Research article

LUMINESCENCE
The Journal of Biological and Chemical Luminescence

Received: 13 February 2011, Revised: 04 May 2011, Accepted: 04 May 2011, Published online in Wiley Online Library: 28 June 2011

(wileyonlinelibrary.com) DOI 10.1002/bio.1324

Serum inverts and improves the fluorescence response of an aptamer beacon to various vitamin D analytes

John G. Bruno,* Maria P. Carrillo, Taylor Phillips and Allison Edge

ABSTRACT: A dominant aptamer loop structure from a library of nearly 100 candidate aptamer sequences developed against immobilized 25-hydroxyvitamin D_3 (calcidiol) was converted into a 5′-TYE 665 and 3′-Iowa black-labelled aptamer beacon. The aptamer beacon exhibited a mild 'lights on' reaction in buffer as a function of increasing concentrations of several vitamin D analogues and metabolites, with a limit of detection of approximately 200 ng/mL, and was not specific for any particular congener. In 10% or 50% human serum, the same aptamer beacon inverted its fluorescence behaviour to become a more intense 'lights off' reaction with an improved limit of detection in the range 4–16 ng/mL. We hypothesized that this drastic change in fluorescence behaviour was due to the presence of creatinine and urea in serum, which might destabilize the quenched beacon, causing an increase in fluorescence followed by decreasing fluorescence as a function of vitamin D concentrations that may bind and quench increasingly greater fractions of the denatured beacons. However, the results of several control experiments in the presence of physiological or greater concentrations of creatinine and urea, alone or combined in buffer, failed to produce the beacon fluorescence inversion. Other possible mechanistic hypotheses are also discussed. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: aptamer; beacon; fluorescence; serum; vitamin D

Introduction

Accurate assessment of vitamin D levels in serum is important to avoid nutritional deficiencies leading to rickets, osteoporosis or other bone diseases, and may be important for research in the areas of immune system and cardiovascular health (1). Historically, vitamin D assays have involved the use of vitamin D-binding proteins (DBP), receptors or antibodies and have required a high level of technical skill. We set out to determine whether we could develop a DNA aptamer beacon (2–4) or competitive aptamer assay (5–7) for NASA to rapidly and simply assess the vitamin D status of astronauts aboard spacecraft, where laboratory space and labour must be minimized.

This paper focuses on surprising results from the implementation of our aptamer beacon in diluted human serum. Although a useful detection limit of approximately 4–16 ng/mL was obtained within 20–30 min, which may be of interest to the clinical diagnostics community (1,8), our beacon is unable to discriminate the various major analogues and metabolites of vitamin D. The beacon does appear to be specific for the general family of vitamin D analytes. The reported prototype vitamin D assay is not intended as a competitor for standard radioimmunoassay, HPLC or mass spectral means of quantifying vitamin D analogues and metabolites (1,8). We simply wish to report the serendipitous finding that our weak 'lights on' aptamer beacon assay, as observed in binding buffer, becomes inverted ('lights on' converts to 'lights off') as well as becoming much more robust and sensitive in serum. This is especially intriguing, because even in the relatively low fluorescence far-red spectral region of serum that we targeted (≥650 nm), the vitamin D aptamer beacon should have been overwhelmed by serum autofluorescence (i.e. beacon fluorescence may have been lower than background fluorescence). Instead, it appears that the overall fluorescence of the assay increased > five-fold. We discuss these amazing results in light of a hypothesis that hydrogen bond-disruptive substances, such as creatinine and urea or other substances in serum, may be denaturing and dequenching the 5′ and 3′ end-labelled aptamer beacon's small duplex stem region to maximize fluorescence light output in the absence of vitamin D. Also, in the presence of varied levels of vitamin D analogues and metabolites, some of the aptamer beacons may bind their target analyte and adopt a renatured conformation, leading to some level of quenching and an apparent 'lights off' fluorescence response as a function of vitamin D concentration that may be useful for assay development. This observation also suggests that the aptamer beacon may have a greater affinity for vitamin D analogues and metabolites than DBP or albumin, which bind and transport vitamin D in serum (9). We also test the vitamin D aptamer beacon's fluorescence behaviour in the absence of serum but in the presence of high physiological and non-physiological levels of creatinine and urea, with and without varied levels of calcidiol, and offer interpretations of our results.

* Correspondence to: J. G. Bruno, Operational Technologies Corporation, 4100 NW Loop 410, Suite 230, San Antonio, TX 78229, USA. E-mail: john.bruno@otcorp.com

Operational Technologies Corporation, San Antonio, TX 78229, USA

Copyright © 2011 John Wiley & Sons, Ltd.

Materials and methods

Vitamin D analogues and metabolites, serum and other reagents

All vitamin D analogues and metabolites, including vitamin D_2, vitamin D_3 (cholecalciferol), 25-hydroxyvitamin D_3 (calcidiol) and 1α,25-dihydroxyvitamin D_3 (calcitriol), were obtained from Calbiochem/EMD Chemicals (Gibbstown, NJ, USA) and dissolved in 1 mL methanol prior to use. Any unused portions were stored in opaque containers at −20°C for future use. Human serum was obtained from Mediatech Inc. (Manassas, VA, USA) and Lonza Inc. (BioWhittaker® brand; Walkersville, MD, USA) and stored frozen at −20°C until thawed in a 37°C water bath for use in experiments. Single-strength binding buffer (1×BB; 0.5 mol/L NaCl, 10 mmol/L Tris–HCl and 1 mmol/L $MgCl_2$, pH 7.5–7.6 in nuclease-free deionized water) was used for all aptamer and serum dilutions. Bone collagen crosslinkers [pyridinoline (Pyd) and deoxypyridinoline (DPD)] were obtained from Quidel Corp. (San Diego, CA, USA). All other chemical reagents, including nuclease-free deionized water, were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Vitamin D SELEX aptamer development

For brevity, the reader is referred to previous descriptions of calcidiol or related small molecule aptamer development in the literature (5,10). In summary, calcidiol was immobilized by the Mannich reaction onto PharmaLink™ affinity columns (Pierce, Thermo-Fisher Scientific, Co., Pittsburgh, PA, USA) according to the manufacturer's instructions for aptamer selection (5,10). Seventy-two base aptamers from each round were eluted from the column with 3 mol/L sodium acetate, pH 5.2, then dialysed overnight against nuclease-free deionized water and concentrated by a Speed Vac® apparatus, as previously described (5,10). The presence of full-length 72-base aptamer PCR products was verified by ethidium bromide-stained 2% agarose gel electrophoresis against standard DNA ladders after each round of selection, and aptamers from the fifth round of selection were cloned into chemically competent *Escherichia coli*, using a Lucigen GC kit (Middleton, WI, USA). All aptamers were sequenced by rolling circle amplification at Sequetech Corp. (Mountain View, CA, USA).

