Optimization of Diamond Nucleic Acid Dye for quantitative PCR

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Here, we evaluate Diamond Nucleic Acid Dye (DD) for use in quantitative PCR (qPCR) applications. Although DD is a commercially available stain for detection of DNA separated by gel electrophoresis, its use as a detection dye in qPCR has yet to be described. To determine if DD can be used in qPCR, we investigated its inhibitory effects on qPCR at concentrations ranging 0.1–2.5×. Serial dilution of DNA was used to determine the efficiency, sensitivity, and linearity of DD-generated qPCR data in comparison to other commonly used fluorescent dyes such as SYBR Green (SG), EvaGreen (EG), and BRYT Green (BG). DD was found to be comparable with other dyes for qPCR applications, with an R^2 value >0.9 and an efficiency of 0.83. Mitochondrial DNA (mtDNA) target signals were successfully produced by DD over a DNA dilution range of ~28 ng–0.28 pg, demonstrating comparable sensitivity to the other dyes investigated. C_q values obtained using DD were lower than those using EG by almost 7 cycles. We conclude that Diamond Nucleic Acid Dye is a cheaper, less toxic alternative for qPCR applications.

Fluorescent dyes have been used in quantitative PCR (qPCR) for many years (1). The most commonly used of these dye is the cyanine intercalating dye SYBR Green I (SG) (2). SG is known for its DNA binding sensitivity and is reported to have an ~1000-fold increase in fluorescence when bound to DNA (3,4). Other fluorescent dyes that have been established for use in qPCR include EvaGreen (EG) (5), LCGreen (6), SYTO dyes (7), ResoLight (8), and BRYT Green (BG) (9), amongst others. One well-known issue with the use of SG in the laboratory is its mutagenic and toxic nature, as it can easily penetrate the cell membrane (10). SG is stated to cause mutations above the dosage of 33.3 µg per plate in the Ames Test. The toxicity of another commonly used dye, ethidium bromide (EtBr), is observed at 250–500 µg dosages per plate in the Ames Test.

Diamond Nucleic Acid Dye (DD) is an external groove binding dye shown to have a similar sensitivity as SG and a limit of detection of 0.5 ng when detecting DNA in agarose gels (10). DD can penetrate the cell membrane, leading to interactions with genomic DNA (10); however, studies have shown that DD is less mutagenic and genotoxic when compared with EtBr (11). This reduction in toxicity may be due to the different binding mechanism of DD with DNA compared with other intercalating dyes, since DD does not bind between base pairs of DNA but instead binds externally (10). Currently, DD has not been evaluated for use in qPCR applications.

EG has been established for its use with qPCR and with high-resolution melt (HRM) curve analysis, as it has been shown to be stable in PCR (5). SG is known to inhibit PCR at high concen-

trations and, with 25 ng of DNA, was shown to inhibit the reaction at concentrations >1×. In comparison, EG still shows amplification at a concentration of 2.5×, indicating that EG inhibits PCR less than SG (12).

Here, we investigate the use of DD in qPCR and compare the effectiveness of DD to the other fluorescent qPCR dyes: SG, EG, and BG. We determined the optimal concentration of DD within the reaction, the level of amplification inhibition, and the sensitivity and efficiency of the reaction. To determine how robust DD is, various primers were chosen that generated different product lengths. As DD is a much cheaper dye than SG (~5-fold cheaper) and is stated to be less toxic and mutagenic than EtBr (www.promega.com.au/resources/pubhub/diamond-nucleic-acid-dye-is-a-safe-and-economical-alternative-to-ethidium-bromide/), DD presents a

METHOD SUMMARY

Application of Diamond Nucleic Acid Dye for the quantification of DNA using real-time PCR and a mitochondrial target sequence is presented. Comparisons are made with established fluorescent dyes, and the benefits of using the Diamond dye are detailed.

Figure 1. Effect of dye concentration on cycle number and level of inhibition of Diamond Nucleic Acid Dye (DD) on the reaction (A) Average cycling curve of normalized fluorescence intensity against cycle number with varying DD concentration (0.1–1.5X). (B) The line of best fit for DD dye concentration against average C_q value with the threshold set at 0.12; error bars indicate the 95% confidence intervals. Complete inhibition was observed at concentrations >1.5X.

suitable alternative fluorescent dye for use in qPCR provided its sensitivity and efficiency are comparable to the dyes used currently.

