BOXTO as a real-time thermal cycling reporter dye

ASHRAF I AHMAD

Department of Chemical and Biological Engineering-Molecular Biotechnology, Chalmers University of Technology, 405 30 Göteborg, Sweden

(Fax, 46 31 773 3910; Email, ashraf.ahmad@molbiotech.chalmers.se)

The unsymmetrical cyanine dyes BOXTO (4-[6-(benzoxazole-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride) and its positive divalent derivative BOXTO-PRO (4-[(3-methyl-6-(benzoxazole-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-(3-trimethylammonium-propyl)-quinolinium dibromide) were studied as real-time PCR reporting fluorescent dyes and compared to SYBR GREEN I (SG) (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium). Unmodified BOXTO showed no inhibitory effects on real-time PCR, while BOXTO-PRO showed complete inhibition. Sufficient fluorescent signal was acquired when 0.5–1.0 μM BOXTO was used with RotorGene and iCycler platforms. Statistical analysis showed that there is no significant difference between the efficiency and dynamic range of BOXTO and SG. BOXTO stock solution (1.5 mM) was stable at –20°C for more than one year and 40 μM BOXTO solution was more stable than 5x SG when both were stored at 4°C for 45 days.

[Ahmad A I 2007 BOXTO as a real-time thermal cycling reporter dye; *J. Biosci.* **32** 229–239]

1. Introduction

Symmetrical cyanine dyes typically contain two benzazole moieties connected by a polymethine chain, whereas unsymmetrical cyanine dyes consist of a benzazole group and a quinoline or a pyridine heterocycle connected by a methine bridge. The fluorescence enhancement of unsymmetrical cyanine dyes has been attributed to restriction of the internal motion of the dye molecule upon binding to double-stranded DNA (dsDNA). Typically, the quantum yield of fluorescence increases when torsional motion around the methine bridge is diminished which, in turn, reduces the non-radiative relaxation of the excited singlet state (Serpone and Sahyun 1994; Khairutdinov and Serpone 1997).

BEBO (4-[(3-methyl-6-(benzothiazol-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium iodide) and BOXTO (4-[6-(benzoxazole-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride) are unsymmetrical monovalent cyanine dyes (figure 1) that bind to the minor groove of dsDNA with minimal perturbation of the DNA helix compared with intercalators, and they have very low affinity for single-stranded DNA. These dyes have a crescent shape that fits the curvature of the minor groove, and are cations which stabilize binding to the negatively charged DNA (Karlsson *et al* 2003; Karlsson *et al* 2003; Eriksson *et al* 2003).

The chemical difference between BEBO and BOXTO is the benzoxazole moiety in place of pyridinium

Keywords. BOXTO; BOXTO-PRO; real-time PCR; unsymmetrical cyanine dyes

Abbreviations used: BEBO, (4-[(3-methyl-6-(benzothiazol-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-pyridinium iodide); BOXTO, (4-[6-(benzoxazol-2-yl-(3-methyl-)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride); BOXTO-PRO, (4-[(3-methyl-6-(benzoxazole-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-(3-trimethylammonium-propyl)-quinolinium dibromide); DMSO, dimethyl sulphoxide; dsDNA, double-stranded DNA; LNA, locked nucleic acid; NTC, non-template control; PCR, polymerase chain reaction; SYBR GREEN I, (SG) (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium).

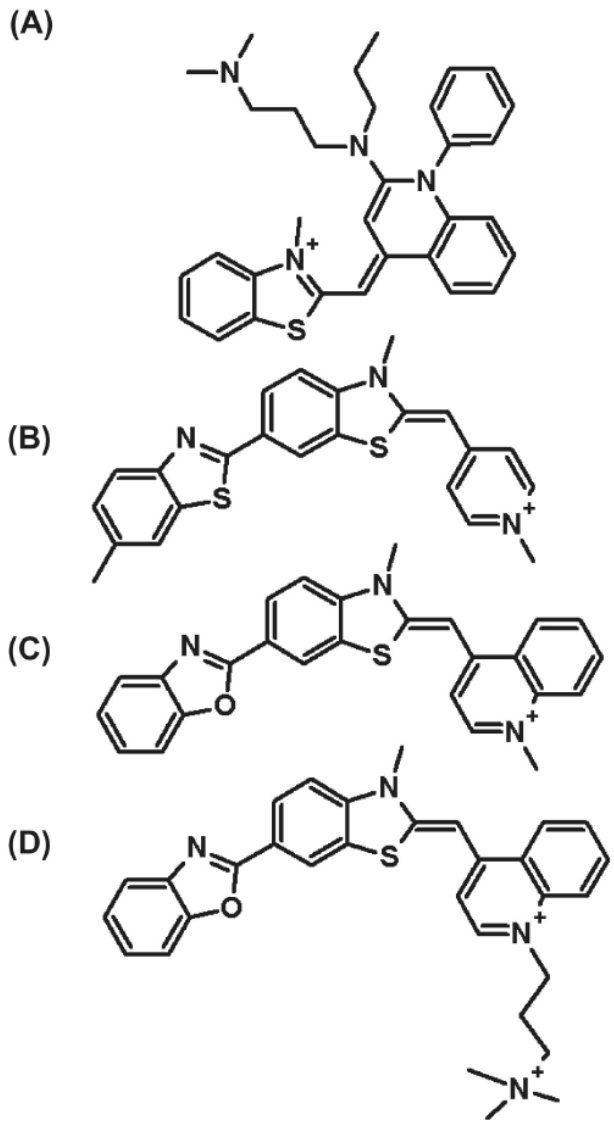

Figure 1. Chemical structures of **(A)** SG, **(B)** BEBO, **(C)** BOXTO and **(D)** BOXTO-PRO.