Aptamer beacon candidates and loop C beacon

A total of 98 candidate vitamin D aptamer sequences (not shown) were obtained and analysed for common full-length consensus sequences and common sequence segments of five or more bases. These sequences were also subjected to secondary stem–loop structure analyses, using internet-based Vienna RNA software (11) with DNA parameters (12) at 25°C to look for common loop structures. Several common stem–loop structures were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) with 5′-TYE 665 fluorophore and 3′-Iowa black quencher labels, as indicated in Fig. 1, and HPLC-purified for testing of their assay potential vs various levels of vitamin D analogues and

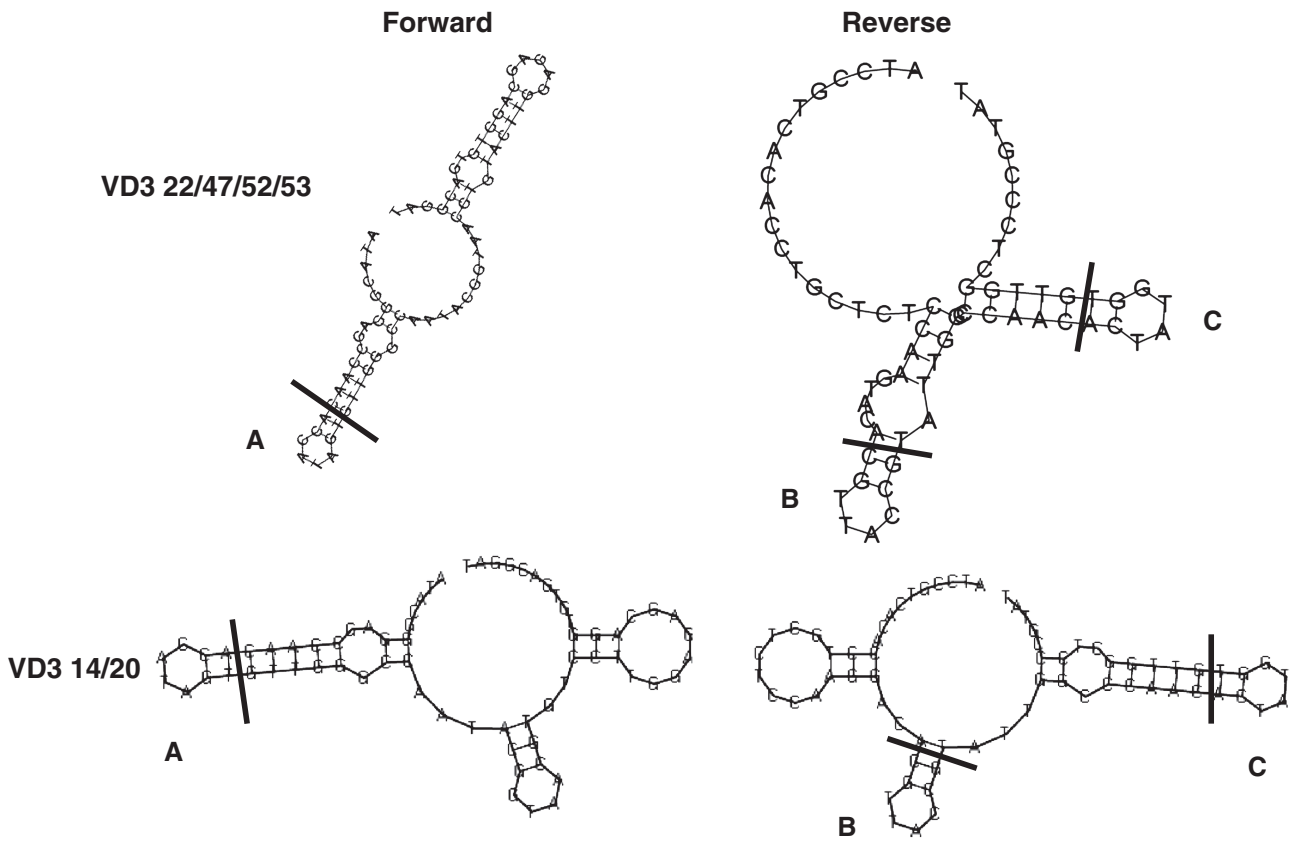

Figure 1. Secondary structures of the most common vitamin D-binding aptamers in the sequenced population, as determined by Vienna RNA software using DNA parameters at 25°C. Cut-off boundaries for the eight-base stem–loop structures projected to represent potential aptamer beacons are indicated by the lines.

 Copyright © 2011 John Wiley & Sons, Ltd.

LUMINESCENCE
The Journal of Biological and Chemical Luminescence

metabolites. The optimum eight-base loop for vitamin D detection designated loop C was: 5′-TYE 665-ACT ATG GT-Iowa black RQSp-3′.

Beacon titration assays, spectrofluorometry and Picofluor™ handheld fluorescence measurements

Vitamin D aptamer beacons were typically dissolved in 1×BB, 10%, 50% or nearly 100% serum diluted in 1×BB at 200 ng/mL and used in 4 mL volumes in cuvette assays, following serial two-fold dilutions of vitamin D analogues and metabolites as shown in the figures. Given that the optimum loop C beacon weighed ~3.6 kDa, this equates to using 0.23 nmol beacon/4 mL cuvette test, or an final beacon concentration of 57.5 nmol/L. The 4 mL polystyrene cuvettes were obtained from Thermo-Fisher Scientific, capped and gently mixed at room temperature (~25°C) for 20–30 min prior to fluorescence assessment. Fluorescence measurements were carried out on a Varian Cary Eclipse spectrofluorometer with 800–1000 V PMT settings, as indicated in each figure, excitation at 645 ± 5 nm and emission scanning in the range 660–720 nm. A custom-made optically modified Picofluor™ handheld fluorometer (Turner Biosystems Inc.; now the Quantifluor™ by Promega Biosystems, Sunnyvale, CA, USA) was fitted with a red LED excitation source centred at 650 nm, with a 660–720 nm bandpass emission filter. Sensitivity of the Picofluor™ (STD VAL button) was set at 999.0 and relative fluorescence units (RFUs) were recorded in an EXCEL spreadsheet.

