodini, *Br. J. Biomed. Sci.*, 2002, **59**, 228.

16 R. Kirby, E. J. Cho, B. Gehrke, T. Bayer, Y. S. Park, D. P. Neikirk, J. T. McDevitt and A. D. Ellington, *Anal. Chem.*, 2004, **76**, 4066.

17 R. Piper, J. Lebras, L. Wentworth, A. Hunt-Cooke, S. Houzé, P. Chiodini and M. Makler, *Am. J. Trop. Med. Hyg.*, 1999, **60**, 109.

18 (a) K. Stepniewska, E. Ashley, S. J. Lee, N. Anstey, K. I. Barnes, T. Q. Binh, U. D'Alessandro, N. P. J. Day, P. J. de Vries, G. Dorsey, J.-P. Guthmann, M. Mayxay, P. N. Newton, P. Olliaro, L. Osorio, R. N. Price, M. Rowland, F. Smithuis, W. R. J. Taylor, F. Nosten and N. J. White, *J. Infect. Dis.*, 2010, **201**, 570; (b) A. M. Dondorp, S. Yeung, L. White, C. Nguon, N. P. J. Day, D. Socheat and L. von Seidlein, *Nat. Rev. Micro.*, 2010, **8**, 272.

19 (a) S. Centi, S. Tombelli, M. Minunni and M. Mascini, *Anal. Chem.*, 2007, **79**, 1466; (b) C. H. Wang, K. Y. Lien, J. J. Wu and G. B. Lee, *Lab Chip*, 2011, **11**, 1521; (c) M. Herrmann, E. Roy, T. Veres and M. Tabrizian, *Lab Chip*, 2007, **7**, 1546; (d) K. Tram, P. Kanda, B. J. Salena, S. Huan and Y. Li, *Angew. Chem., Int. Ed.*, 2014.

20 (a) S. Jahanshahi-Anbuhi, K. Pennings, V. Leung, M. Liu, C. Carrasquilla, B. Kannan, Y. Li, R. Pelton, J. D. Brennan and C. D. Filipe, *Angew. Chem., Int. Ed.*, 2014, **53**, 6155; (b) S. Ramachandran, E. Fu, B. Lutz and P. Yager, *Analyst*, 2014, **139**, 1456; (c) E. Garcia, J. R. Kirkham, A. V. Hatch, K. R. Hawkins and P. Yager, *Lab Chip*, 2004, **4**, 78.

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2015