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Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis

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Abstract

Following the initial report of the use of SYBR Green I for real-time polymerase chain reaction (PCR) in 1997, little attention has been given to the development of alternative intercalating dyes for this application. This is surprising considering the reported limitations of SYBR Green I, which include limited dye stability, dye-dependent PCR inhibition, and selective detection of amplicons during DNA melting curve analysis of multiplex PCRs. We have tested an alternative to SYBR Green I and report the first detailed evaluation of the intercalating dye SYTO9. Our findings demonstrate that SYTO9 produces highly reproducible DNA melting curves over a broader range of dye concentrations than does SYBR Green I, is far less inhibitory to PCR than SYBR Green I, and does not appear to selectively detect particular amplicons. The low inhibition and high melting curve reproducibility of SYTO9 means that it can be readily incorporated into a conventional PCR at a broad range of concentrations, allowing closed tube analysis by DNA melting curve analysis. These features simplify the use of intercalating dyes in real-time PCR and the improved reproducibility of DNA melting curve analysis will make SYTO9 useful in a diagnostic context.
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Real-time PCR is rapidly becoming a standard method in many diagnostic and research laboratories. The market for this technology continues to expand and there have been many probe-based systems developed to meet this demand, including Taqman probes [1], molecular beacons [2], FRET[1] probes [3], Scorpions [4], and iFRET [5]. An alternative to probe-based detection is the use of fluorescent double-stranded DNA (dsDNA)-specific intercalating dyes. Such a dye, ethidium bromide, was used in the first description of real-time PCR by Higuchi et al. [6,7]. Since then, a relatively small number of dyes, including YO-PRO-1 [8], SYBR Green I [9,10], BEBO [11], and LC Green [12], have been evaluated for use in real-time PCR applications. While probe-based detection methods continue to be developed and improved, progress for intercalating dyes has been relatively slow.

SYBR Green I has become the preferred choice for real-time PCR because it is cost effective compared to probe-based systems, allows the generic detection of amplified DNA, and can be used to differentiate DNA by DNA melting curve analysis [9,10]. However, SYBR Green I also has disadvantages that limit its ease of use. It cannot generally be used in standard PCR buffers without further optimization of conditions and the inclusion of additional reagents to improve reaction efficiency, such as DMSO [13], bovine serum albumin,

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[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; DMSO, dimethyl sulfoxide.

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and Triton X-100 [14]. Depending on the reaction conditions, the dye also appears to be inhibitory to PCR in a concentration-dependent manner [9,15] which can be overcome by increasing the concentration of $MgCl_2$ in the reaction [14,15]. The degradation products of the dye have also been reported to be inhibitory to PCR [14].

DNA melting curve analysis using SYBR Green I is widely applied for the identification of a variety of organisms (e.g., *Leptonema* [16], *Borellia* [17,18], *Legionella* [19], and *Cryptosporidium* [20]). However, the melting temperature (T_m) obtained using this dye can be affected by the concentration of dye [10] and the concentration of DNA [21]. For example, in the study of Ririe et al. [10] a 3 log-fold difference in the number of starting copies of target DNA used in real-time PCR resulted in a T_m difference of more than 1 °C for the same amplicon.

A further limitation of SYBR Green I is that it appears to have limited application for the analysis of multiplex PCR. In a study by Giglio et al. [22], multiplex PCRs for *Vibrio cholerae* and *Legionella pneumophila* were analyzed by DNA melting curve analysis using SYBR Green I. It was found that only one amplicon could be detected by melting curve analysis but that both amplicons were being amplified as determined by agarose gel electrophoresis. This effect appeared to be related to the relative concentrations of the amplified DNA and dye because time-based analysis demonstrated that both amplicons could be detected by melting curve analysis after 20 cycles of PCR but only one amplicon could be detected after 40 cycles of PCR.

The SYTO family of dyes have many properties that make them useful for labeling live cells, including good membrane permeability, low cytotoxicity, and enhanced fluorescence in the presence of DNA (US patent 5,436,134 [23]). Although they are ostensibly DNA-binding dyes, their low toxicity suggests that they may not interfere with cellular processes, such as DNA and RNA synthesis, at the concentrations that are used for staining cells. Little information with regard to their specificity for dsDNA versus single-stranded DNA (ssDNA) or their suitability as dyes for real-time PCR is available. Evaluation of the mode of action of the BacLight Live/Dead kit (Molecular Probes) in our laboratory suggested that SYTO9 is a dsDNA-specific dye. These factors, together with the similarity in excitation/emission maxima in SYTO9 and the FAM channel of the Rotor Gene 3000, have led us to select SYTO9 for evaluation in real-time PCR.

We report herein the novel use of SYTO9 in real-time PCR. This dye supports PCR amplification over a wide range of dye concentrations and produces robust DNA melting curves that are not affected by DNA concentration. In addition, we investigated the inhibitory effect of dye concentration on PCR and the effect of dye concentration on DNA melting curves. These data extend upon the work of Giglio et al. [22] and demonstrate that, unlike SYBR Green I, SYTO9 readily allows the detection of multiple amplicons in a multiplex PCR. The utility of SYTO9 is demonstrated using both prokaryote and eukaryote systems.

Materials and methods

Source of isolates and DNA template preparation

The source of *V. cholerae* O1 El Tor biotype and *L. pneumophila* serotype 1 have been described previously by Giglio et al. [22]. DNA was extracted from cultures using Qiagen DNA-mini spin columns following the manufacturer's methods. The origins and characterization of *Giardia duodenalis* isolates Ad-1, Ad-19, and Ad-23, representative of Assemblages A, B, and F, respectively, and of a *Giardia ardeae* isolate have been described previously [24]. DNA samples from barley (*Horedum vulgare*) varieties Atlas, Atlas68, Proctor, and Shannon were kindly provided by Dr. Brendon King. DNA concentrations for purified extracts were determined by measuring UV absorbance at 260 nm.