Results

Figure 1 illustrates the forward and reverse-primed secondary structures of the four most common full-length aptamers (clones 22, 47, 52 and 53) in the 98-sequence pool of candidate aptamers selected against calcidiol. Two other clones (14 and 20) from the same selected pool demonstrated identical A, B or C stem–loop structures, as indicated in the figure. Each of these candidate beacon stem–loop structures were truncated to the eight-base forms shown in Fig. 1, and these short sequences were synthesized with 5′-TYE 665 fluorophore and 3′-Iowa black quencher appendages, followed by HPLC purification at Integrated DNA Technologies Inc.

Each of the candidate aptamer beacons (loops A–C from Fig. 1) was subjected to spectrofluorometric analysis vs serial two-fold dilutions of calcidiol in 1×BB, as shown in Fig. 2. As Fig. 2C illustrates, loop C was the optimal aptamer beacon because it yielded the greatest spectral spread or separation of emission spectra as a function of calcidiol concentration. Fig. 2B also illustrates that little or no fluorescence could be accounted for by the calcidiol itself ('VD3 only' curve). Finally, Fig. 2D

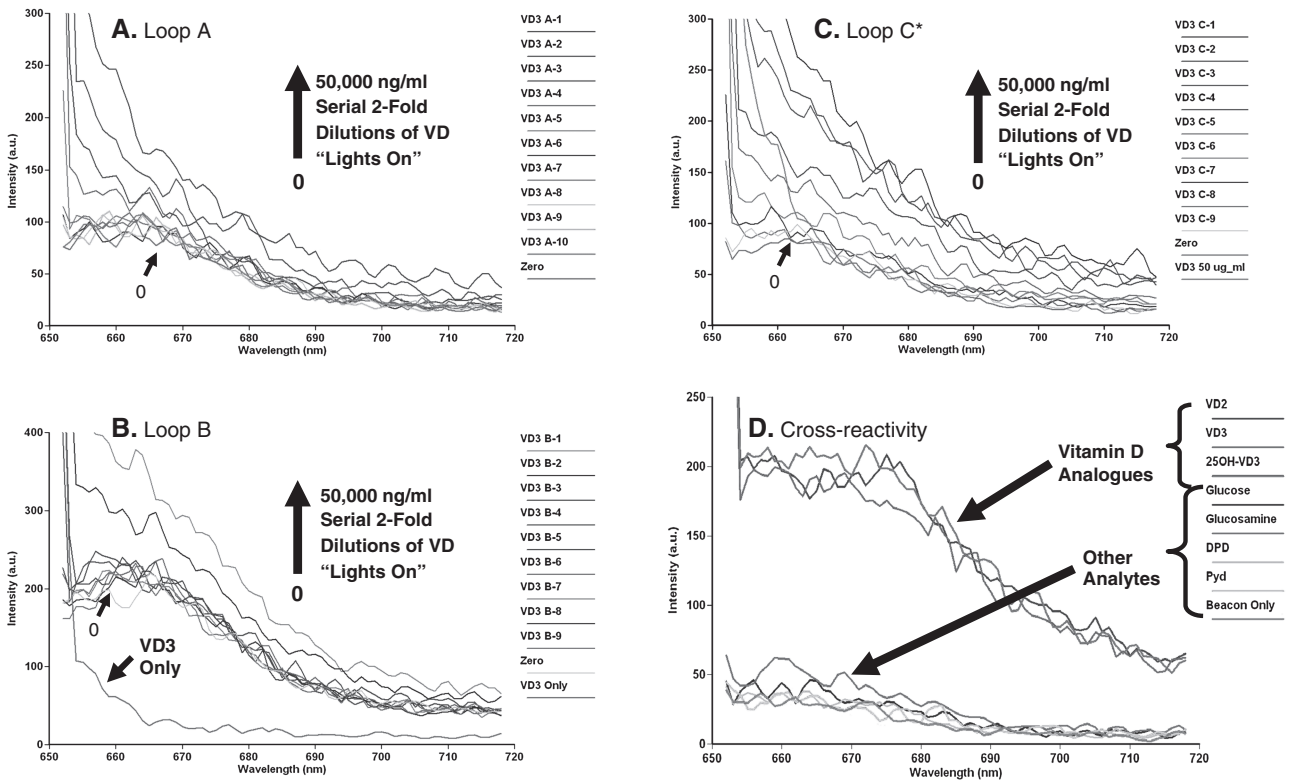

Figure 2. (A–C) 'Lights on' fluorescence responses of each candidate aptamer beacon loop, beginning with 50 µg or 50 000 ng/mL calcidiol, with serial two-fold dilutions and a negative (no calcidiol) control, typically represented by the spectrum or trace labeled "0". *Loop C was the optimal aptamer beacon, having the greatest separation of spectra as a function of calcidiol concentration. PMT setting for all panels was 1000 V. (D) Specificity of the loop C aptamer beacon for vitamin D analogues and metabolites (all at 1500 ng/mL) vs other analytes that could be encountered in serum at typical physiological concentrations [glucose at 100 mg/dL, glucosamine, pyridinoline (Pyd) and deoxypyridinoline (DPD), all at 1000 ng/mL] and the loop C beacon at 200 ng/mL in 1×BB. The VD3-only spectrum in (B) refers to analysis of 50 000 ng/mL calcidiol in 1×BB by itself, without any candidate aptamer beacon as a control.

 Copyright © 2011 John Wiley & Sons, Ltd.

shows that the loop C beacon could specifically recognize, but not discriminate, a number of relevant vitamin D analogues and metabolites (including calcitriol, not shown) vs other analytes, including glucose, glucosamine and bone crosslinkers such as pyridinoline (Pyd) and deoxypyridinoline (DPD), at physiologically relevant levels that could be found in serum (see Fig. 2 caption).

The remarkable data displayed in Fig. 3 illustrate that the relatively mild 'lights on' fluorescence response of the loop C vitamin D aptamer beacon in 1×BB (Fig. 3A) inverts to become a more robust 'lights off' assay as a function of calcidiol levels (Fig. 3B–D) when 10% and 50% human serum are added. The near-100% serum addition is sufficient to swamp the aptamer beacon's fluorescence (i.e. flatten the titration curve) almost completely and destroy the vitamin D assay, as shown by comparison of Picofluor™ fluorescence values in Fig. 3D.