(figure 1). This benzoxazole moiety increases the quantum yield of BOXTO to 0.52 when bound to mixed-sequence DNA, and increases fluorescence intensity 300-fold more than that exhibited when BEBO is bound to the same DNA molecules. Unsymmetrical cyanine dyes (e.g. BEBO and BOXTO) have low solubility at high concentrations; therefore, the positive divalent version BOXTO-PRO (4-[(3-methyl-6-(benzoxazole-2-yl)-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene)]-1-(3-trimethylammo-nium-propyl)-quinolinium dibromide) (figure 1) was synthesized to enhance solubility in stock solutions without exerting any effect on spectral behaviour (Karlsson *et al* 2003**,** 2005).

SG (2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium) carries a phenyl group at position one of the quinolinium ring and an N-(3-dimethylaminopropyl)-N-propylamino residue at another position of the quinolinium ring (figure 1); therefore, SG carries two positive charges under standard conditions. Positive charges contribute to the high binding affinity for dsDNA, which was another reason to modify BOXTO to the positive divalent form BOXTO-PRO (Zipper *et al* 2004). A comparison study between SG and SYTO9 showed that SYTO9 can be used in real-time PCR assays over a broader range of dye concentrations without causing PCR inhibition; in addition, its stability was comparable with that of SG (Paul *et al* 2005).

The detection of nucleic acids by fluorescent dyes during PCR in a real-time format has become increasingly important for a variety of analytical and diagnostic applications (Wittwer *et al* 1997; Vitzthum and Bernhagen 2002). BEBO was recently tested as a DNA reporter dye in real-time PCR, and was compared with SG (Martin *et al* 2003). BEBO has disadvantages: (i) the dsDNA-bounded BEBO complex has maximum absorbance at 467 nm and emission at 492 nm, for which most of the present commercial real-time PCR platforms are not optimized, and (ii) the Ct value measured by BEBO was 4 cycles higher than that measured by SG (Martin *et al* 2003).

Recently, BOXTO was used as quality control dye (not for quantification) to distinguish between false-positive (primer dimers) and actual products by combining a sequence-specific probe and BOXTO in the same reaction. Results showed that BOXTO can be used together with both TaqMan probes and locked nucleic acid (LNA) probes without any inhibitory effect on PCR. Signals acquired by the probe were used to quantify the PCR product while that acquired by BOXTO dsDNA binding was used to generate a melting curve (Lind *et al* 2006).

Since the past decade, SG is considered the best fluorescent dye that can be used to quantify PCR products in real-time format (Wittwer *et al* 1997; Morrison *et al* 1998); therefore, SG is considered the standard and reference dye in the present study, which focused on studying the ability of BOXTO and its derivative BOXTO-PRO to be used in detecting and quantifying amplified DNA during PCR in a real-time format, and how close the new dye's efficiency and dynamic range was to that of SG.

2. Materials and methods

2.1 *Primers construction*

Table 1 shows all the primer sequences, amplicon sizes, targeted genes and templates used in this study. Primer 3

Table 1. Primers used in the study.

Gene	Primer sequence	Template	Product size
Factor B	5′-CCATGGCCAGCTCTCCTAAC-3′	PCR product	229 bp
	5′-CATTCCGCAGACTTGGATGC-3′		
ADH3	5′-CGACCGCTGATGCTATTC-3′	PCR product	169 bp
	5′-AAGGAACCCAAGCCACCT-3′		
Hb-β	5′-GCACCTTTGCCACACTGAG-3′	Human genomic DNA	288 bp
	5′-ATGCTCAAGGCCCTTCATAATA-3′		
ALAS-5	5′-CTGGAGCCCTGGAGTCTGT-3′	Human cDNA	298 bp
	5′-ATCTGGGGTGTGTGGTGAG-3′		
TEF-1α	5′-GCTATGCCCCTGTATTGGAT-3′	Human cDNA	309 bp
	5′-GAGCTTTCTGGGCAGACTTG-3′		
GAPD	5′-AAGGGCATCCTGGGCTAC-3′	Human cDNA	304 bp
	5′-AGGGGAGATTCAGTGTGGTG-3′		
Cathepsin D	5′-CTGCCACCCTACCTGTTCA-3′	Human cDNA	253 bp
	5′-ATGTCAGCTGGGGCTCTCA-3′		
FOXD-3	5′-GCATCGAGAACATCATAGGTG-3′	Human cDNA	246 bp
	5′-CTGCGGGCTGCAGAAACT-3′		

Factor B, mouse platelet-derived growth factor B chain exons 3 and 4; ADH3, *Saccharomyces cerevisiae* aldehyde dehydrogenase 3; Hb-β, haemoglobin beta; ALAS-5, 5-aminolaevulinate synthase; TEF-1α, translation elongation factor 1 alpha; GAPD, glyceraldehyde-3-phosphate dehydrogenase; FOXD-3, Forkhead box protein D3

(*http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi*) was used to design all primers, groups of primers were further analysed and tested by an oligoanalyser (*http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx*) and netprimer (*http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html*) to select the best primers for lowest self- and cross-reactivity, in addition to other criteria.

2.2 *Real-time PCR*

Thermal cycling set-ups varied according to the template used in each of 3 groups: (i) PCR products template; 15 s at 95°C (denaturation), 20 s at 60°C (annealing) and 25 sec at 72°C (extension) for the factor B template; and for the ADH3 template; 15 s at 95°C, 20 sec at 58°C and 20 s at 72°C; (ii) human genomic DNA; 20 s at 95°C, 25 s at 58°C and 35 s at 72°C; and (iii) human cDNA; 15 s at 95°C, 20 s at 58°C and 30 s at 72°C. The melting curve was generated (for all types of templates) by thermal denaturation from 60°C to 95°C by increasing the temperature in steps of 0.5°C, with a 10 s gap between measurements.