Real-time PCR and melting curve analysis of prokaryotic DNA

Real-time reactions were conducted in 100-μl thin-walled tubes and monitored using a Rotor Gene 3000 (Corbett Research, Sydney, Australia). The multiplex PCRs for *V. cholerae* and *L. pneumophila* were performed as described previously [20], except that the *V. cholerae* multiplex assay was modified to include DMSO (5% v/v final concentration), primer H3F was excluded, and the primer concentrations of the remaining four primers were reduced to 0.5 μM each (final concentration). The primer set 27f(5′-AGAGTTTGATYMTGG CTCAG-3′) and 1492r (5′-TACGGYTACCTTGTT ACGACT-3′), modified from Suzuki and Giovannoni [25], was used to amplify a 1.46-kb fragment of the 16S rRNA gene from *V. cholerae* DNA. Each 20-μl reaction mixture contained 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, 2.5 mM $MgCl_2$, 1× PCR buffer II (Applied Biosystems Foster City, CA, USA), 5% v/v DMSO, 1 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 5 μl of genomic DNA. Thermal cycling consisted of an initial denaturation at 95 °C for 10 min followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min.

Dye concentrations of SYTO9 (Molecular Probes, OR) or SYBR Green I used in reactions ranged from 10 to 0.5 μM. The molar concentration of SYBR Green I was calculated using the values determined by Zipper et al. [26], who estimated that a 1× SYBR Green 1 solution represents a dye concentration of 2 μM. Fluorescence

data were acquired on the FAM channel (excitation at 470 nm, detection at 510 nm) at the end of each 72 °C extension step of the PCR. The FAM channel closely matches the excitation and emission maxima for SYTO9 (485 and 498 nm, respectively, according to the manufacturer's specifications). Fluorescence was measured over a range of gain settings to accommodate the detection of different dye concentrations within a single experiment. This was achieved using a feature of the RotorGene 3000 that allows the creation of multiple FAM channels, each with different gains ranging from 10 to −1.

Following amplification, melting curves were acquired on the FAM channel using 1 °C steps with a hold of 60 s at each step from 70 to 99 °C. The differentiated data were analyzed using the Rotor Gene software (v6.0 build 14) with the digital filter set as "none." When required, samples were analyzed by 1% agarose gel electrophoresis with the addition of Gelstar nucleic acid stain (Cambrex Bio Science, Rockland, IN) using standard methods [27].

Determination of dye stability and between-run reproducibility

Master mixes were prepared for the three bacterial PCR assays described above and 20-µl aliquots were dispensed into 100-µl tubes using a CAS-1200 liquid handling system (Corbett Research). Each 20-µl aliquot contained 25,000 or 25 pg of DNA and either 2 or 0.5 µM dye. Aliquots were stored in the dark at −20 °C until used. Real-time PCR was conducted on a Rotor-Gene 3000 (Corbett Research) as described above. A threshold value of 0.02 was used to determine the C_t (cycle that crosses the threshold) value for each reaction. For the multiplex reactions, the C_t values represent the combined signal for both amplicons. This information cannot be used for quantitation, but it does provide a measure of assay reproducibility because it would be expected that changes in the amplification of either fragment will affect the total fluorescence and therefore affect the C_t.

Real-time PCR and melting curve analysis of eukaryotic DNA

The *YLM* locus was amplified from barley DNA using the YLMF (5′ CAGGAGCTGGTGAAATAGT GCCT 3′) and YLMR (5′ TTAAAGGGCTCCGT GAAGC 3′) primers of Paltridge et al. [28]. PCRs were conducted on a Rotor-Gene 3000 using the following conditions: 95 °C for 10 min and 50 cycles of 94 °C for 10 s, 58 °C for10 s, and 72 °C for 15 s. The reaction mixture (25 µl) contained 100 ng of genomic DNA, 1× PCR buffer II, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 1 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), 0.5 µM each primer, and 3.3 µM SYTO9. DNA melting curve analysis was monitored from 70 to 95 °C in 1 °C increments using a 30-s hold at each step on the FAM channel. Amplification products were characterized by gel electrophoresis (as described above) to confirm amplification of the expected sized fragment.

A 660-bp fragment of the gene encoding glutamate dehydrogenase was amplified from *Giardia* DNA using primers GDHGENF (5′ CGYGTNCCNTGGNTGG AYGAYGCYGG 3′) and GDHGENR (5′ AGYTT CTCCTCGKTGAASCC 3′) and the following conditions: 95 °C for 5 min and 55 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The reaction mixture (20 µl) contained 2 µl of DNA extract, 1× PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 1.25 mM $MgCl_2$, 200 µM each deoxynucleoside, 1 U of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA USA), 0.25 µM each primer, 5% DMSO, and 3.3 µM SYTO9. Data were acquired at 72 °C on the FAM channel. DNA melting curve analysis (using version 5.0 build 60 of the Rotor-Gene software) was monitored from 60 to 99 °C in 1 °C increments using a 10-s hold at each step on the FAM channel. Amplification products were characterized by gel electrophoresis (as described above) to confirm amplification of the expected sized fragment.

Statistical analysis

Statistical analyses (two-sample *t* test for independent samples) were conducted using Origin version 7 (OriginLab, MA, USA).

Results and discussion

Effect of dye concentration on DNA melting curves

The available product information for the SYTO family of dyes states that they are nucleic acid stains and provides absorption and emission spectra for each dye in the presence of DNA and RNA (http://www.probes.com/media/pis/mp07572.pdf). However, no information on dye specificity with regard to dsDNA or ssDNA is provided. To investigate this for SYTO9, DNA melting curve experiments were conducted using genomic DNA amplified by the *Legionella* multiplex PCR in the absence of any dye. A small volume of SYTO9 was added post-PCR to provide a concentration of 3.3 µM, which was selected because it is the working concentration used to stain live cells. The volume of dye added was kept small to minimize any change to the buffer or $MgCl_2$ concentrations. For comparison, SYBR Green I was added to replicate samples at the same concentration. The detection of distinct melting peaks (Fig. 1) demonstrates that SYTO9 is specific for dsDNA

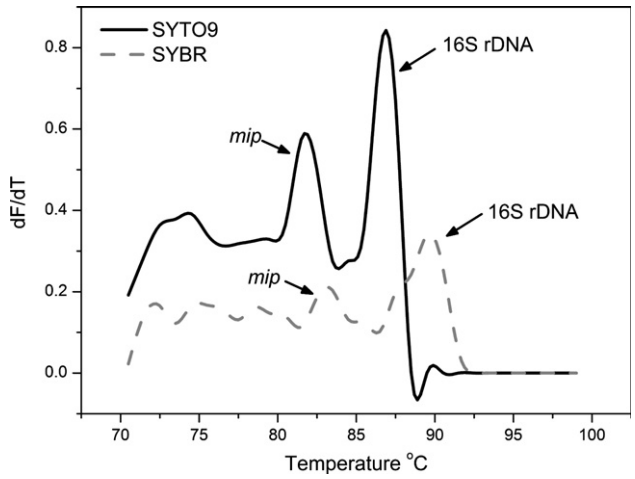

Fig. 1. DNA melting curve analysis of amplified *mip* and 16S rDNA fragments using 3.3 μM either SYTO9 or SYBR Green I that has been added post-PCR.

and that it produces results comparable with SYBR Green I, although the T_m values determined by each dye differed from each other by 2–3 °C.