Figures 4A, B, illustrates controls in which 10% and 50% human serum were diluted with 1×BB and even higher levels of calcidiol (in the range 2500–10 000 ng/mL) were added and compared to the fluorescence of 10% and 50% human serum alone, to demonstrate that serum itself without the loop C aptamer beacon is not tremendously fluorescent in the mid to far red region of the spectrum. Figures 4A and 4B also demonstrate that the addition of vitamin D to serum does not somehow enhance or quench serum autofluorescence in the red region in the absence of the loop C aptamer beacon (i.e. the aptamer beacon is a necessary component for the 'lights off' response). These data combined with those presented in Figs 2, 3, led to the hypothetical model shown in Fig. 4 C, which attempts to explain the fluorescence inversion and enhanced assay sensitivity observed herein. The simple model in Fig. 4 C shows the loop C beacon population being initially and completely denatured (dequenched and fluorescing strongly) by substances in serum and then partially renaturing as a function of increasingly available vitamin D that is added exogenously. As more and more members of the aptamer beacon population become renatured by higher vitamin D levels, the greater the percentage of beacons that become quenched again (right side of Fig. 4 C), leading to an apparent 'lights off' fluorescence response to increased vitamin D levels.

To determine how reproducible the beacon inversion phenomenon is, we repeated the experiment shown in Fig. 3 with two different lots of human serum from a second supplier and obtained the data shown in Fig. 5 for 10% and 50% human serum. Although the absolute values were not the same between experiments, the fluorescence inversion and 'lights off' trend was reproducible, as clearly demonstrated in Figs. 3, 5.

We next tested the hypothesis that the highest physiological serum levels of creatinine and urea, alone or combined, could

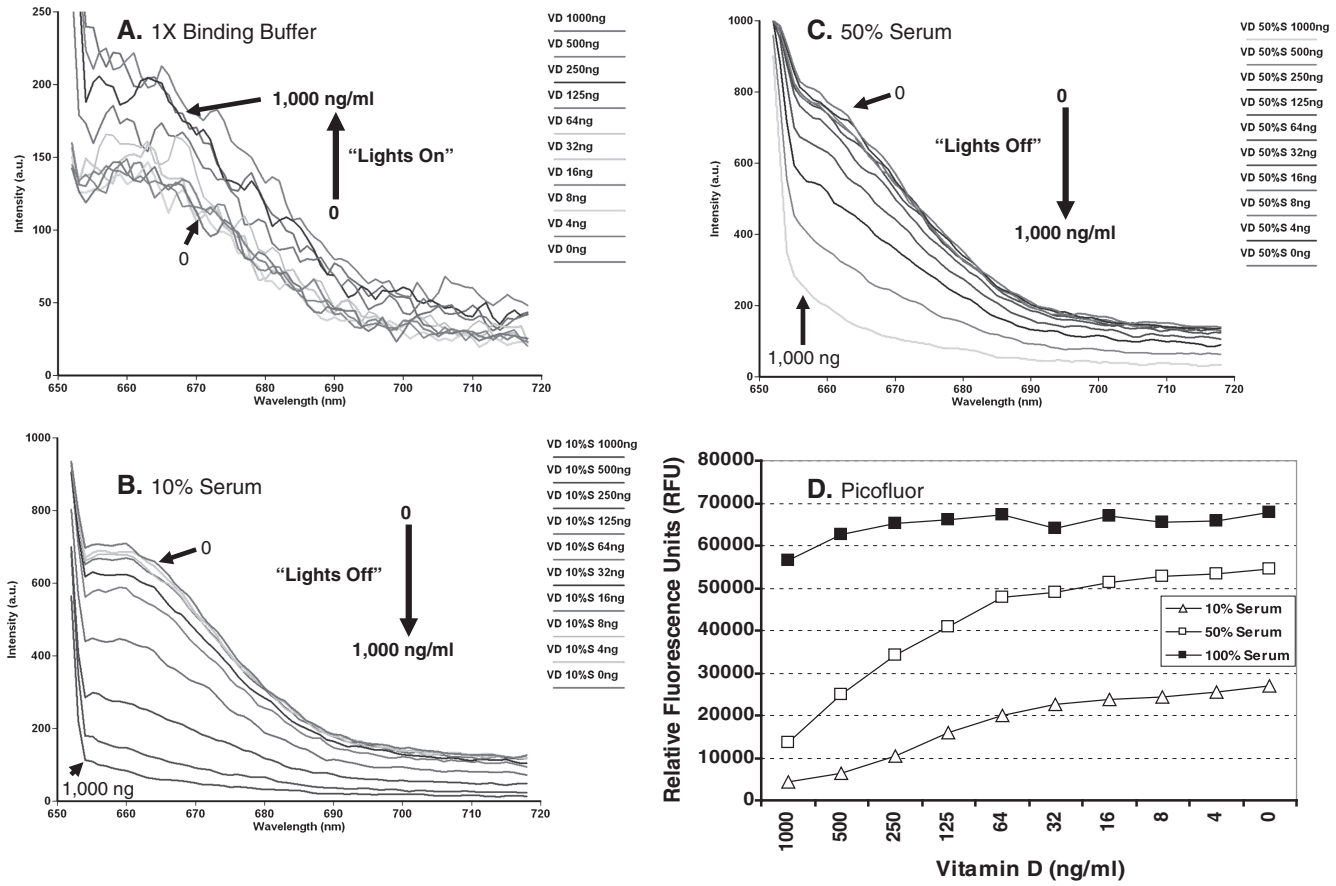

Figure 3. Comparison of fluorescence response for the loop C beacon in 1×BB (A) vs 10% and 50% human serum (B, C). PMT setting was 1000 V in (A) and 800 V in (B, C). (D) comparison of values obtained for the same cuvettes, using a custom red-emitting Picofluor™ handheld fluorometer for the loop C beacon in 10%, 50% and ~ 100% human serum as a function of calcidiol concentration in the range 0–1000 ng/mL.

Copyright © 2011 John Wiley & Sons, Ltd.