Materials and methods

Optimization of fluorescent dye concentration for qPCR

Diamond Nucleic Acid Dye (DD) (Promega, Sydney, Australia) was prepared as a 20X solution in a buffer of 1X tris-acetate (TA) by a 1:500 dilution of the stock concentrate (10,000X). The primer sequences and amplicon properties for this study are shown in Table 1 and described in

Reference 13. Fragment 1 primers were used to determine the inhibition effect of DD on PCR amplification. Five different reactions were prepared in quadruplicate; the quantity of DNA remained constant at 20 ng in each reaction, but the final dye concentration varied (0.1X, 0.5X, 1X, 1.5X, 2X, 2.5X) in a total reaction volume of 20 μ L. Amplifications were performed in a 72 Rotor-Disc in a Rotor-Gene Q (Qiagen, Qiagen, Chadstone Centre, Australia). The channels selected for fluorescence detection were green (excitation 470 ± 10 nm, emission 510 ± 5 nm) and a modified channel labeled “diamond” (excitation 470 ± 10 nm,

emission 557 ± 5 nm) to account for the differences in the excitation (494 nm) and emission (558 nm) of DD compared with the excitation (495 nm) and emission (520 nm) of SG. The PCR program began with an initial hold at 95°C for 2 min, then 50 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. This was followed by a melt from 72°C to 95°C in 1°C increments.

The median C_q value was calculated for each dye concentration, and the slope of the trendline generated by plotting the median C_q value against the dye concentration was used as an indicator of the degree of amplification inhibition.

Table 1: qPCR primer sequences used in this study and properties of the resulting amplicons.

Primer sets	5'—Sequence—3'	Amplicon length (bp)	Tm (°C)
1	Fragment 1 F-GACCAATGATATGAAAAACCATCGTTGT R-CAAGCATACTCCTAGTAAGGATCCG	170	68.8 64.67
2	Fragment 2 F-TGAGGACAAATATCATTYTGAAGRG R-ATCGGAATGGGAGGTATTCTAGG	246	67.6 71.3

Primer sets 1 and 2 targeted the end of the mitochondrial genes for tRNA-Glu and cytochrome b, respectively. These primer sets, which were used to test the suitability of Diamond Nucleic Acid Dye (DD) relative to other qPCR dyes, were selected based on the product size and also to ensure that there was a minimal chance of contamination from the operator between the different dyes tested. This was accomplished using non-human (Primer set 1) and human-specific primers (Primer set 2). Primer design published in Tobe and Linacre (13).

Determination of DD efficiency and sensitivity compared with SYBR Green, EvaGreen, and BRYT Green

A series of dilutions was prepared to determine the efficiency of DD, EG, and BG in qPCR, and to compare these to SG. Amplifications were performed using Fragment 2 primers, which produce a 246-bp amplicon (Table 1). Five 10-fold serial dilutions to a final dilution of $1/10^5$ were prepared from neat DNA (whole genomic DNA from a buccal swab prepared by a solid phase extraction) quantified by a Qubit dsDNA HS Assay at 28.4 ng/ μ L (Thermo Fisher Scientific, Scoresby, Australia). A no-template control was prepared for quality control to determine if any contamination was present in any of the reagents.

Based on the results of part 1 of this study investigating optimal dye concentration, DD was used at a final

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Figure 2. Average qPCR cycling with a 5-dilution series over 50 cycles using 0.5x Diamond Nucleic Acid Dye (DD). Detection was done using the diamond channel, with 1 being the neat DNA concentration at 28.4 ng/ μ L, followed by 1/10 serial dilutions. Reactions were done in quadruplicate.

concentration of 0.5x. The DD reactions consisted of 10 μ L KAPA Taq ReadyMix 2x (GeneWorks Pty Ltd, Thebarton, Australia), 0.5 μ L of each primer pair (10 μ M/ μ L), 0.5 μ L 20x DD, 1 μ L DNA solution, and 8 μ L TA buffer, in a total reaction volume of 20 μ L. The SG reactions consisted of 10 μ L KAPA SYBR FAST qPCR Master Mix 2x (Kapa Biosystems), 0.5 μ L of each primer pair, 1 μ L DNA solution, and 8.5 μ L TA buffer, in a total reaction volume of 20 μ L. The BG reaction consisted of 10 μ L GoTaq qPCR Master Mix 2x (Promega), 0.5 μ L of each primer pair, 1 μ L DNA solution, and 8.5 μ L TA buffer, in a total reaction volume of 20 μ L. The EG reaction consisted of 10 μ L KAPA Taq ReadyMix 2x, 0.5 μ L of each primer pair, 1 μ L EG at 20x, 1x concentration as suggested by the manufacturer's protocol (Jomar Life Research, Scoresby, Australia), 1 μ L DNA solution, and 7.5 μ L TA buffer, in a total reaction volume of 20 μ L. A negative control (NTC) was prepared for each of the dyes tested to determine if any contamination was

present; the reactions were prepared as above with an extra 1 μ L 1x TA buffer added instead of DNA.