To examine the effect of dye concentration on melting curves, similar experiments using a broad range of concentrations (0.5–33 μM) of either SYTO9 or SYBR Green I and either *Legionella* multiplex PCR or *Vibrio* multiplex PCR amplification products were conducted. Comparison of amplicon T_m versus dye concentration for the *mip*, *Legionella* 16S rDNA, and *hlyA* amplicons demonstrated that the T_m increases with increasing dye concentration (Fig. 2), suggesting that the dyes have a stabilizing effect on the dsDNA. This effect was much more pronounced for SYBR Green I, where the rate of increase in the T_m was higher than that for SYTO9 for dye concentrations above 2 μM. In the case of SYTO9 the T_m plateaued for concentrations of dye at or below 2 μM, and the maximum difference in T_m between low ($\leqslant$2 μM) and high (33 μM) dye concentrations was 2 °C for the three amplicons analyzed. In contrast, the T_m values determined using SYBR Green I did not plateau until the dye concentration was 0.5 μM, and the difference in T_m between the lowest and the highest dye concentrations was 7 °C for *hlyA*, 10 °C for *mip*, and >10 °C for 16S (the T_m for the latter being >99 °C in the presence of 33 μM dye). The observation that SYBR Green I has a greater enhancement on T_m compared to SYTO9 under the same conditions suggests either that SYBR Green I binds more tightly to dsDNA or that more SYBR Green I molecules can bind per unit DNA. This finding is supported by the recent work of Zipper et al. [26], which suggests that SYBR Green I has a dual mode of binding to dsDNA which is dependant on the dye/base pair ratio. The effect of the dye concentration on the T_m was reflected in the shape of the melting peaks (Fig. 3). For SYTO9, which had relatively little effect on T_m, the shape of the melting peaks was not affected by

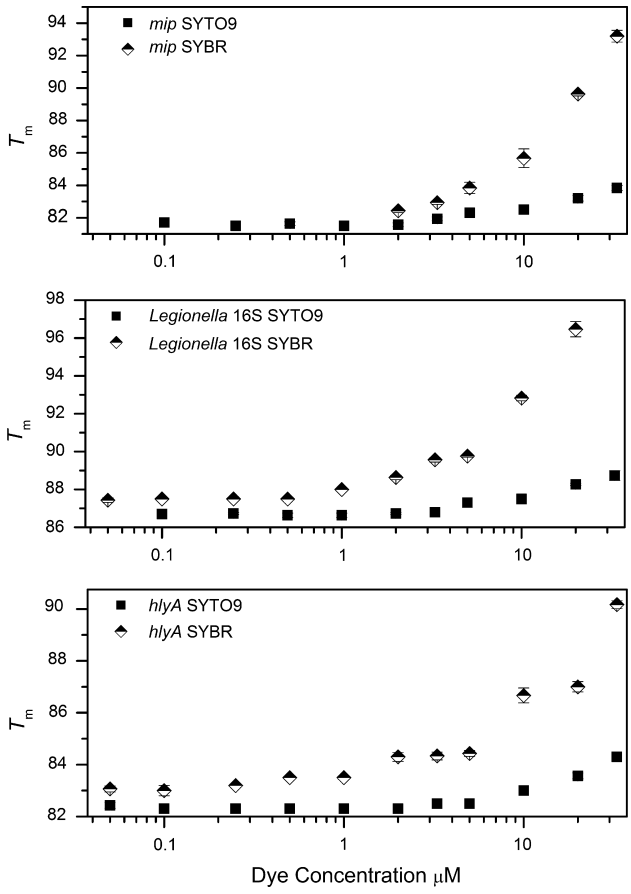

Fig. 2. Comparison of dye concentration versus T_m for *mip*, *Legionella* 16S rDNA, and *hlyA* amplicons.

dye concentration, as illustrated for the *mip* and 16S rDNA amplicons in Fig. 3. In the case of SYBR Green I, both amplicons could be detected at high dye concentrations but the peaks were broad and difficult to interpret. As the concentration of SYBR Green I decreased, the melting peaks became sharper, with best peak resolution obtained for concentrations below 2 μM. At lower concentrations of the SYBR Green I, the peak for *mip* became more difficult to detect or could not be detected (Fig. 3).

Comparison of SYTO9 with SYBR Green I for real-time PCR and DNA melting curve analysis

The utility of SYTO9 was assessed by using it to monitor DNA amplification in real-time for the *Legionella* multiplex, *Vibrio* multiplex, and 16S rDNA PCRs. These assays amplify fragments of 114 bp (*mip*) and 386 bp (*Legionella* 16S rDNA), 385 bp (*ctxAB*) and 497 bp (*hlyA*), and approximately 1.5 kb (*Vibrio* 16S rDNA), respectively. The *Vibrio* 16S rDNA fragment would normally be considered too large for real-time PCR and represents a challenge for amplification if any of the conditions affect the reaction efficiency. All reactions were conducted in triplicate, using 250,000–25 pg of