The PCR mixture contained 3 mM $MgCl_2$ (Sigma), 200 mM dNTPs (Sigma), 0.4 mM primers (MWG Biotech), 0.03 U/μl Taq polymerase (Sigma), and 0.2 x SG (Molecular Probes). BOXTO and BOXTO-PRO were recently synthesized at the Department of Chemistry and Bioscience, Chalmers University of Technology (Karlsson *et al* 2003; Karlsson *et al* 2003; Eriksson *et al* 2003). The dyes were dissolved in dimethyl sulphoxide (DMSO) as 1.5 mM first stock solution then further diluted with milliQ water to 40 μM as second stock for daily use. Different concentrations of BOXTO (0.1 μM, 0.5 μM, 1.0 μM, 2.5 μM) and BOXTO-PRO (2.0 μM, 4.0 μM, 8.0 μM) were tested for determination of optimal concentration.

RotorGene (Corbett Research) and iCycler (BioRad) were used for real-time thermal cycling and denaturation for amplification and melting curve generation. For all templates mentioned, the signal was acquired at the end of the extension step; SG signal was acquired by the FAM channel and BOXTO signal was acquired by the JOE (carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein) channel.

3. Results

3.1 *Determination of optimal dye concentration*

Real-time PCR conditions for the factor B template were used to test several concentrations of BOXTO (0.1 μM, 0.5 μM, 1.0 μM, 2.5 μM), BOXTO-PRO (2.0 μM,

Table 2. Optimal concentration determination for BOXTO and BOXTO-PRO using the factor B PCR system.

Concentration	Ct	Ct NTC	Efficiency	Correlation coefficient
SG				
0.2x	14.51	26.41	0.91	0.9999
BOXTO				
2.5 μM	18.20	31.73	0.76	0.9844
1.0 μM	**16.38**	**30.65**	**0.95**	**0.9995**
0.5 μM	**16.40**	**30.47**	**0.94**	**0.9996**
0.1 μM	16.25	29.5	0.91	0.9995
BOXTO-PRO				
8.0 μM	32.32	40.00	0.61	0.9970
4.0 μM	**16.54**	**32.72**	**0.97**	**0.9999**
2.0 μM	17.21	32.19	0.97	0.9968

4.0 μM, 8.0 μM) and SG (reference dye) with a 10-fold diluted factor B template (replica of 3 concentrations). Efficiency and correlation coefficient were calculated by the software provided with the RotorGene platform used. Efficiency, Ct and correlation coefficient calculated by the SG signal were used as reference values to determine the optimal concentration for BOXTO and BOXTO-PRO. Recommended BOXTO and BOXTO-PRO concentrations are 0.5–1.0 μM and 4.0 μM, respectively (table 2).

BOXTO (0.75 μM) and BOXTO-PRO (4.0 μM) were tested with one concentration of the ADH3 template to check the compatibility of the dyes with another template. BOXTO-PRO has a higher Ct value than BOXTO (figure 2a). Melting curve analysis showed that BOXTO-PRO inhibited amplification of the ADH3 template; the only amplified form was primers and the only product was primer dimers (figure 2b); therefore, BOXTO was chosen to be compared with SG as a real-time PCR reporter dye.

3.2 *BOXTO as a quantitative PCR reporter dye*

BOXTO was tested with 10-fold serial dilutions of the factor B template (2×10^3 – 2×10^7 copies/μl with RotorGene and 2.5×10^3 – 2.5×10^6 copies/μl with iCycler). Signals acquired were analysed by the platform's software to generate a standard curve and the PCR efficiency and correlation coefficient were calculated. Figures 3 and 4 show that BOXTO can be used efficiently in quantitative real-time PCR with high efficiency (95% by RotorGene and 95.6% by iCycler) and correlation coefficient (0.9999 by RotorGene and 0.999 by iCycler), in addition to the ability of BOXTO in real-time thermal denaturation to generate a melting curve that distinguishes positive results from false-positive (primer dimers) ones. At the same time, these results show the reproducibility of BOXTO with different real-time PCR platforms (RotorGene and iCycler).

3.3 *BOXTO reproducibility*

Real-time PCR for factor B was repeated 13 times on RotorGene; for each repetition a duplicate of 3 serially diluted factor B templates were used. The RotorGene software was used to analyse signals acquired and to calculate PCR efficiency, correlation coefficient and Ct for the template and non-template control (NTC). Data generated were statistically analysed to calculate the average, variance and standard deviation by the Microsoft Office Excel 2003 software. Table 3 shows that the results for BOXTO had good reproducibility; the average efficiency and correlation coefficient were 94.2% ± 3.9% and 0.9992 ± 0.0006, respectively, with low variance, while a higher standard deviation and variance were observed for the NTC Ct value. This is because the NTC was random and varied from one run to another (Wittwer *et al* 1997; Ball *et al* 2003).

3.4 *Comparing BOXTO with SG*

All previously mentioned results showed that BOXTO worked with quantitative and qualitative real-time PCR as a reporter dye, but the important question is: is it possible for a researcher to use BOXTO instead of SG? And how close are the results generated by BOXTO to those generated by SG?

Real-time PCR was repeated 9 times (for both BOXTO and SG) with duplicates of 3 dilutions of the factor B template. The results were then analysed by the RotorGene software, where all experimental parameters were the same except the dye. Both sets of samples were tested at the same time. PCR efficiency, Ct (template) and Ct (NTC) were measured at a fixed threshold. The results with BOXTO were compared with those with SG, which was considered a standard and reference real-time PCR dye. Efficiency and dynamic range (difference between Ct [NTC] and Ct [template]) for both dyes were statistically compared by the Fisher *F*-test (comparing significant differences between variances) followed by the Student *t*-test (comparing significant differences between means) using the data analysis routine of Microsoft Office Excel 2003 software. Table 4 shows that experimental *F* and *t* values are lower than tabulated *F* and *t* values (at 95% confidence interval) for the efficiency and dynamic range; therefore, we have no evidence to reject the null hypothesis and, as a result, there is no significant difference between the variances and the means for both dyes.