All reactions were performed in quadruplicate. Amplifications were performed on a 72 Rotor-Disc in a Rotor-Gene Q. The channels selected for fluorescence detection were green and diamond (see above). The PCR cycle began with an initial hold at 95°C for 2 min, then 50 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. This was followed by a melt from 72°C to 95°C in 1°C increments.

The lower limit of sensitivity of the reaction was determined by preparing a 1/10⁶ dilution to determine if a signal from any of the dyes could be detected. This was performed in quadruplicate.

Results and discussion

We first assessed the impact of DD concentration on PCR amplification. Our data showed that the optimal concentration of DD for qPCR was 0.5x (Figure

Table 2: Average C_q values for dilution series of each fluorescent dye.

Dilution	Diamond Nucleic Acid Dye (DD)	SYBR Green I (SG)	EvaGreen (EG)	BRYT Green (BG)
1	13.82 (0.02)	12.33 (0.03)	17.45 (0.03)	14.02 (0.03)
1/10	15.53 (0.02)	15.26 (0.05)	20.97 (0.07)	17.40 (0.07)
1/100	19.37 (0.20)	19.33 (0.12)	25.48 (0.11)	21.72 (0.08)
1/10 ³	23.17 (0.08)	22.99 (0.08)	29.84 (0.17)	25.68 (0.05)
1/10 ⁴	27.03 (0.71)	26.28 (0.20)	34.50 (0.42)	29.24 (0.12)
1/10 ⁵	32.93 (1.47)	31.93 (0.26)	39.92 (0.41)	34.51 (0.48)

Each dilution series started with an initial DNA concentration of 28.4 ng per reaction, with the C_q threshold set at 0.09 and the standard deviation shown in parentheses. Results from the 1/10⁶ dilutions were not reproducible.

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1). The slope of the trendline (Figure 1B) indicates the degree of amplification inhibition (15.9) as the concentration of the DD increases. Complete inhibition occurred at concentrations $>1.5\times$ (data not shown), and partial inhibition occurred at $1.5\times$ (2 of 4 replicates had an amplification product).

A previous study (12) showed that at lower DNA quantities (25 ng), SG can inhibit the reaction when concentrations are $>0.5\times$. This is in contrast to DD, which showed inhibition at concentrations $>1.5\times$. In the same study, EG showed no inhibition of qPCR at concentrations $>2\times$, with DNA amounts ranging 25–100 ng (12), indicating EG has less of an inhibitory effect on qPCR when compared with DD.

Average C_q values for DD were 0.04–1.49 cycles higher than SG (Table 2); a higher C_q value indicates a lower initial copy number for the target amplification product. It should be noted that DD was compared with the SG kit (KAPA SYBR FAST qPCR Kit), which has an engineered *Taq* polymerase that is more resistant to the inhibitory effects of SG within the reaction, as stated by the manufacturer. We expect that with a similar manufacturing process, DD would likely show improved C_q values from those determined in this study. In comparison to EG, DD has lower C_q values, ranging 3.63–7.47 fewer cycles. When comparing DD to BG, the C_q values for DD are on average 0.20–2.51 cycles lower than BG.

Total human genomic DNA was quantified using the Qubit dsDNA HS Assay (28.4 ng/ μ L) and was used as an approximation of the total amount of DNA present in the sample. The manufacturer states the assay is able to accurately detect between 0.2 pg/ μ L and 100 ng/ μ L DNA. As the primers used in these reactions are for the mitochondrial genome, the detection limit would be expected to be lower than for primers used to target nuclear DNA. Due to the multi-copy nature of mitochondrial DNA (mtDNA) within a single mitochondrion and the variation in the number of mitochondria per cell, it is not possible to determine the exact number of mitochondrial genomes per cell; however an average of 500 per cell has been suggested (14). Based on this average value, the neat sample (28.4

Figure 3. Standard curves of DNA concentration against C_q values for the different fluorescent dyes. SYBR Green (SG), BRYT Green (BG), and EvaGreen (EG) were analyzed through the green channel, and Diamond Nucleic Acid Dye (DD) was analyzed through the diamond channel. The efficiency of the reaction and the R^2 values indicating the linearity of the dyes' fluorescent signals are also shown.

ng/ μ L) contains ~ 2.1 million copies of mtDNA/ μ L, and the lowest dilution ($1/10^6$) contains ~ 2 copies of mtDNA/ μ L. This is comparable to the sensitivity claimed for SG.