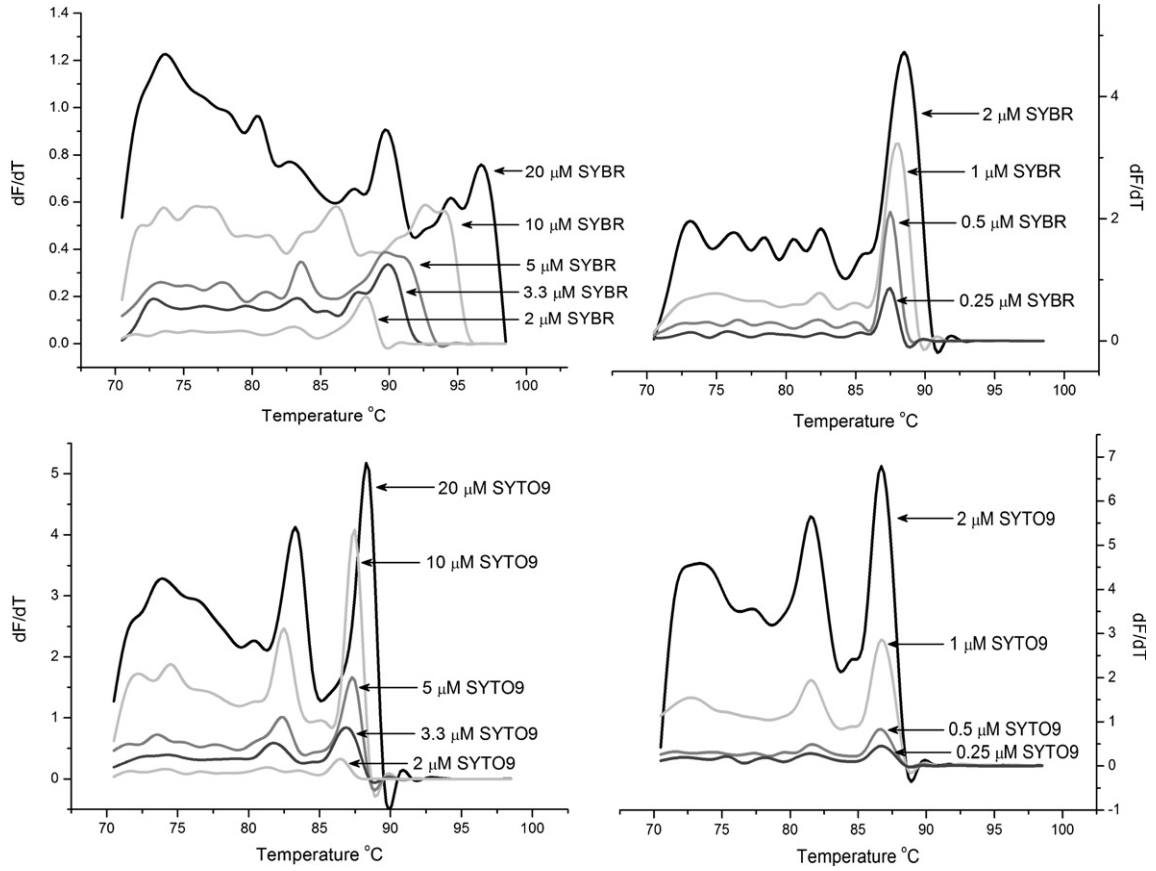

Fig. 3. Comparison of DNA melting curves obtained using *Legionella* DNA amplified with *mip* and 16S rDNA primers and different concentrations of SYTO9 or SYBR Green I.

DNA per reaction. For comparison, PCRs were also conducted using SYBR Green I. Based on the results of the initial melting curve experiments, SYTO9 concentrations of 10, 5, 2, and 0.5 μM and SYBR Green I concentrations of 10, 2, 1, and 0.5 μM were investigated. These values represent concentrations across the range where the dyes had different effects on melting temperature.

The results, summarized in Table 1, demonstrate that SYTO9 can be used in PCR over a broader range of concentrations than can SYBR Green I. The *Legionella* multiplex PCR was inhibited by SYBR Green I at dye concentrations of 10 and 2 μM. The smaller *mip* fragment was amplified at a SYBR Green I concentration of 1 μM, and both fragments were amplified using 0.5 μM dye. The amplification of *mip* and 16S rDNA using 0.5 μM SYBR Green I was affected by the amount of starting template in the assay. When starting with 250,000 pg of template DNA only the *mip* fragment amplified (Fig. 4). However, both fragments amplified using 25,000 pg of template DNA, suggesting that the *mip* amplicon preferentially amplifies at higher template concentrations. As the amount of starting template decreased, the height of the 16S rDNA peak increased and the *mip* peak height decreased. Using 25 pg of template DNA the peak heights for the two amplicons were similar. These results are consistent with the findings of Giglio et al. [22] and suggest that in the presence of lower amounts of amplicon (expected for reactions starting with less template DNA) both amplicons can be detected by melting curve analysis, but at higher amounts of amplicon the dye becomes limiting and the amplicon with the higher T_m is preferentially detected. In contrast, SYTO9 concentrations of 5, 2, and 0.5 μM allowed the amplification of both fragments in the *Legionella* multiplex assay. All reactions using 10 μM SYTO9 and reactions with 5 and 2 μM SYTO9 starting with 250,000 pg of template DNA amplified only the *mip* fragment. This suggests that high SYTO9 concentrations or a combination of dye concentration and high starting template causes the preferential amplification of the smaller *mip* fragment.

The *Vibrio* multiplex PCR was less affected by dye concentration than was the *Legionella* muliplex PCR. Amplification was inhibited by SYBR Green I at a concentration of 10 μM, but the assay worked for the lower dye concentrations. Only the *hlyA* amplicon could be detected by DNA melting curve analysis although both fragments amplified (data not shown). This result is consistent with the findings of Giglio et al. [22]. In the case of SYTO9, both *hlyA* and *ctxAB* were amplified in the

Table 1
Summary of results for *Legionella* multiplex, *Vibrio* multiplex, and 16S rDNA (27f/1492r) PCRs using different dye concentrations and amounts of starting DNA template