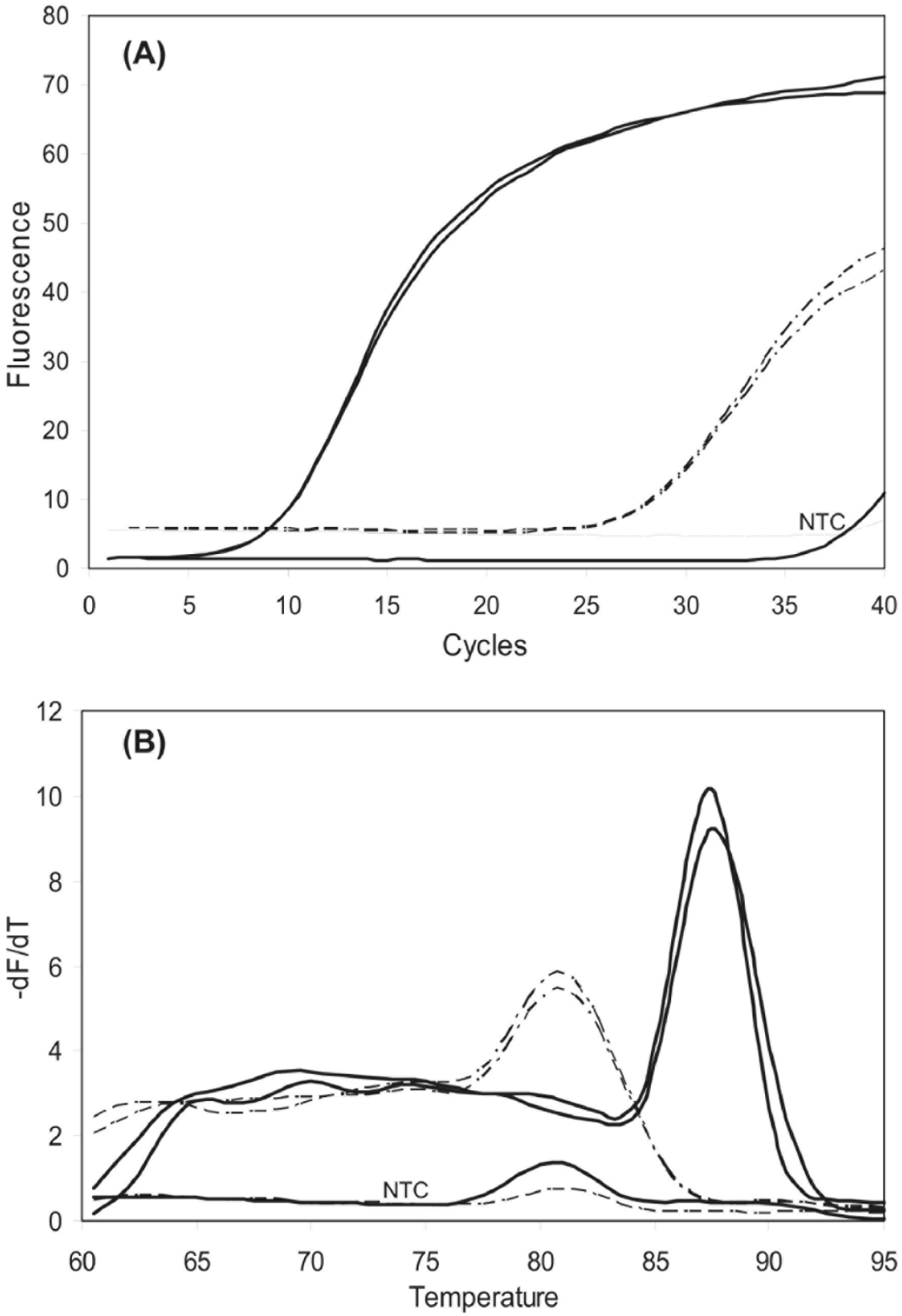

Figure 2. **(A)** Raw amplification data of the ADH3 template with BOXTO and BOXTO-PRO as reporter dyes. **(B)** Inverted first derivative melting curve. (------) BOXTO-PRO, (——) BOXTO