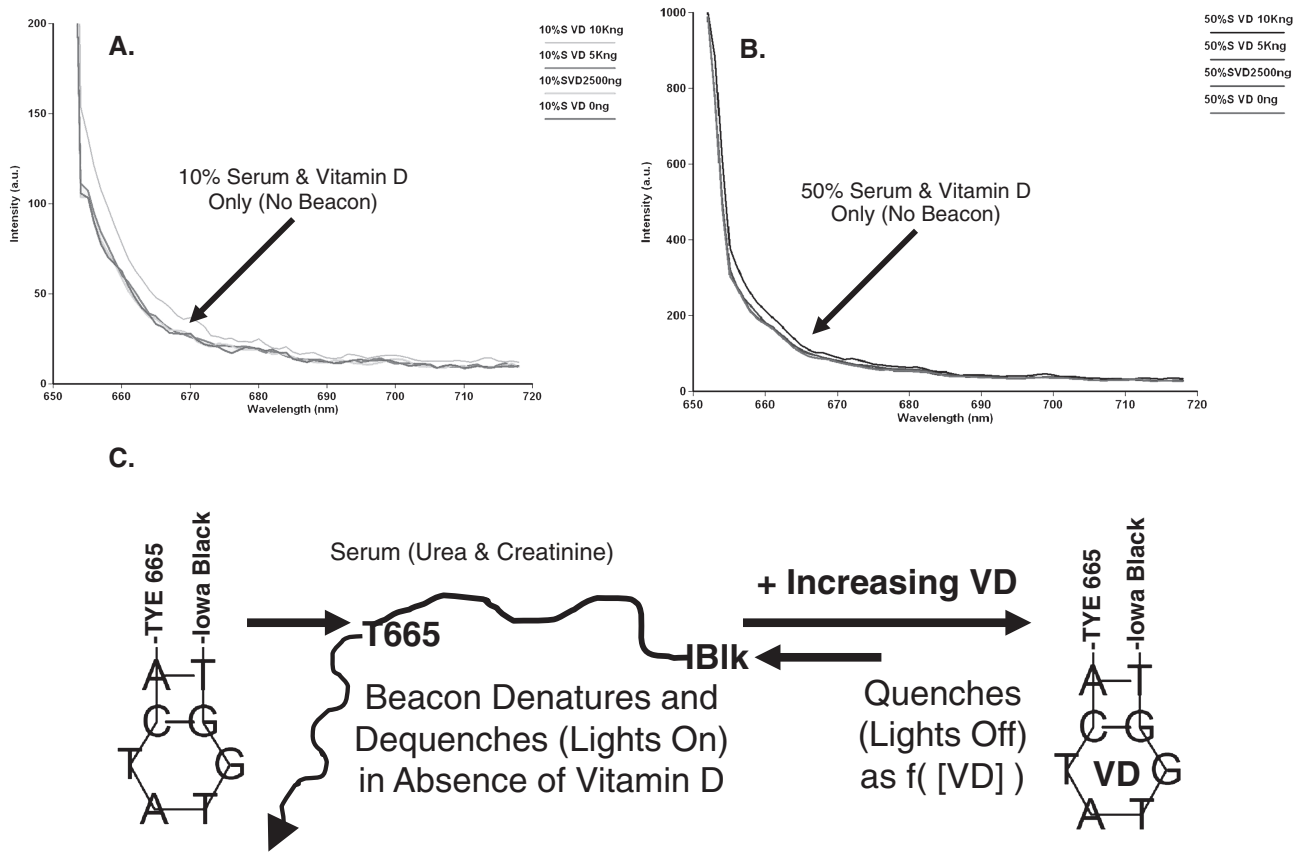

Figure 4. (A, B) Fluorescence spectra for 10% and 50% human serum controls with 2500–10 000 ng/mL calcidiol in the absence of the loop C beacon. (C) Hypothesized mechanism for the fluorescence assay inversion from 'lights on' to 'lights off' in serum, which denatures (dequenches) the beacon via hydrogen bond disruption of base pairing (possibly from creatinine and urea in serum), followed by renaturation and quenching of an increasing proportion of the loop C aptamer population as a function of vitamin D concentration.

denature our vitamin D aptamer beacon. Figure 6 illustrates the results of the isolated creatinine- and urea-denaturing experiments in 1×BB without serum proteins. In healthy individuals, serum levels of creatinine range up to ~1 mg/dL and serum levels of urea range up to ~40 mg/dL, and much higher levels are seen in various disease states (16). We made stock solutions of creatinine and urea at 100× these maximal human serum concentrations and exposed the same amount of our vitamin D aptamer beacon used in previous experiments to these stock creatinine and urea solutions or serial 10-fold dilutions of the stocks for 30 min at room temperature to obtain the results shown in Fig. 6, from which it is clear that not even the 100× stock solution of creatinine or urea environment causes the beacon to fluoresce significantly brighter. Various combinations of high, but physiological, levels of creatinine and urea (data not shown) also could not significantly elevate the beacon's fluorescence intensity to the high levels seen in the presence of 10–50% human serum, thereby refuting our denaturation hypothesis. However, 10× and 100× levels caused some detectable increases in fluorescence, as shown in Fig. 6, and we have noted significant background fluorescence elevation for this and other aptamer beacon assays conducted in undiluted urine samples, which might possibly be attributed to more highly concentrated creatinine and urea in urine vs serum (14–16).

Finally, we tested the addition of varied levels of vitamin D (calcidiol) to the 1× creatinine and urea in 1×BB without serum, alone and combined, as shown in Fig. 7, to determine whether an odd synergistic effect could account for the fluorescence inversion and 'lights off' response. However, as Fig. 7 clearly shows, we again obtained only a mild 'lights on' response to titration with 4–1000 ng/mL calcidiol in 1× creatinine or 1× urea, alone or combined, in buffer. The experiment in Fig. 7 suggests strongly that there is an unknown substance or property of human serum that is responsible for the fluorescence inversion and robust 'lights off' conversion of our vitamin D experiments.

Discussion

As indicated in the introduction to this paper, it was not our intent to describe a fully characterized aptamer beacon assay for vitamin D. Instead we have described the rather amazing phenomenon of fluorescence inversion from 'lights on' to 'lights off' behaviour of a prototype aptamer beacon system, which is clearly mediated by the addition of serum. While aptamer beacons or competitive aptamer systems have been described which exhibit either 'lights on' or 'lights off' behaviour in various buffers against macromolecular and small molecular targets (2–7,13), to the best of our knowledge this is the first report of a switch in that basic trend due to serum addition. The sheer magnitude of the fluorescence turnaround (Figs 3, 5, 7) from the buffer to serum environment makes this phenomenon noteworthy. In fact, we were forced to drop the PMT setting from 1000 V (in buffer) to 800 V (in 10–50% serum) when this phenomenon was first observed, to prevent the

Copyright © 2011 John Wiley & Sons, Ltd.