	Dye (μM)	Amplicon	pg DNA/reaction				
			250,000	25,000	2500	250	25
SYBR Green I	10	16S	–[a]	–	–	–	–
		mip	–	–	–	–	–
	2	16S	–	–	–	–	–
		mip	–	–	–	–	–
	1	16S	–	–	–	–	–
		mip	+[b]	+	+	+	+
	0.5	16S	–	+	+	+	+
		mip	+	+	+	+	+
SYTO9	10	16S	–	–	–	–	–
		mip	+	+	+	+	+
	5	16S	–	+	+	+	+
		mip	+	+	+	+	+
	2	16S	+	+	+	+	+
		mip	+	+	+	+	+
	0.5	16S	+	+	+	+	+
		mip	+	+	+	+	+
SYBR Green I	10	*hlyA*	–	–	–	–	–
		ctxAB	–	–	–	–	–
	2	*hlyA*	+	+	+	+	+
		ctxAB	–	–	–	–	–
	1	*hlyA*	+	+	+	+	+
		ctxAB	–	–	–	–	–
	0.5	*hlyA*	+	+	+	+	+
		ctxAB	–	–	–	–	–
SYTO9	10	*hlyA*	+	+	+	+	+
		ctxAB	+	+	+	+	+
	5	*hlyA*	+	+	+	+	+
		ctxAB	+	+	+	+	+
	2	*hlyA*	+	+	+	+	+
		ctxAB	+	+	+	+	+
	0.5	*hlyA*	+	+	+	+	+
		ctxAB	+	+	+	+	+
SYBR Green I	10	27f/1492r	–	–	–	–	–
	2	27f/1492r	–	–	–	–	–
	1	27f/1492r	+	+	+	+	+
	0.5	27f/1492r	+	+	+	+	+
SYTO9	10	27f/1492r	+	+	+	+	–
	5	27f/1492r	+	+	+	+	+
	2	27f/1492r	+	+	+	+	+
	0.5	27f/1492r	+	+	+	+	+

[a] No specific melt peak detected.
[b] Specific melt peak detected.

presence of 10–0.5 μM dye. The results for the large 16S rDNA fragment were similar, with the fragment being amplified using all concentrations of SYTO9 tested, although reactions containing 10 μM SYTO9 and 25 pg DNA failed. Amplification of the large 16S rDNA fragment was inhibited in the presence of 10 and 2 μM SYBR Green I but not for lower dye concentrations. The results suggest that the PCR inhibition is dependent not only on the dye concentration but also on other factors such as the primers and amplicons.

The DNA melting curve data from these experiments allowed the investigation of the combined effect of dye concentration and starting template DNA concentration on T_m. The results for *mip* and *hlyA* are presented in Fig. 5. The T_m for *mip* was not affected by the amount of starting template DNA using 10 μM SYTO9, although there was an approximate 1 °C decrease in T_m for the lower SYTO9 concentrations. For 0.5 μM SYTO9 there was a small increase (0.1 °C) in *mip* T_m as the amount of starting template decreased. The T_m for *mip* underwent a similar trend using 1 μM SYBR Green I, with an increase by 0.5 °C between reactions with 25 ng compared with 25 pg DNA template. However, the trend was reversed for *mip* PCRs using 0.5 μM SYBR Green I. The T_m of the *hlyA* fragment was more consistent when amplified with a range of SYTO9 concentrations. The T_m was the same for reactions amplified with 10–2 μM SYTO9 (Fig. 5). Similarly, reactions amplified with 2 μM SYBR Green I had the same T_m irrespective of the amount of starting template. The T_m for *hlyA* using 0.5 μM either SYTO9 or SYBR Green I underwent a trend similar to that with *mip* and 1 μM SYBR Green I. These results support the initial DNA melting curve experiments and show that the relative DNA:dye ratio affects the T_m. For reactions with high starting DNA template and low dye concentration, the dye is limiting and so the T_m is lower than those for reactions that had less starting template DNA and the same dye concentration. An exception to this was the *mip* T_m values with 0.5 μM SYBR Green I. In this case, the T_m decreased with decreasing starting template DNA concentration. This can be explained by considering the overall results for the *Legionella* multiplex PCR using 0.5 μM SYBR Green I. At the highest starting template concentration only *mip* amplified, but at the lower concentrations both *mip* and 16S rDNA amplified. This means that there will be more total amplified DNA in reactions starting with lower amounts of starting template. In addition, preferential binding of SYBR Green I to the higher-T_m 16S rDNA fragment would make less dye available to bind to the *mip* product. These factors would account for the decrease in T_m observed for *mip*.

Determination of dye stability and between-run reproducibility

The stability of SYTO9 and SYBR Green I and the reproducibility of amplification and DNA melting curves were examined by testing replicate aliquots ($n = 4$) of master mixes stored at −20 °C over a 3-week period. Different dye concentrations (2 and 0.5 μM) and starting amounts of DNA template (25,000 and 25 pg) were investigated. The resulting T_m and C_t values for the various assays are summarized in Tables 2 and 3, respectively. Any detrimental changes in the master mixes due to dye degradation because of storage should be reflected by changes in either of these values. The results for the fresh master mixes (week 0) were consistent with

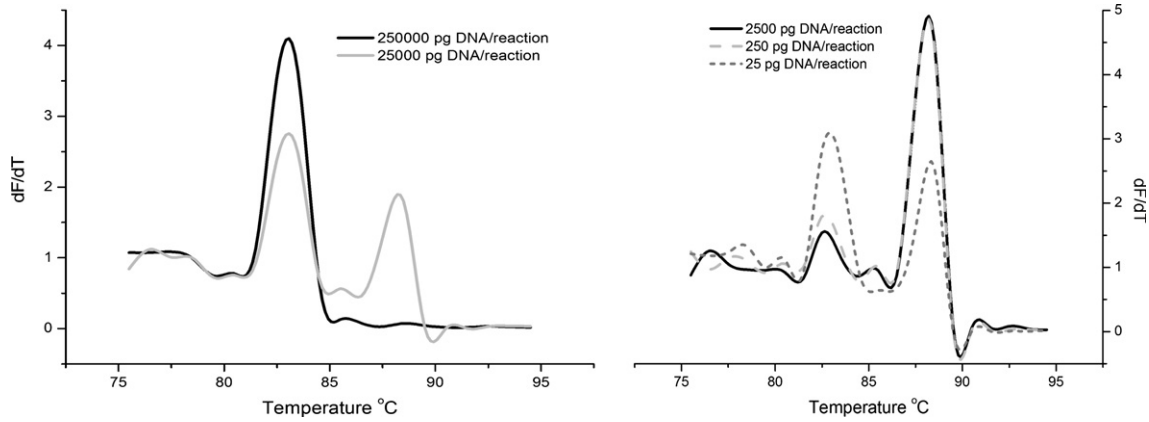

Fig. 4. Comparison of DNA melting curves for the *Legionella* multiplex PCR incorporating 0.5 μM SYBR Green I and different amounts of starting DNA template.