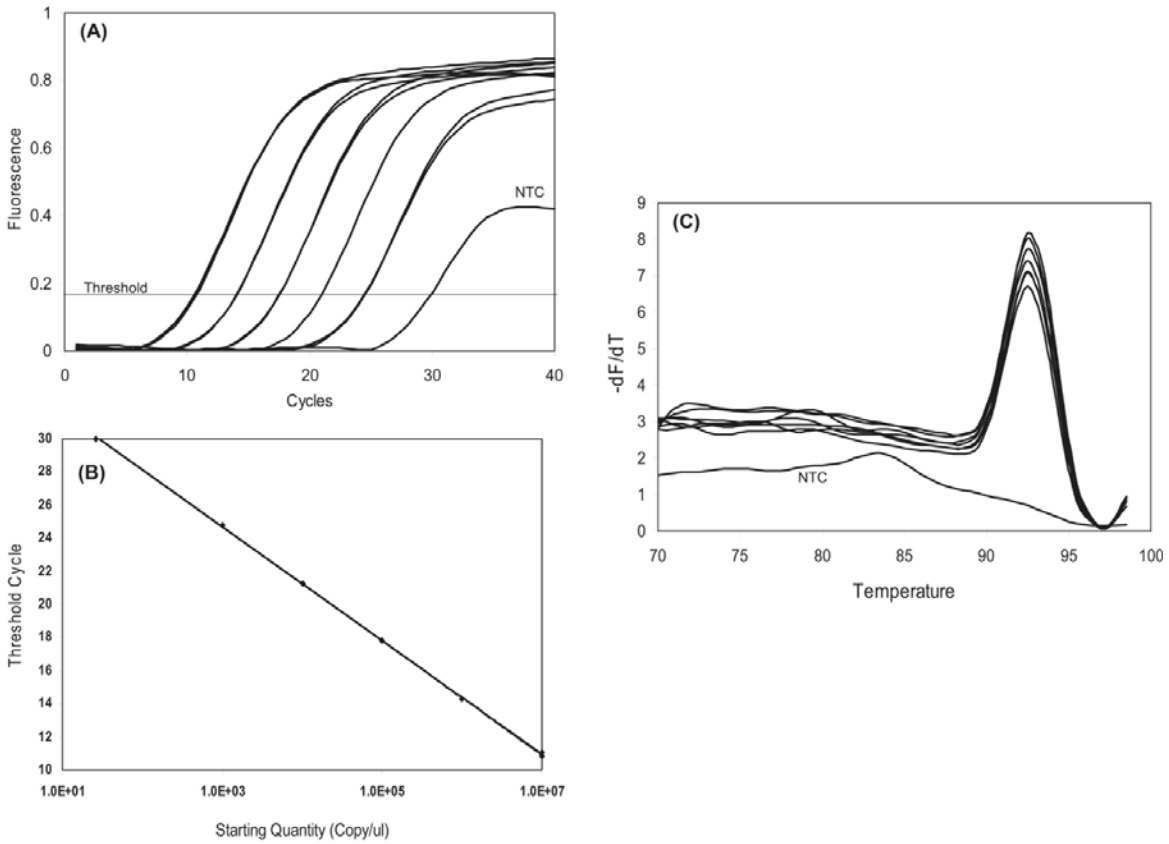

Figure 3. **(A)** Testing serial dilutions (2×10^3 – 2×10^7 copy/μl) of template B using BOXTO with RotorGene. **(B)** Standard curve indicating reaction efficiency = 0.95 and $r^2 = 0.9999$. **(C)** Inverted first derivative melting curve with a melting peak maximum at 92.5ºC.

3.5 *BOXTO compatibility with genomic and cDNA*

Human genomic and cDNA were used as starting materials (templates) for several genes (table1) with BOXTO and a previously mentioned PCR mix. RotorGene was used for real-time thermal cycling and data analysis. BOXTO was efficient for genomic and cDNA with a low background signal that can be overcome by PCR product accumulation (figure 5).

3.6 *Stability study*

BOXTO stock solutions (5x SG and 40 μM) were prepared in milliQ water and both stocks were used in standard real-time PCR at two time intervals, first at zero incubation time (fresh stock) and the second after 45 days' incubation at 4°C. Analysis of the results by RotorGene's software showed that 40 μM BOXTO stock solution is more stable than 5x SG for (at least) 45 days' incubation at 4°C (figure 6).

4. Discussion

BOXTO and its positive divalent derivative BOXTO-PRO have been studied as thermal amplification and denaturation reporter dyes in a real-time format. BOXTO and BOXTO-PRO succeeded as real-time PCR reporter dyes with a factor B template, according to Ct, efficiency and correlation coefficient values compared with those calculated with SG (table 2). The calculated Ct value for BOXTO-PRO, when used with the ADH3 template, was very high compared with that generated from BOXTO (figure 2A). As shown in figure 2B, the negative first derivative melting curves of the ADH3 template, the melting peak maximum for BOXTO-PRO showed that the only amplified product is primer dimers and obviously BOXTO-PRO inhibits template denaturation, probably by strong electrostatic binding due to the presence of two positive charges on the dye molecule (figure 1D). At the same time, this could be sequence specific, because BOXTO-PRO did not work in the same way with the factor B template. In contrast, the two positive charges gave