Our data showed that DD exhibited a curve shape that is seen with other commonly used dyes for qPCR, such as SG, EG, and BG (Figure 2), demonstrating its suitability for use in qPCR.

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Figure 4. Average melt curve of serial dilutions of DNA (A) SYBR Green (SG); (B) EvaGreen (EG); (C) Diamond Nucleic Acid Dye (DD); and (D) BRYT Green (BG). SG, EG, and BG were analyzed through the green channel, and DD was analyzed through the diamond channel. The value for the neat DNA at 28.4 ng/μL is 1, followed by the 1/10 serial dilutions. All of the replicates for the 1/10⁶ dilution are shown in order to illustrate their variability. NTC = no-template control.

The linearity (R^2) of the standard curves was >0.97 for all dyes (Figure 3). BG had the highest R^2 value (0.996) and DD had the lowest value (0.970). Based on the values generated from the standard curve in relation to the slope (15), DD had the highest reaction efficiency at 0.83 and EG had the lowest at 0.67 (Figure 3). Combining all of the aspects of qPCR (15), it can be seen that DD is comparable with other currently commercially available dyes.

At genomic DNA concentrations less than the 1/10⁵ dilution, the dyes were not capable of generating a reproducible signal, and at a 1/10⁶ dilution, the samples failed to amplify. Some replicates that did not produce an amplification signal produced a broad melt peak at ~75–77°C (Figure 4). One of the 4 replicates of the 1/10⁶ dilution SG sample (Figure 4A) produced the target melt peak. The other 3 replicates had a broad peak ranging ~75–77°C, and these peaks were 5-fold smaller than the peak seen for DD (Figure 4C). Similarly, for BG (Figure 4D), 1 of 4 replicates for the 1/10⁶ dilution produced the target melt product; the other 3 replicates and the NTC produced no products. For EG, the 1 of 4 replicates for the 1/10⁶ dilution produced the target melt peak, along with a broad peak at 77°C; another of the replicates produced only a broad peak at 77°C; and the remaining 2 replicates and the NTC produced no products (Figure 4B).

For DD, there were no amplification products in the 1/10⁶ dilution sample and the NTC samples (Figure 4C). The observed broad minor melt peaks are ~77°C, which would likely correspond to the expected melting point of the primer-dimer. All of the other DNA dilutions for DD had peaks at ~85°C. The results from the 1/10⁶ dilution may be due to differences in the binding mechanism of DD in compared to the other dyes typically used. DD is an external groove binding dye, which means the DNA does not need to be double-stranded for the dye to bind. The molecular structure of DD is proprietary, which limits any further comment on mechanism. In comparison, SG, which intercalates between the base pairs of DNA and has electrostatic interactions, only binds double-stranded DNA (dsDNA) (3). This would be a plausible explanation as to why DD produces a signal in the NTC and in dilutions that are too low for detectable amplification.

A further study was conducted to determine if DD binds to single-stranded DNA (ssDNA), but there were no ssDNA

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amplification products (data not shown); therefore, this was not the cause of the variability in DD at the lowest dilutions (Figure 3). Furthermore, the disassociation of DD from ssDNA occurs at a relatively low temperature of ~73°C (data not shown), which is ~4 degrees lower than the temperature at which the primer dimer was observed in the reactions (77°C) (Figure 4). The binding of DD to ssDNA is therefore not likely to be an issue for normal qPCR reactions.

This is the first study to examine the use of DD, which to date has only been used for staining agarose gels, for qPCR. The inhibitory effect DD has on PCR was investigated, and DD was found to completely inhibit the reaction at concentrations >1.5x. The efficiency, linearity, and sensitivity of the reaction were also investigated using serial dilutions of DNA. DD allowed detection of as little as 0.28 pg of DNA (~20 copies of mtDNA target). This sensitivity is comparable to SG, EG, and BG, which are currently used for qPCR. DD is a suitable, and cheaper (by approximately 5 fold), alternative to the dyes currently used for qPCR detection.

Author contributions

A.M.H., S.S.T., and A.L. conceived the idea for the study. A.M.H. performed all laboratory work. A.M.H., S.S.T., and A.L. analyzed the data and drafted the manuscript. All authors approved of the final manuscript prior to submission.

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Competing interests

The authors declare no competing interests

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