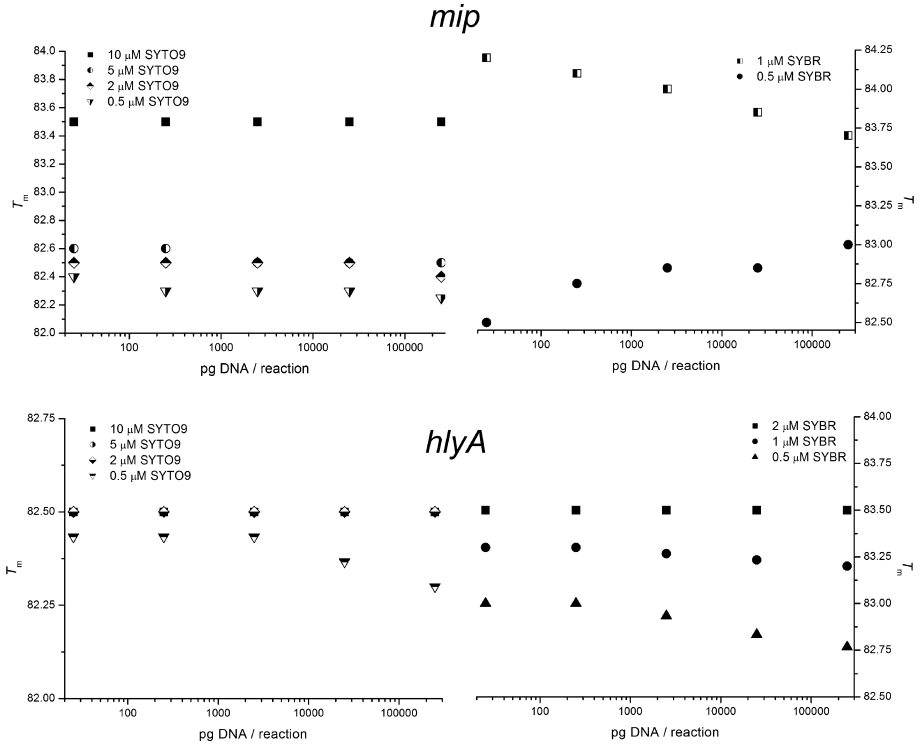

Fig. 5. T_m values for *mip* and *hlyA* determined from PCRs incorporating different dye concentrations and different amounts of starting DNA template.

the previous results, with 2 μM SYBR Green I inhibiting all of the *Legionella* multiplex PCRs and the 16S rDNA PCRs with 25 pg of DNA template. In addition, the *ctxAB* amplicon could not be detected by DNA melting curve analysis using either concentration of SYBR Green I. The T_m values of the amplicons from the *Legionella* multiplex and *Vibrio* multiplex PCRs were highly consistent over the 3-week period for reactions with either SYTO9 concentrations or 0.5 μM SYBR Green I. Although the variation was small (generally 0.1 °C), the average T_m values for some weeks were significantly different from those obtained at week 0. The T_m values for *hlyA* using 2 μM SYBR Green I were the most variable and consistently had larger standard deviations than those observed under other reaction conditions (Table 2). The results for the 27f/1492r PCR were more variable between each week for both dyes but there was marginal variation between replicates amplified on the same week. Amplification for this assay was variable for reactions containing 2 μM SYTO9 and 25 pg DNA after week 0, with some reactions failing to amplify the large 16S rDNA fragment. Similar variation was observed for reactions containing 2 μM SYBR Green I and 25,000 pg DNA (with reactions starting with 25 pg DNA failing). The reactions that failed had large amounts of nonspecific product (data not shown).

Table 2
Summary of T_m values for amplicons from *Legionella* multiplex, *Vibrio* multiplex, and 16S rDNA (27f/1492r) PCRs using different concentrations of SYTO9 or SYBR Green I and different storage times

Assay	Dye conc. (μM)	pg DNA/ reaction	SYTO9				SYBR Green I			
			Week				Week			
			0	1	2.0	3.0	0	1	2.0	3.0
Legionella 16S	2	25,000	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	—[a]	—	—	—
	2	25	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	—	—	—	—
	0.5	25,000	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.7 ± 0.1	87.5 ± 0.0	88.0 ± 0.0	—
	0.5	25	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.5 ± 0.0	87.7 ± 0.1	87.6 ± 0.1	87.7 ± 0.0	87.5 ± 0.0
Legionella mip	2	25,000	82.5 ± 0.0	82.5 ± 0.0	82.4 ± 0.1	82.3 ± 0.0	—	—	—	—
	2	25	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	82.3 ± 0.0	—	—	—	—
	0.5	25,000	82.3 ± 0.0	82.2 ± 0.0	82.3 ± 0.0	82.1 ± 0.1	82.6 ± 0.1	82.6 ± 0.1	83.0 ± 0.2	83.1 ± 0.1
	0.5	25	82.3 ± 0.0	82.2 ± 0.0	82.4 ± 0.1	82.1 ± 0.1	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.1
Vibrio hlyA	2	25,000	82.7 ± 0.0	82.7 ± 0.0	82.7 ± 0.0	82.8 ± 0.1	84.6 ± 0.3	85.3 ± 0.1	84.8 ± 0.2	84.8 ± 0.5
	2	25	82.7 ± 0.0	82.7 ± 0.0	82.7 ± 0.0	82.7 ± 0.0	85.0 ± 0.3	85.4 ± 0.2	85.4 ± 0.2	85.5 ± 0.0
	0.5	25,000	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0
	0.5	25	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	82.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0	83.5 ± 0.0
Vibrio ctxAB	2	25,000	76.5 ± 0.1	76.5 ± 0.0	76.4 ± 0.1	76.5 ± 0.0	—	—	—	—
	2	25	76.3 ± 0.1	76.5 ± 0.0	76.2 ± 0.2	76.5 ± 0.1	—	—	—	—
	0.5	25,000	76.5 ± 0.0	76.5 ± 0.1	76.5 ± 0.1	76.5 ± 0.0	—	—	—	—
	0.5	25	76.3 ± 0.0	76.5 ± 0.0	76.4 ± 0.1	76.5 ± 0.0	—	—	—	—
27f/1492r	2	25,000	85.4 ± 0.1	85.3 ± 0.0	85.5 ± 0.0	85.5 ± 0.0	87.4 ± 0.1	86.8 ± 0.0	87.3 ± 0.2	87.5 ± 0.0
16S rDNA	2	25	85.5 ± 0.0	85.4 ± 0.1	85.5 ± 0.0	85.5 ± 0.0	—	—	—	—
	0.5	25,000	85.1 ± 0.1	85.2 ± 0.0	85.2 ± 0.1	85.3 ± 0.0	85.5 ± 0.1	85.6 ± 0.1	85.5 ± 0.0	85.7 ± 0.0
	0.5	25	85.1 ± 0.1	85.1 ± 0.3	85.2 ± 0.1	85.2 ± 0.2	85.5 ± 0.0	85.6 ± 0.1	85.5 ± 0.0	85.5 ± 0.0