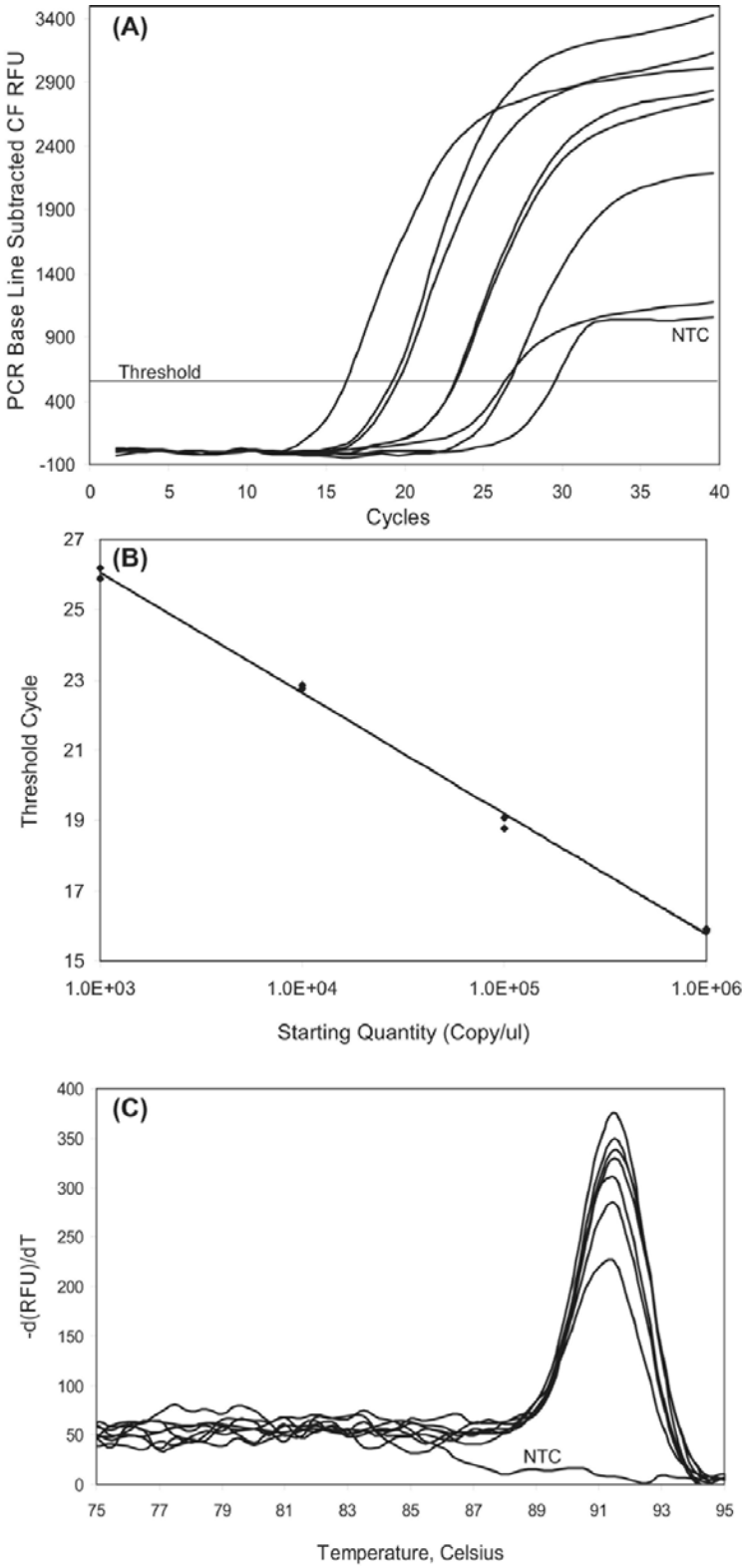

Figure 4. **(A)** Testing serial dilutions of template B (2.5×10^3 – 2.5×10^6 copies/μl) using BOXTO with iCycler. **(B)** Standard curve indicating reaction efficiency = 95.6% and $r^2 = 0.999$. **(C)** Inverted first derivative melting curve with a melting peak maximum at 91.5ºC.

Table 3. Reproducibility study.

Test no.	Ct	Ct NTC	Efficiency (%)	Correlation coefficient
1	16.38	29.75	95	0.9989
2	16.58	30.65	95	0.9995
3	16.59	31.45	90	0.9996
4	17.00	29.80	98	0.9979
5	16.73	30.90	95	0.9983
6	16.89	30.60	91	0.9997
7	16.91	31.61	92	0.9997
8	17.07	31.35	91	0.9996
9	17.00	31.00	93	0.9992
10	16.95	30.50	92	0.9993
11	16.63	31.00	91	0.9992
12	16.52	27.93	104	0.9983
13	16.61	27.54	98	0.9999
Average	**16.75**	**30.31**	**94.2**	**0.9992**
Standard deviation	**0.221**	**1.27**	**3.9**	**0.0006**
Variance	**0.049**	**1.62**	**0.15**	**4.0×10^{-7}**

SG its unique behaviour as a real-time PCR reporter dye (figure 1A).

Fluorescent signals generated by BOXTO binding to dsDNA is detectable by both RotorGene (figure 3) and iCycler (figure 4) due to its moderate molar absorption coefficient (e) and quantum yield, which equals 37,800 M-1cm-1 and 0.52, respectively (Karlsson *et al* 2003).

Significant increase in the fluorescence signal is observed when a suitable dye/base pair ratio (dbprs) is applied. SG starts intercalation with dsDNA, followed by surface binding at dbprs above ~0.15 to generate a significant increase in fluorescence (Zipper *et al* 2004), while the increase in fluorescence quantum yield on BOXTO binding to DNA is 50-fold at dbprs 0.01 (Karlsson *et al* 2003). On comparing the 2 ratios of the dyes it is obvious that SG is more sensitive to shorter DNA sequences, while lower concentrations of BOXTO can be used to achieve significant signals. Generating a signal during real-time PCR depends on the physical and chemical features of an individual dye. SG has a higher molar absorption coefficient (73,000 M-1cm-1) and quantum yield (0.8) than BOXTO (Zipper *et al* 2004), and SG is positive divalent. These unique characteristics of SG lead to a lower Ct value for the template and, at the same time, a lower Ct value for the NTC (earlier detection);

Table 4. Comparison between BOXTO and SG.

SYBR Green I				BOXTO			
Efficiency	Ct	Ct^{NTC}	Dynamic range ($Ct^{NTC} - Ct$)	Efficiency	Ct	Ct^{NTC}	Dynamic range ($Ct^{NTC} - Ct$)
0.95	14.33	29.50	15.17	0.95	16.58	31.00	14.42
0.97	14.49	30.14	15.65	0.93	16.60	31.15	14.55
0.96	14.55	26.08	11.53	0.98	16.41	27.30	10.89
0.92	14.43	26.74	12.31	0.94	16.20	27.90	11.7
1.05	13.32	23.44	10.12	0.94	16.05	26.78	10.73
0.97	14.37	26.34	11.97	0.97	16.15	28.50	12.35
0.98	14.51	26.03	11.52	0.96	16.10	27.68	11.58
0.92	14.17	26.00	11.83	0.93	15.90	27.13	11.23
1.03	14.01	27.76	13.75	0.98	15.62	28.67	13.05
Statistical analysis							
Statistical test			Experimental value			Tabulated value (95%)	
F-test for efficiency			0.038			4.43	
F-test for dynamic range			0.515			4.43	
t-test for efficiency			0.259			2.12	
t-test for dynamic range			0.638			2.12	

