



Blending DNA binding dyes to improve detection in real-time PCR



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ABSTRACT

The success of real-time PCR (qPCR) analysis is partly limited by the presence of inhibitory compounds in the nucleic acid samples. For example, humic acid (HA) from soil and aqueous sediment interferes with amplification and also quenches the fluorescence of double-stranded (ds) DNA binding dyes, thus hindering amplicon detection. We aimed to counteract the HA fluorescence quenching effect by blending complementary dsDNA binding dyes, thereby elevating the dye saturation levels and increasing the fluorescence signals. A blend of the four dyes EvaGreen, ResoLight, SYBR Green and SYTO9 gave significantly higher fluorescence intensities in the presence and absence of HA, compared with the dyes applied separately and two-dye blends. We propose blending of dyes as a generally applicable means for elevating qPCR fluorescence signals and thus enabling detection in the presence of quenching substances. © 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Real-time PCR (qPCR) is a powerful analytical tool, used for diagnostic and research applications in various scientific fields. However, its success is partly limited by the presence of inhibitory compounds from the samples or the analysis process [1]. PCR inhibitors can disturb amplification by directly affecting the DNA polymerase, by binding to nucleic acids or by altering the ion composition in the reactions [2]. Humic substances, in particular humic acid (HA), from soil and aqueous sediment are well-known PCR inhibitors shown to interfere with the DNA polymerase [3–5]. We recently found that HA also obstructs qPCR amplicon detection, i.e. causes lowered fluorescence signals in reactions although amplification is unaffected [5]. HA molecules quenched the fluorescence of four common double-stranded (ds) DNA binding dyes, most likely through static quenching via binding between HA and dye.

Increased dye concentrations could be applied to counteract the HA-induced fluorescence quenching. However, high concentrations of dyes such as SYBR Green I and EvaGreen inhibit PCR amplification [6–8]. Blending dsDNA binding dyes has been proposed as a means to improve PCR detection in pure reactions,

by elevating the fluorescence signals and lowering quantification cycle (Cq) values [9].

The objective of this study was to counteract the detection inhibition caused by HA in qPCR, by applying different concentrations of the dsDNA binding dyes SYBR Green I (SG), EvaGreen (EG), ResoLight (RL) and SYTO9 (SY), individually or as blends. We propose blending of dyes as a generally applicable means for elevating qPCR fluorescence signals and thus enabling detection in the presence of quenching substances.

2. Materials and methods

Humic acid standard (product number 53680, Sigma-Aldrich, Taufkirchen, Germany) was dissolved in TE buffer to 500 ng/μL (10 mM Tris, 0.1 mM EDTA, pH 8) (Medicago, Uppsala, Sweden) and diluted to the following final amounts per reaction: 100 ng, 200 ng and 500 ng. Template DNA (Quantifiler Human DNA Standard, Life Technologies), was quantified with Biodrop μLITE (BioDrop) and 2 ng per reaction was used.

A LightCycler Nano instrument with LightCycler Nano Software v 1.1 (Roche Diagnostics, Basel, Switzerland) was used for all qPCR experiments. Total reaction volume was 20 μL. The following reagents were included in the reactions: 1× Immobuffer (Bioline Reagents Ltd., London, United Kingdom), 0.2 mM dNTP (Roche Diagnostics), 0.5 μg/μL BSA (Roche Diagnostics), 4 mM MgCl₂ (Roche Diagnostics) and 0.3 μM of each primer, RB1_80F and RB1_235R, (Life Technologies, New York, NY, USA) [10] and 1 U Immolase DNA polymerase (Bioline Reagents Ltd.). Reactions were run in technical duplicates or triplicates.