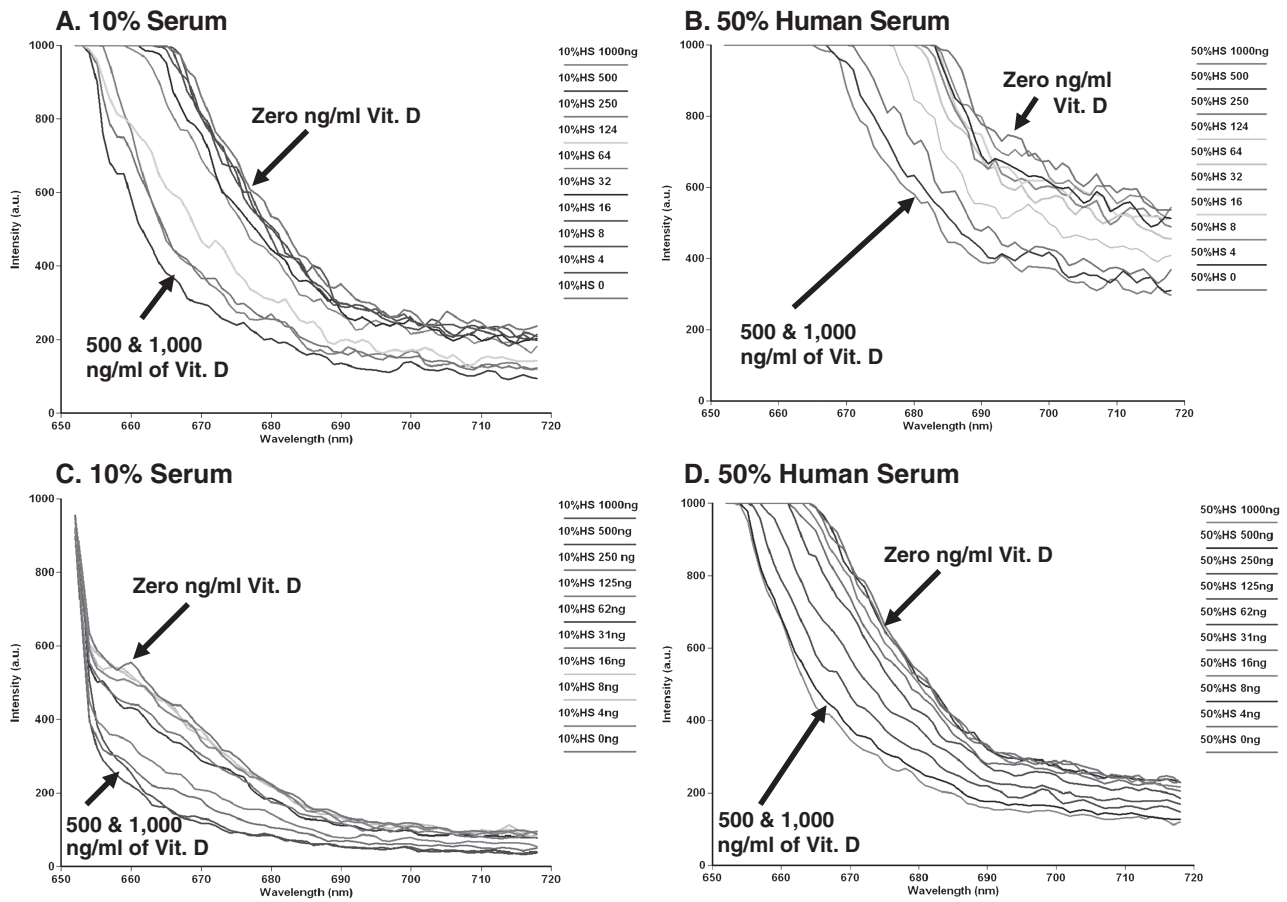

Figure 5. Reproducibility of the fluorescence inversion trend, using two different lots of human serum diluted to 10% and 50% in 1×BB. Data in (A, B) were from the same lot of human serum, as were the data in (C, D). PMT setting was 800 V for all experiments.

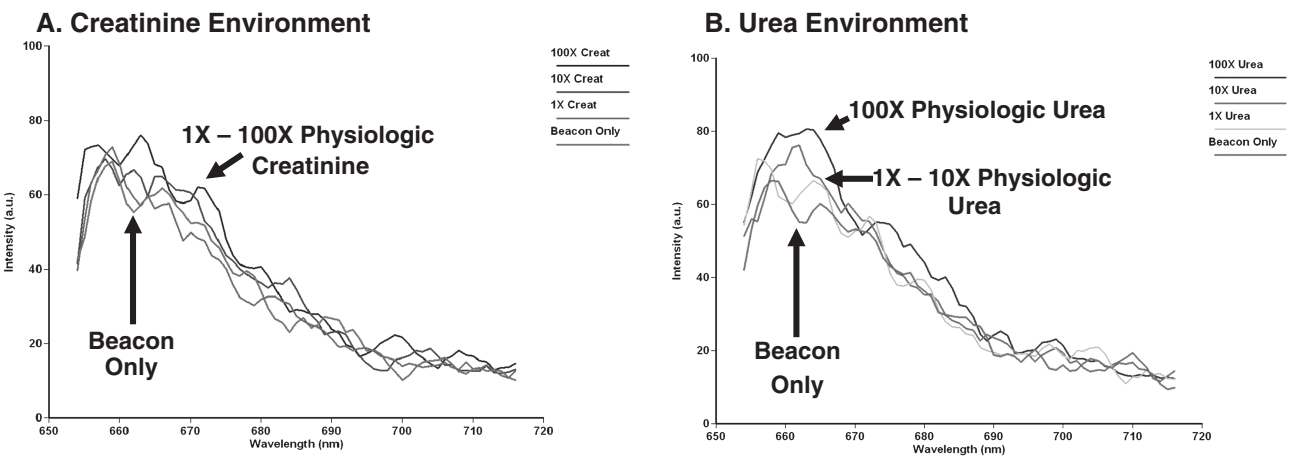

Figure 6. General lack of fluorescence effect on the vitamin D aptamer beacon by (A) 1–100× physiological levels of creatinine and (B) 1–100× physiological levels of urea (levels defined in the text) in buffer (1×BB) and in the absence of human serum. PMT setting was 1000 V for all experiments.

spectra from shooting off-scale. That fact represents at least a five-fold increase in overall fluorescence of the same loop C aptamer beacon system due to serum. While the inverted beacon 'assay' is clearly not very reproducible in terms of absolute values for vitamin D quantification (i.e. comparing results of Fig. 3 with Fig. 5), the same trends were observed in fluorescence behaviour as a function of calcidiol concentrations in three different lots of human serum.