T_m values significantly different ($p < 0.05$) from week 0 are shaded.
[a] Specific peak not detected.

Table 3
Summary of C_t values for *Legionella* multiplex, *Vibrio* multiplex, and 16S rDNA (27f/1492r) PCRs using different concentrations of SYTO9 or SYBR Green I and different storage times

Assay	Dye conc. (μM)	pg DNA/ reaction	SYTO9				SYBR Green I			
			Week				Week			
			0	1	2	3	0	1	2	3
Legionella multiplex	2	25,000	5.0 ± 0.3	5.1 ± 0.6	4.7 ± 0.3	6.1 ± 0.1	—[a]	—	—	—
	2	25	12.8 ± 0.1	13.1 ± 0.2	13.1 ± 0.3	13.3 ± 0.1	—	—	—	—
	0.5	25,000	4.4 ± 0.0	5.0 ± 0.0	5.0 ± 0.0	5.1 ± 0.1	6.1 ± 0.0	6.1 ± 0.0	6.2 ± 0.1	6.4 ± 0.1
	0.5	25	13.7 ± 0.1	13.9 ± 0.3	14.0 ± 0.3	14.0 ± 0.2	13.8 ± 0.2	13.8 ± 0.6	13.7 ± 0.5	14.1 ± 0.7
Vibrio multiplex	2	25,000	4.4 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.4 ± 0.0	20.7 ± 2.1	23.4 ± 1.1	20.8 ± 1.8	20.6 ± 2.2
	2	25	14.5 ± 0.2	14.7 ± 0.3	14.5 ± 0.2	14.7 ± 0.2	24.8 ± 0.5	26.5 ± 0.9	24.4 ± 1.0	24.2 ± 1.1
	0.5	25,000	6.4 ± 0.3	6.7 ± 0.0	6.5 ± 0.3	6.4 ± 0.3	4.5 ± 0.0	4.5 ± 0.0	4.5 ± 0.0	4.5 ± 0.0
	0.5	25	16.5 ± 0.2	16.7 ± 0.3	16.6 ± 0.2	16.8 ± 0.1	14.7 ± 0.1	14.7 ± 0.1	14.6 ± 0.0	14.6 ± 0.1
27f/1492r	2	25,000	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	5.3 ± 0.4	14.8 ± 0.3	12.8 ± 0.4	13.3 ± 1.0	19.4 ± 0.9
16S rDNA	2	25	15.4 ± 0.5	15.4 ± 0.2	15.3 ± 0.6	17.8 ± 0.9	24.4 ± 0.6	23.0 ± 0.3	21.7 ± 5.9	26.3 ± 2.8
	0.5	25,000	3.5 ± 0.0	3.4 ± 0.2	3.4 ± 0.2	6.3 ± 0.2	2.9 ± 0.0	2.9 ± 0.0	2.9 ± 0.0	5.5 ± 0.5
	0.5	25	16.1 ± 0.5	16.4 ± 0.2	15.9 ± 0.4	24.6 ± 4.1	15.0 ± 0.1	15.1 ± 0.4	14.8 ± 0.5	21.1 ± 0.5

C_t values significantly different from week 0 ($p < 0.05$) are shaded.
[a] No amplification of specific product.

The reaction stability was further investigated by comparing the average C_t values for the master mixes over time. The C_t values for the *Legionella* multiplex assay were consistent over time and did not differ significantly for the first 2 weeks for either dye, with the exception of the master mix with 0.5 μM SYTO9 and 25,000 pg DNA, which had a significantly lower C_t value at week 0 compared to later weeks (Table 3). At week 3 the C_t values for reactions with 2 μM SYTO9 or 0.5 μM SYBR Green I were significantly larger than those at week 0, with the exception of the 0.5 μM SYBR reactions with 25 pg DNA, which were not significantly different but had large standard deviations. The *Vibrio* multiplex reactions with SYTO9 were highly reproducible and the C_t values did not change over the 3 weeks of storage. Similar results were obtained for the *Vibrio* multiplex using 0.5 μM SYBR Green I, but the results were highly variable using 2 μM SYBR Green I. The C_t values for the amplification using the 27f/1492r primers were consistent for the first 2 weeks when using either concentration

of SYTO9 or 0.5 μM SYBR Green I. In all cases the C_t values were significantly worse at week 3. Collectively, the DNA melting curve and C_t data suggest that master mixes containing SYTO9 or 0.5 μM SYBR Green I are stable for 2–3 weeks and that the absolute time may be a function of the PCR assay. Amplicon size may be a factor, considering that reactions amplifying the large 16S rDNA fragment demonstrated the most dramatic change in C_t values at week 3. However, the nature of the amplicons may also play a role considering that the *Vibrio* multiplex assay was still functional after 3 weeks of storage, whereas the *Legionella* multiplex assay was starting to show signs of deterioration, yet the amplicons are of similar size. These results contrast with the report of Karsai et al. [14], who found that SYBR Green I is unstable when stored in TE (pH 8.0) at −20 °C. The reaction buffer used in the experiments described herein also had a pH of 8.0.