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The dsDNA binding dyes were used in the following concentrations: EvaGreen (Biotium Inc., Hayward, CA, USA): $0.5\times$ ($0.665\ \mu\text{M}$ [7]), $1\times$ ($1.33\ \mu\text{M}$), $2\times$ ($2.66\ \mu\text{M}$), $3\times$ ($3.99\ \mu\text{M}$); SYBR Green I (Life Technologies): $0.25\times$ ($0.17\ \mu\text{M}$ [7]), $0.5\times$ ($0.34\ \mu\text{M}$), $1\times$ ($0.68\ \mu\text{M}$), $2\times$ ($1.36\ \mu\text{M}$); SYTO9 (Life Technologies): $2\ \mu\text{M}$, $4\ \mu\text{M}$, $6\ \mu\text{M}$, $10\ \mu\text{M}$; and ResoLight (Roche Diagnostics): $0.5\times$, $1\times$ and $2\times$. The dyes were applied individually and in combinations of two, three and four dyes. Excitation and emission spectra for the dyes, and recommended dye concentrations, are presented in Supplementary Table S1.

qPCR run conditions were: initial heat-activation of DNA polymerase at 95°C for 10 min, 45 cycles of 10 s at 95°C and 50 s at 60°C . Melt curve analysis was performed with an increase of $0.1^\circ\text{C}/\text{s}$, starting from 60°C and ending at 97°C .

Amplification of the correct product (156 bp) was verified with 1% agarose gel electrophoresis, stained with $1\times$ GelRed (Biotium). Gel bands were visualized using BioOne Quantity (Bio-Rad).

Data are presented as the average end-point fluorescence intensity (FI), and the dyes/dye blends are statistically compared using Student's t test with a significance level of 5%.

3. Results and discussion

Initially, we investigated at which concentrations individual dyes could be applied without causing dye-induced amplification inhibition, defining inhibition as a C_q value shift >1 cycle with respect to the lowest concentration tested for each dye.

We found that $1\times$ SYBR Green I (SG, $0.68\ \mu\text{M}$) and $2\times$ EvaGreen (EG, $2.66\ \mu\text{M}$) inhibited amplification (C_q value shifts of 4.2 and 1.1 cycles, respectively) (data not shown). A five-fold increase of SYTO9 (SY, from $2\ \mu\text{M}$ to $10\ \mu\text{M}$, did not disturb amplicon generation (C_q value shift of 0.2 cycles). These results coincide with previous findings on dye-induced amplification inhibition [6,7]. $2\times$ ResoLight (RL) was also inhibitory (C_q shift of 2.3 cycles). Thus, the maximum applied dye concentrations in the blends used were $1\times$ EG, $0.5\times$ SG ($0.25\times$ SG in the four-dye blend due to partial inhibition), $10\ \mu\text{M}$ SY and $1\times$ RL.

End-point fluorescence intensity (FI) and normalised fluorescence intensity for single dyes and three representative dye blends are presented in Fig. 1 and Supplementary Table S2. In pure reactions without HA (positive controls, PC), the dye blends gave significantly higher FI values compared with each dye used individually ($p < 0.05$). Similarly to the results in Kavanagh et al.

[9], the fluorescence intensities of the blends were approximately additive compared with the individual dyes. The mean fluorescence intensities for the dyes were 1.16 fluorescence units for $1\times$ EG, 0.15 for $0.25\times$ SG, 0.93 for SY and 0.85 for RL, whereas the four-dye blend showed a value of 2.29.

HA lowered the fluorescence intensity for all dyes and blends, but the blends generally gave higher FI values compared with each dye used separately (Fig. 1 and Supplementary Table S2). The highest FI values, both in positive controls and with addition of HA, were reached by blending all four dyes. For example, for 100 ng HA the four-dye blend gave significantly higher fluorescence intensities compared with the individual dyes and two-dye blends ($p < 0.05$, Supplementary Table S2). Interestingly, HA-induced fluorescence quenching was least pronounced in reactions with SY (Fig. 1 and Supplementary Table S2). SG was also quite HA-tolerant, although the fluorescence levels were lower than for SY. With SY alone, the fluorescence intensity was unaffected by 200 ng HA ($p = 0.88$), whereas EG and RL were severely quenched by 100 ng HA ($p < 0.01$).