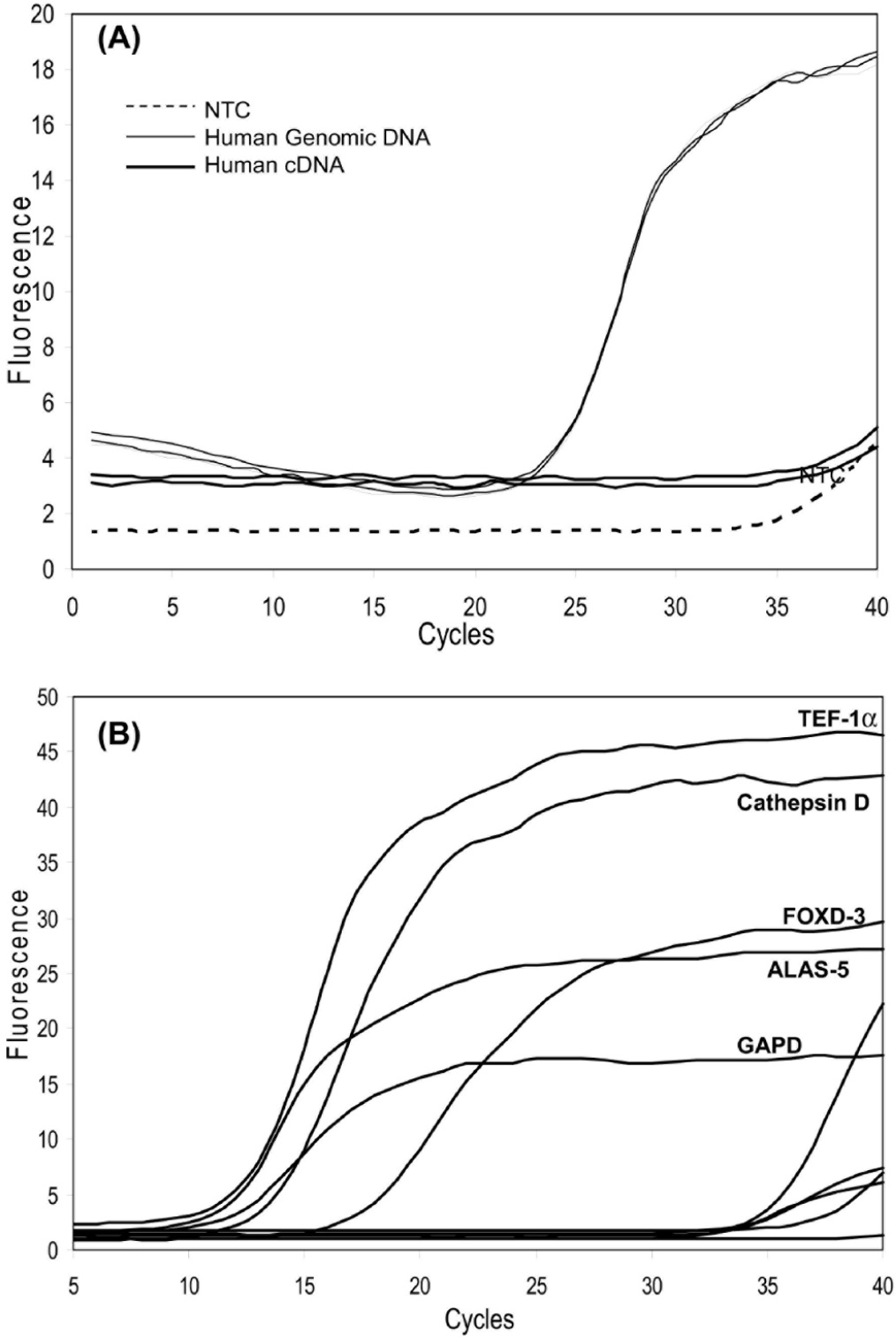

Figure 5. Testing BOXTO with **(A)** human genomic DNA and **(B)** human cDNA as PCR templates. ALAS-5, 5-aminolaevulinate synthase; TEF-1α, translation elongation factor 1 alpha; GAPD, glyceraldehyde-3-phosphate dehydrogenase; FOXD-3, Forkhead box protein D3.