These remarkable observations beg the question of what is causing this drastic increase in overall fluorescence of the beacon system, and how serum leads to an inverted 'lights off' system. Using the principle of Occam's razor, we postulated the

 Copyright © 2011 John Wiley & Sons, Ltd.

LUMINESCENCE
The Journal of Biological and Chemical Luminescence

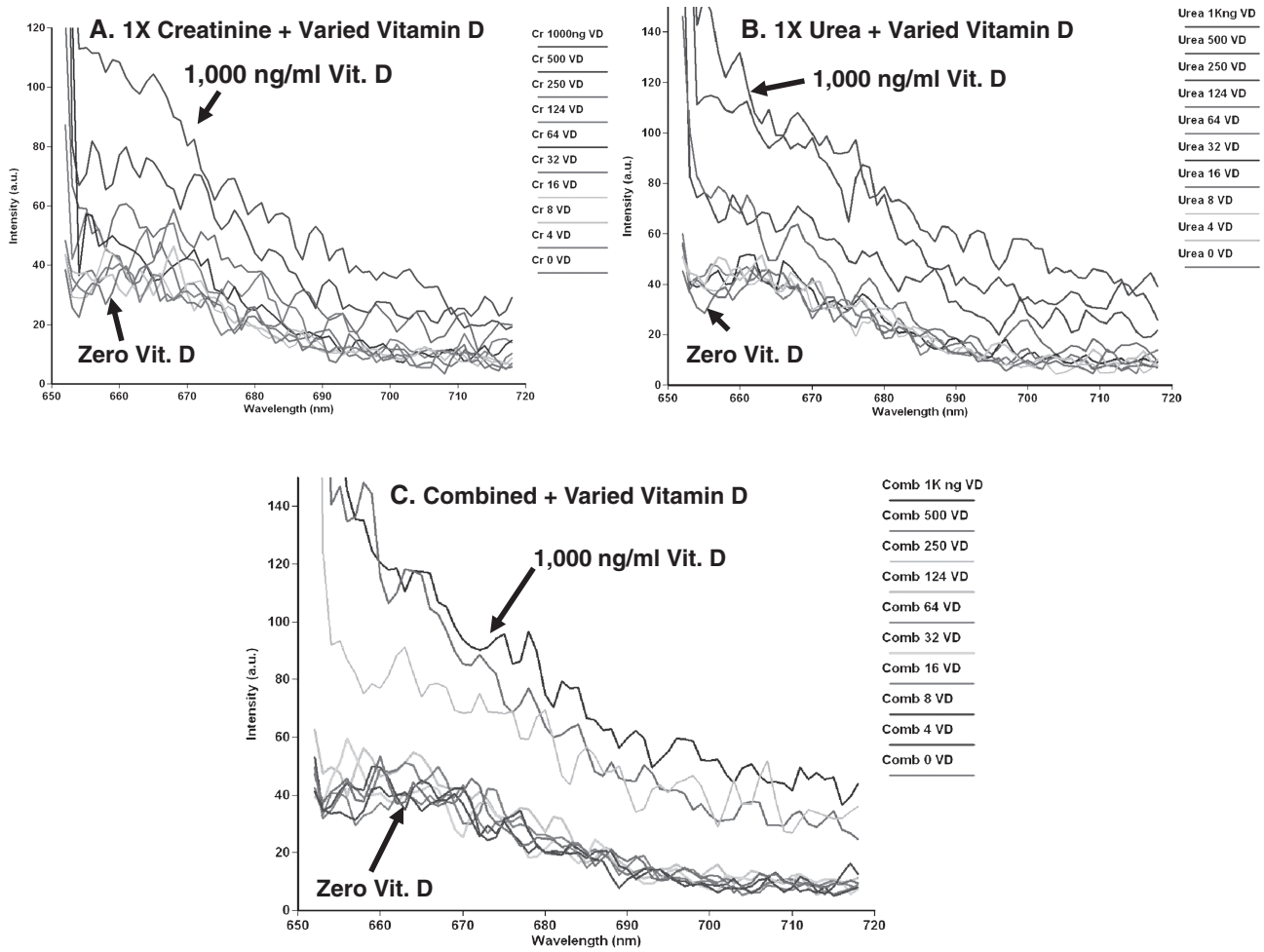

Figure 7. Results of the aptamer beacon vitamin D (calcidiol) titration studies in the range 4–1000 ng/mL, repeated in the presence of: (A) 1× creatinine; (B) 1× urea; and (C) combined 1× creatinine and 1× urea in buffer without human serum. PMT setting was 1000 V for all experiments.

simplest and most logical explanation to be opening and dequenching of the aptamer beacon's duplex stem region by hydrogen bond-destabilizing substances. Serum contains low mg/dL levels of both creatinine and urea, which we postulated might compete with the nucleotides in the duplex region of a beacon for hydrogen-bonding and base-pairing opportunities. Low levels of creatinine and urea have both been shown to destabilize double-stranded stem regions of oligonucleotides (14,15) and disrupt primer–template annealing in PCR systems at levels as low as 50 mmol/L (14). If one examines the structure of creatinine, it becomes apparent that it has an entire 'face' or side that closely mimics that of guanine and cytosine (due to its guanido- and imidazole-like nature) and should therefore readily compete with G or C in the very short duplex stem region of our aptamer beacon, thereby leading to fluorophore and quencher separation and increased light output.

The empirical evidence presented in Fig. 6, in the absence of serum proteins, indicates that high-range physiological (1×) levels of creatinine and urea, alone or in combination, while possibly enhancing fluorescence slightly, cannot account for the drastic fluorescence inversion phenomenon we report here. Levels of creatinine and urea in urine are much higher and should be considered as factors in aptamer assays conducted in urine. These small molecules can be removed from urine by size-exclusion desalting columns prior to aptamer-based analyses, if necessary, but were not a factor at levels found in human serum for our vitamin D aptamer beacon experiments.