Clearly, the investigated dyes have different properties in terms of fluorescence, interaction with HA and negative effect on amplification. This is likely, in part, due to different structures and modes of binding to dsDNA. The majority of dyes used for DNA visualization are cyanine dyes [11] interacting with dsDNA through two ways of non-covalent binding: intercalation and minor-groove binding [12]. SG is an asymmetrical cyanine dye [13], and EG is presumably a symmetrical cyanine dye [9], although the exact structure is a trade secret. The high HA-tolerance of SY is likely linked to its high concentration ($10\ \mu\text{M}$) compared with EG ($1\times - 1.33\ \mu\text{M}$) and SG ($0.5\times - 0.34\ \mu\text{M}$). However, it is also plausible that SY has lower affinity for binding to HA molecules compared with the other dyes, since $2\ \mu\text{M}$ SY also showed high HA-tolerance (Supplementary Table S2).

Apart from disturbing the fluorescence, HA may interfere with the DNA polymerase and thus obstruct amplification [3,5]. For 500 ng HA, amplification was inhibited as seen by increased C_q values for all dyes used separately (data not shown). On the other hand, C_q values were lowered by adding this amount of HA to the four-dye blend and the SG/SY blend (data not shown). This is due to the complex interactions between HA, the dyes and amplification: the high amount of dye molecules in the blends presumably lowers the amplification efficiency. The increased level of HA leads to a lowered amount of free dye. Thus, for high amounts of HA,

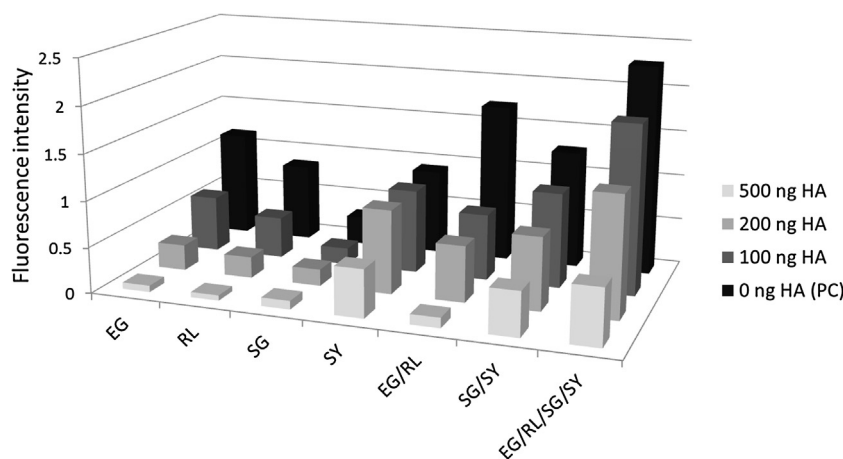


Fig. 1. Mean end-point fluorescence intensities for individual dyes and dye blends in the presence and absence of humic acid (HA). The concentrations of the dyes are: individual dyes: $1\times$ EG; $1\times$ RL; $0.5\times$ SG; $10\ \mu\text{M}$ SY; two-dye blends: $1\times$ EG/ $1\times$ RL; $0.5\times$ SG/ $10\ \mu\text{M}$ SY; four-dye blend: $1\times$ EG/ $1\times$ RL/ $0.25\times$ SG/ $10\ \mu\text{M}$ SY. Values are presented as mean FI of three replicates. For standard deviations, see Supplementary Table S1. PC: Positive control. $n = 2-3$.

Table 1

The amplicon melt temperatures (T_m) decrease with increasing concentrations of HA, for single dyes as well as for dye blends. The smallest difference in T_m between 0 and 500 ng HA was seen with the blend SG/SY followed by SY alone. The concentrations of the dyes are; individual dyes: 1 × EG; 1 × RL; 0.5 × SG; 10 μM SY; two-dye blends: 1 × EG/1 × RL; 0.5 × SG/10 μM SY; four-dye blend: 1 × EG/1 × RL/0.25 × SG/10 μM SY. Values are presented as mean T_m ± STD, n=2.