Real-time PCR and melting curve analysis of eukaryote systems

Having established the utility of SYTO9, the dye was used to convert a conventional PCR assay for barley yellow dwarf virus resistance to a real-time format. Initial experiments found that the expected sized fragments, which were 90 bp for the barley yellow dwarf virus resistance-associated allele and 101 bp for the susceptible-associated allele, were amplified for SYTO9 concentrations of 16.5 μM and lower but that 33 μM SYTO9 was inhibitory (data not shown). The GC compositions of these amplicons were 42.0 and 44.5%, respectively, and DNA melting curve analysis identified a single peak for each allele with T_ms of 79.8 ± 0.1 °C ($n = 16$) for the resistance-associated allele and 81.4 ± 0.2 °C ($n = 16$) for the susceptible-associated allele. Based on a comparative quantitation analysis of the amplification data, the reaction efficiencies for samples with the susceptible-associated allele (0.57 ± 0.02, $n = 16$) were significantly poorer ($p < 0.0001$) than those for samples with the resistance-associated allele (0.72 ± 0.06, $n = 16$). The results suggest that DNA melting curve analysis can be readily applied to the differentiation of these allelic variants and should be simpler and easier to interpret than electrophoretic analysis of small amplicons.

The utility of SYTO9 was further evaluated by applying it to a conventional PCR assay for the parasitic protozoan *Giardia.* Sequence data from the *gdh* locus amplified by this assay have previously been used to determine the evolutionary relationships of the major assemblages of *G. duodenalis* [24], and it was predicted that the level of diversity may allow differentiation of some of these groups by DNA melting curve analysis. Isolates representing three of these assemblages and another species of *Giardia*, *G. ardeae*, were analyzed by real-time PCR and DNA melting curve analysis in two replicate experiments. In each case, the expected sized products were amplified (data not shown) and the first derivative profiles of the DNA melting curves were diagnostic for each assemblage or species of *Giardia* (Fig. 6). The GC composition for these samples was relatively high (58.3–62.8%, Table 4), demonstrating that SYTO9 is also useful for the amplification and analysis of GC-rich DNA. With the exception of the Assemblage B sample, all amplicons produced two peaks. The average T_m values from the two experiments for each sample are summarized in Table 4. The data suggest that the overall GC composition of a DNA fragment is not necessarily a good predictor of the T_m. The *G. ardeae* and Assemblage F amplicons possess similar GC compositions (62.8% versus 62.6%), yet the T_m values are 2 °C different for each peak. In contrast, the GC composition of *G. ardeae* differs from Assemblage A by 2.8% yet the T_m values are similar (although the relative heights of the two peaks for each sample are different—see Fig. 6). These data suggest that DNA melting analysis can detect finer-scale differences in sequences, such as localized domains of different GC composition. However, additional experimental data are required to support this hypothesis.

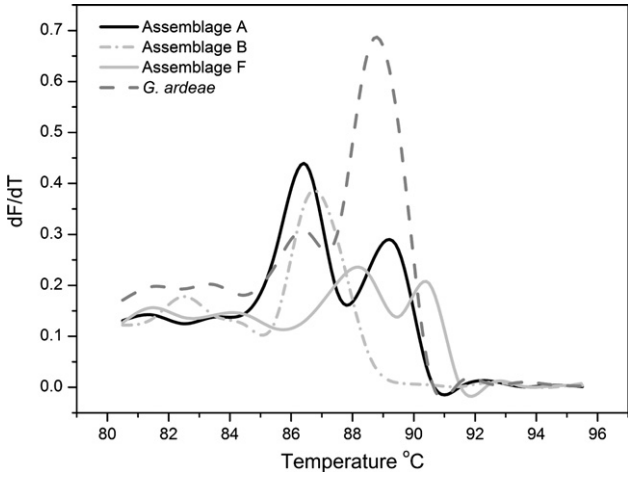

Fig. 6. DNA melting curve analysis of the amplified *Giardia gdh* fragment using SYTO9.

Table 4
Summary of T_m values obtained by DNA melting curve analysis of the *gdh* amplicon using SYTO9 and GC% of the amplicon for each *Giardia* isolate

Sample	Peak 1 °C	Peak 2 °C	GC%
Giardia duodenalis A	86.7	89.5	60.0
Giardia duodenalis B	87.0		58.3
Giardia duodenalis F	88.3	90.4	62.6
Giardia ardeae	86.4	88.8	62.8

Conclusions

The data clearly show that SYTO9 can be used for real-time PCR and DNA melting curve analysis of both

prokaryote and eukaryote systems and that the results are highly reproducible. Comparison of SYTO9 with SYBR Green I demonstrated that both dyes affect amplicon T_m values in a concentration-dependent manner, but the effect of SYBR Green I is much more extreme, suggesting either that SYBR Green I binds dsDNA more tightly or that more dye molecules can bind per unit DNA compared with SYTO9. The key performance advantages of SYTO9 compared with SYBR Green I are that SYTO9 can be used in real-time PCR assays over a broader range of dye concentrations without causing PCR inhibition, that it produces more robust and consistently shaped DNA melting curves that are less affected by dye concentration, and that it can be more readily used for the detection of multiple amplicons by DNA melting curve analysis without any apparent bias. Both dyes were demonstrated to be stable in master mixes for 2–3 weeks, but the absolute reaction stability and dye concentration that causes PCR inhibition appear to differ between assays. The performance characteristics of SYTO9 make this dye easier to use in real-time PCR applications than SYBR Green I, particularly for adapting conventional assays to a real-time format and for DNA melting curve analysis. These characteristics will extend the scope of using dsDNA-specific dyes in diagnostic applications and make SYTO9 an exciting alternative to SYBR Green I. Further studies using a broad range of PCR assays are required to determine the performance characteristics of SYTO9 for quantitative PCR, particularly in relation to how SYTO9 compares with SYBR Green I for reaction efficiency. Given the demonstrated utility of SYTO9 for real-time PCR, it will be interesting to investigate other SYTO dyes to determine whether the properties of SYTO9 are common to other members of this family of dyes.

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