Serum contains other substances, especially proteins, which also have high affinity for vitamin D, but perhaps these are out-competed by the loop C aptamer beacon, leading to the 'lights off' response. Ray *et al.* (9) cite the range of DBP and albumin dissociation constants for vitamin D analogues and metabolites to be 10^8–10^{11} mol/L. Therefore, if our hypothetical model to explain the fluorescence switch seen herein is correct, then the loop C aptamer beacon should have a comparable or lower K_d for the vitamin D family of compounds, since the aptamer beacon appears to compete for free vitamin D at low levels in human serum samples (e.g. Figs. 3, 5). As Fig. 7 clearly illustrates, human serum is a necessary component to enable the fluorescence inversion phenomenon, because when all other components are added (i.e. the aptamer beacon, 1× creatinine and 1× urea, alone or combined, and varied levels of vitamin D), we cannot reproduce the inversion to 'lights off' fluorescence behaviour without human serum.

Ray *et al.* (9) also describe some interesting hydrophobic binding pocket interactions of a fluorescent dye, 2-*p*-toluidinyl-6-sulphonate (TNS), which dramatically enhances the intrinsic fluorescence of DBP and albumin centred around 510–520 nm. While we cannot rule out a similar TYE 665 dye (bound to the 5′ aptamer beacon's end) competitive effect with vitamin D-like compounds, TYE 665 is not known to bind serum proteins and

 Copyright © 2011 John Wiley & Sons, Ltd.

our observation occurs in the range 660–720 nm vs the lower 510–520 nm peak reported by Ray *et al*. (9).

It has also been suggested that the eight-base beacon may be decomposing rapidly in serum or other biological matrices (17) and that vitamin D may inhibit the responsible serum nuclease, so that when vitamin D levels are low, fluorescence of the system is high, due to disintegrated and dequenched beacons with free TYE 665 dye in solution. Conversely, such a model would allow for fluorescence to be low in the presence of high levels of nuclease-inhibitory vitamin D, because the beacon could exist undamaged and quenched. While we can neither support nor refute this vitamin D nuclease inhibitory hypothesis, it seems unlikely, due to the 3′ and 5′ fluorophore and quencher 'caps', which are known to act as serum exonuclease inhibitors to prevent beacon breakdown (18,19). A specific serum endonuclease activity for this particular beacon sequence might also internally cleave the beacon regardless of protective fluorophore and quencher caps on the ends.

To address these possibilities, we attempted to investigate the effects of 10% and 50% serum on the vitamin D beacon for 30 min at 25°C. We ran unexposed and diluted serum-exposed samples of the beacon on several 4–20% gradient polyacrylamide gels, with and without ethidium bromide staining, but could not visualize the eight-base beacon in any of the lanes. This may be because the beacon is only eight bases in length, folded or quenched, and probably migrates very close to the dye front. Therefore, in the absence of data to confirm or deny this nuclease and vitamin D interaction hypothesis, we consider it to be a potentially viable explanation and continue to investigate it.

In this communication, we are simply reporting a remarkable fluorescence reversal phenomenon and submitting it to the scientific community for further scrutiny. The 'lights on' to 'lights off' switching behaviour induced by serum may be of some use to the scientific community beyond the basic curiosity presented here, and may ultimately lead to useful aptamer beacon assays with enhanced sensitivities and dynamic ranges in serum.

Acknowledgements

This study was funded by a NASA Phase 2 SBIR (Contract No. NNX09CB01C). The authors thank Dr Linda Shackelford and Dr Sara Zwart of the NASA Johnson Space Center (Houston, TX, USA) for advice and guidance throughout the project.

References

1. Hollick MF. Vitamin D status: measurement, interpretation and clinical application. Ann Epidemiol 2009;19:73–8.
2. Hamaguchi N, Ellington A, Stanton M. Aptamer beacons for the direct detection of proteins. Anal Biochem 2001;294:126–31.
3. Stojanovich MN, Prada PD, Landry DW. Aptamer-based folding fluorescent sensor for cocaine. J Am Chem Soc 2001;123:4928–31.
4. Li JJ, Fang X, Tan W. Molecular aptamer beacons for real-time protein recognition. Biochem Biophys Res Commun 2002;292:31–40.
5. Bruno JG, Carrillo MP, Phillips T, Crowell R. Initial development of competitive FRET-aptamer assays for monitoring bone metabolism. J Clin Ligand Assay 2007;30:81–9.
6. Bruno JG, Carrillo MP, Phillips T, Vail NK, Hanson D. Competitive FRET-aptamer-based detection of methylphosphonic acid: a common nerve agent metabolite. J Fluoresc 2008;18:867–76.
7. Bruno JG, Carrillo MP, Phillips T. Development of DNA aptamers to a foot-and-mouth disease peptide for competitive FRET-based detection. J Biomol Technol 2008;19:109–15.
8. Wootton AM. Improving the measurement of 25-hydroxyvitamin D. Clin Biochem Rev 2005;26:33–6.
9. Ray A, Swamy N, Ray R. Cross-talk among structural domains of human DBP upon binding 25-hydroxyvitamin D. Biochem Biophys Res Commun 2008;365:746–50.
10. Bruno JG, Carrillo MP, Phillips T, King B. Development of DNA aptamers for cytochemical detection of acetylcholine. In Vitro Cell Develop Biol Animal 2008;44:63–72.
11. Hofacker IL. Vienna RNA secondary structure server. Nucleic Acids Res 2003;31:3429–31.
12. Santa Lucia J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc Natl Acad Sci USA 1998;95:1460–65.
13. Ozaki H, Nishihira A, Wakabayashi M, Kuwahara M, Sawai H. Biomolecular sensor bases on fluorescence-labeled aptamer. Bioorg Med Chem Lett 2006;16:4381–4.
14. Khan G, Kangro HO, Coates PJ, Heath RB. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. J Clin Pathol 1991;44:360–5.
15. Vallee-Belisle A, Ricci F, Plaxco KW. Thermodynamic basis for the optimization of binding-induced biomolecular switches and structure-switching biosensors. Proc Natl Acad Sci USA 2009;106:13802–7.
16. Kaplan LA, Pesce AJ. *Clinical chemistry: theory, analysis, and correlation*, 2nd edn. St. Louis, MO: CV Mosby, 1989.
17. Chen AK, Behlke MA, Tsourkas A. Avoiding false-positive signals with nuclease-vulnerable molecular beacons in single living cells. Nucleic Acids Res 2007;35:e105.
18. Shaw JP, Kent K, Bird J, Fishback J, Froehler B. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. Nucleic Acids Res 1991;19:747–50.
19. Dougan H, Lyster DM, Vo CV, Stafford A, Weitz JI, Hobbs JB. Extending lifetime of anticoagulant oligodeoxynucleotide aptamers in blood. Nucl Med Biol 2000;27:289–97.

Copyright © 2011 John Wiley & Sons, Ltd.