HA (ng)	EG	RL	SG	SY	EG/RL	SG/SY	EG/RL/SY/SY
0	82.07 ± 0.005	82.65 ± 0.020	83.14 ± 0.026	81.79 ± 0.01	82.85 ± 0.017	82.47 ± 0.016	83.06 ± 0.057
100	81.45 ± 0.071	81.84 ± 0.024	82.28 ± 0.014	81.56 ± 0.019	82.44 ± 0.008	82.43 ± 0.035	82.99 ± 0.167
200	81.12 ± 0.095	81.73 ± 0.016	82.14 ± 0.024	81.40 ± 0.018	82.20 ± 0.018	82.24 ± 0.044	82.51 ± 0.028
500	80.90 ± 0.089	81.59 ± 0.037	81.90 ± 0.016	81.00 ± 0.002	81.92 ± 0.042	82.02 ± 0.092	82.09 ± 0.062
$\Delta T_m(0-500 \text{ ng})$	1.17	1.06	1.24	0.51	0.93	0.45	0.97

quantification may be biased, irrespective of whether individual dyes or blends are applied.

However, qualitative detection in presence of quenching substances can be substantially improved by applying dye blends or high amounts of SY (10 μM). With 800 ng HA, the qPCR fluorescence was completely quenched for 0.5 × SG and 1 × RL respectively, although gel electrophoresis showed that amplification was successful. For 1 × EG, fluorescence was detected in two out of three reactions. Applying the four-dye blend or 10 μM SY alone gave detectable amplification in all replicates (data not shown).

Taken together, blending dyes for analysis of samples containing HA gives the advantage of elevated fluorescence intensity and enhanced detection, which leads to decreased numbers of false negative qPCR results. The HA tolerance of the blend is mainly attributed to the presence of SY. In a practical setting where a commercial master mix is used, containing e.g. EG or SG, SY may be added to make detection of environmental samples more reliable. SYTO dyes, including SYTO9, have been proposed as promising alternatives to the established SG in a couple of earlier publications, due to their lower PCR inhibitory effects [6,14].

Apart from lowered levels of fluorescence, HA also caused lowered amplicon melt temperatures (T_m) (Table 1), presumably by partly hindering the dye molecules from binding to and strengthening the double-stranded form of the PCR products. High levels of DNA saturation is important for melt curve applications, especially high resolution melting analysis (HRM), where small differences in the amplicon melt pattern are used to infer genotypes [15]. Interestingly, RL and EG, the two dyes that showed the greatest losses in FI and substantial changes of T_m in presence of HA, are commonly used and recommended for HRM [16,17]. The blend of SY/SG and SY alone on the other hand, gave the smallest differences in T_m when HA was added. Thus, application of SY could be beneficial also for melt applications. Above all, the results in this study stress that DNA extract purity is essential for accurate HRM analysis.

4. Conclusions

The development of inhibitor-tolerant DNA polymerase-buffer systems has led to a new bottleneck in qPCR analysis of environmental samples: fluorescence quenching by humic acids [5]. We aimed to counteract this effect by preparing blends of complementary dsDNA binding dyes, thereby elevating the dye saturation levels and increasing the fluorescence signals. A blend of four dyes containing 1 × EG, 1 × RL, 0.25 × SG and 10 μM SY gave significantly higher fluorescence intensities in presence and absence of HA compared with the dyes applied separately and two-dye blends. A higher end-point fluorescence may improve analysis by making detection possible also in the presence of quenching substances. Out of the single dyes, SY exhibited the highest resistance to HA-induced detection inhibition, and the smallest differences in T_m due to the presence of HA. This indicates

that SY is suitable also for melt curve applications such as HRM. Blending dsDNA binding dyes is proposed as a general approach for improving qPCR analysis of samples containing substances that cause fluorescence quenching. The most straightforward way of doing this would be to add SY to any commercial master mix that is applied. However, it should be noted that amplification may also be affected by high levels of HA, possibly leading to biased quantification results. Thus, the scope for improved detection with dye blends may be limited to qualitative analysis.

Competing interests statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.btre.2017.02.002>.

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