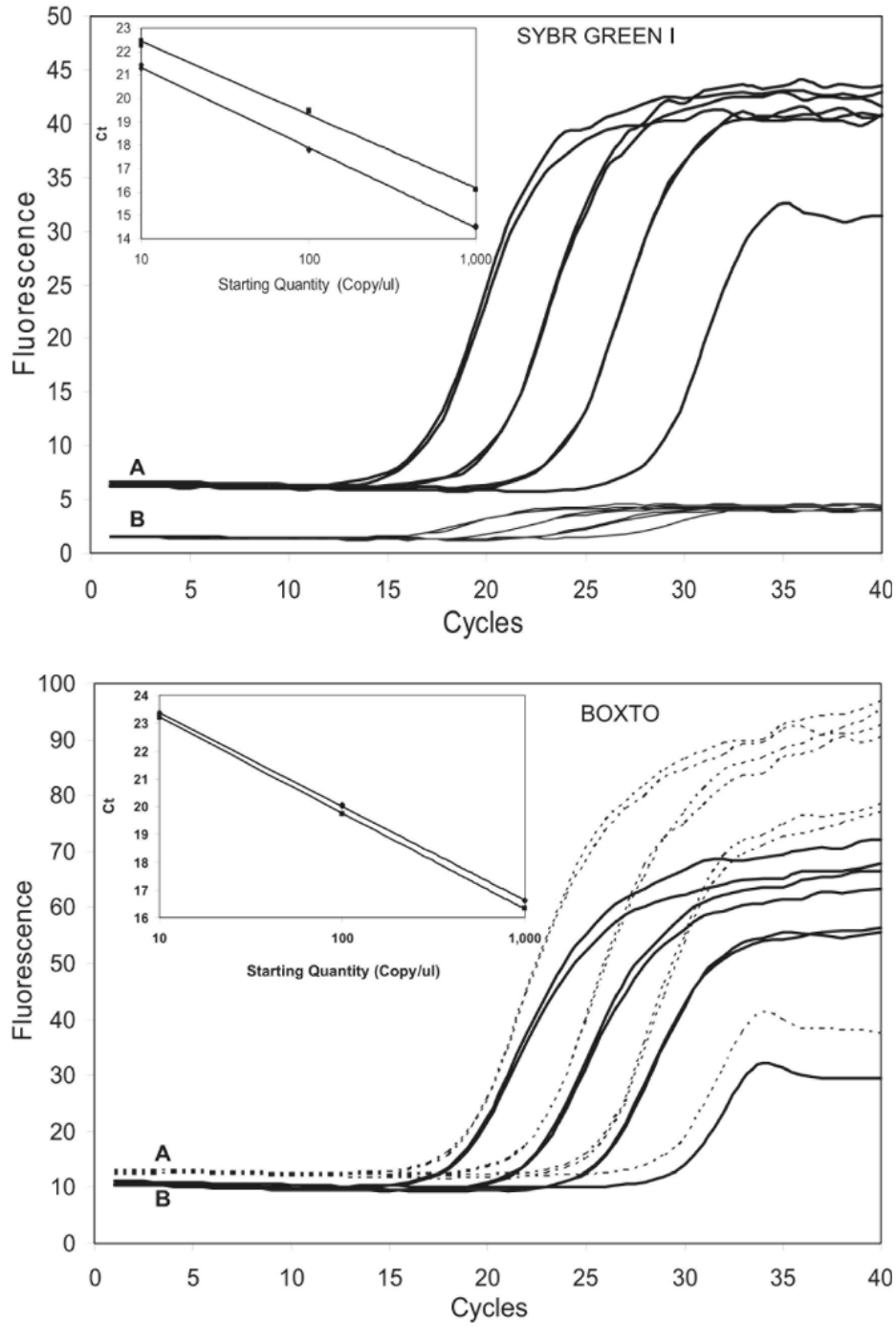

Figure 6. Comparing SG and BOXTO stabilities. (**A)** is zero and (**B)** is 45 days' incubation of stock dyes at 4°C. Curves show the raw data of PCR reaction and insets show the standard curve. For SG; efficiency A = 0.96, r^2 A = 0.9992, efficiency B = 1.1 and r^2 B = 0.9976. For BOXTO; efficiency A = 0.98, r^2 A = 0.9999, efficiency B = 0.93 and r^2 B = 0.9998.

therefore, the dynamic range for a specific amplicon and primers is conserved under fixed concentrations and reaction conditions. The moderate physical and chemical features of BOXTO results in higher Ct values for the template and NTC, and the dynamic range is also conserved in the same manner as that of SG. By comparing the dynamic range for BOXTO with that for SG we found that they are close to each other with some differences; further statistical

analysis showed that these differences are insignificant (table 4). In addition, statistical analysis for PCR efficiencies of SG and BOXTO showed that there is no significant difference between the two dyes; therefore, we conclude that BOXTO can replace SG and can be used for qualitative and quantitative real-time PCR with high stability. We conclude that BOXTO can be efficiently used in real-time thermal cycling with all types of templates (PCR products, genomic DNA and cDNA). The results generated are highly reproducible, with no statistically significant differences between efficiency, correlation coefficient and Ct values generated by BOXTO and SG. In spite of being stable for a long time, BOXTO showed poor stability when it was mixed with the PCR master mixture and incubated at 4°C for 24 h (data not shown). Therefore, further optimization is recommended to enable researchers to prepare a stable PCR master mixture containing BOXTO.

Acknowledgements

Financial support from Chalmers University of Technology is thankfully acknowledged, as well as Dr Jonas H Karlsson and Dr Gunnar Westman for the BOXTO and BOXTO-PRO samples.

References

Ball T B, Plummer F A and HayGlass K T 2003 Improved mRNA quantitation in LightCycler RT-PCR; *Int. Arch. Allergy Immunol.* **130** 82–86

Eriksson M, Karlsson H J, Westman G and Åkerman B 2003 Groove-binding unsymmetrical cyanine dyes for staining of DNA: dissociation rates in free solution and electrophoresis gels; *Nucleic Acids Res.* **31** 6235–6242

Eriksson M, Mehmedovic M, Westman G and Akerman B 2005 Time-resolved electrophoretic analysis of mobility shifts for dissociating DNA ligands; *Electrophoresis* **26** 524–32

Khairutdinov R F and Serpone N 1997 Photophysics of cyanine dyes: subnanosecond relaxation dynamics in monomers, dimers, and H- and J-aggregates in solution; *J. Phys. Chem. B.* **101** 2602–2610

Karlsson H J, Lincoln P and Westman G 2003 Synthesis and DNA binding studies of a new asymmetric cyanine dye binding in the minor groove of [poly(dA-dT)]2; *Bioorg. Med. Chem.* **11** 1035–1040

Karlsson H J, Eriksson M, Perzon E, Åkerman B, Lincoln P and Westman G 2003 Groove-binding unsymmetrical cyanine dyes for staining of DNA: syntheses and characterization of the DNA-binding; *Nucleic Acids Res*. **31** 6227–6234

Lind K, Anders S, Neven Z and Mikael K 2006 Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis; *BioTechnique* **40** 315–319

Martin B, Jonas K, Gunnar W and Mikael K 2003 A new minor groove binding asymmetric cyanine reporter dye for real-time PCR; *Nucleic Acids Res.* **31**, e45

Morrison T M, Weis J J, Wittwer C T 1998 Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification; *Biotechniques* **24** 954–962

Paul T M, Steven G and Christopher P S 2005 Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the eVect of dye concentration on amplification and DNA melting curve analysis; *Anal. Biochem.* **340** 24–34

Serpone N and Sahyun M R V 1994 Photophysics of dithiacarbocyanine dyes: subnanosecond relaxation dynamics of a dithia-2,2'-carbocyanine dye and its 9-methyl-substituted meso analog; *J. Phys. Chem.* **98** 734–737

Vitzthum F and Bernhagen J 2002 SYBR Green I: an ultrasensitive fluorescent dye for double-stranded DNA quantification in solution and other applications; *Recent Res. Devel. Anal. Biochem*. **2** 65–93

Wittwer C T, Herrmann M G, Moss A A and Rasmussen R P 1997 Continuous fluorescence monitoring of rapid cycle DNA amplification; *Biotechniques* **22** 130–139

Wittwer C T, Ririe K M, Andrew R V, David D A, Gundry R A and Balis U J 1997 The LightCycler: a microvolume multisample fluorimeter with rapid temperature control; *Biotechniques* **22** 176–181

Zipper H, Brunner H, Bernhagen J and Vitzthum F 2004 Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.* **32** e103

MS received 15 September 2006; accepted 1 December 2006

*e*Publication: 17 January 2007

Corresponding editor: Reiner A Veitia