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Fluorescent cyanine dyes for the quantification of low amounts of dsDNA

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ABSTRACT

In this research six cyanine fluorophores for the quantification of dsDNA in the pg-ng range, without amplification, are compared under exactly identical conditions: EvaGreen, SYBR Green, PicoGreen, AccuClear, AccuBlue NextGen and YOYO-1. The fluorescence intensity as a function of the amount of dsDNA is measured at the optimal wavelengths for excitation and emission and for each dye the limit of detection and the response linearity at low levels of dsDNA are determined. No linear range was found for SYBR Green and YOYO-1 for pg-ng quantities of dsDNA. EvaGreen, PicoGreen, AccuClear and AccuBlue NextGen show good linearity in the pg-ng range. AccuClear exhibits the widest linear range of 3 pg −200 ng, whereas AccuBlue NextGen turned out to have the highest sensitivity of the tested dyes with a limit of detection of 50 pg.

1. Introduction

Quantification of low amounts of dsDNA is important, in particular in forensic DNA-analysis. To quantify dsDNA without amplification the absorbance at 260 nm can be measured by UV-spectrophotometry, but such measurements are rather insensitive and influenced by the contribution of nucleotides and single-stranded DNA or contaminants (such as proteins or phenol) [1,2]. Therefore, of the known methods for the detection and quantification of dsDNA, such as nucleic acid stains (e.g. Methyl Violet and Thiazole dyes) and nucleic acid labels (e.g. Texas Red, Cy5 and Carboxy-X-Rhodamine (ROX)), the majority is based on fluorescence detection [3]. Three main classes of nucleic acid stains exist: intercalating dyes (e.g. ethidium bromide (EtBr) and propidium iodide (PI)), minor-groove binders (e.g. DAPI and Hoechst dyes) and other nucleic acid stains (e.g. acridine orange). The possible binding options, i.e. (bis-)intercalation, major and minor groove binding and external binding, are depicted in Fig. 1 [4].

In the past the classic intercalating dyes EtBr and PI were often used, which both bind at a stoichiometry of 1 dye molecule per 4 to 5 base pairs [4]. These dyes have a limited sensitivity and high background fluorescence.

The sensitivity can be increased by adding positively charged side chains to a dye, which increases the electrostatic interaction between dye and DNA. Another possibility is to add a (positively charged) linker to form a dimeric or trimeric dye [5,6]. Cyanine dyes have a high affinity for nucleic acids and show 100- to 1000-fold fluorescence enhancement upon binding to DNA and cyanine dyes are also less mutagenic than the classic dyes [4]. In this research the spectral properties of cyanine fluorescent dyes EvaGreen (EG), SYBR Green (SG), PicoGreen (PG), AccuClear (AC), AccuBlue NextGen (AB) and YOYO-1 (YO) are investigated for direct detection and quantification of low amounts of dsDNA, without any amplification. These dyes are selected, because they are commonly used as nucleic acid stains within real-time PCR, gel analysis, DNA quantification kits (e.g. Qubit PicoGreen Assay) and melting curve analysis. EvaGreen and SYBR Green are dyes from two different manufacturers widely used for real-time PCR and melt curve analysis, but also for agarose gel staining [7,8]. PicoGreen and AccuClear/AccuBlue NextGen are also from two different manufacturers and are developed for the quantification of dsDNA dyes, whereas the last two are specifically designed to quantify low amounts of dsDNA without any amplification step [9]. These newly developed dyes are compared with YOYO-1, a dye that is already available for some decades and used for (counter)staining DNA [10].

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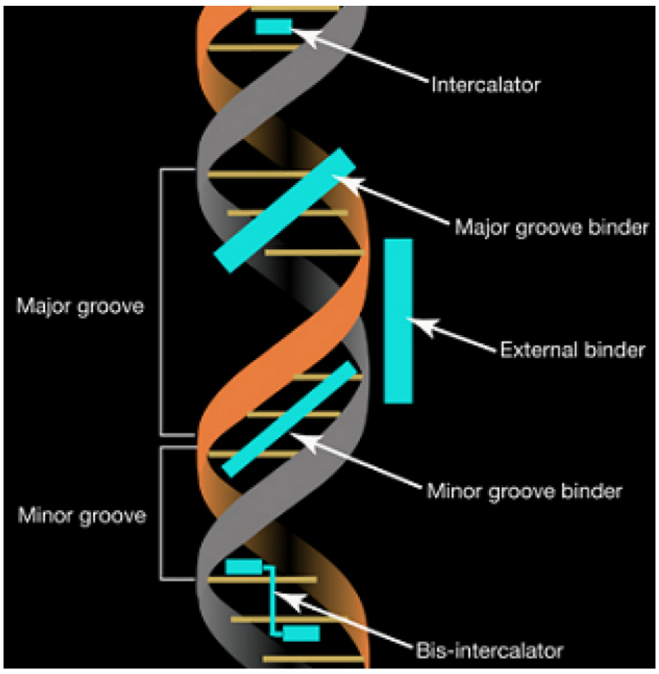

Fig. 1. Schematic diagram showing the possible binding modes of dsDNA dyes, used with permission from Thermo Fisher Scientific, copyrighted 2015 (*www.lifetechnologies.com*) [4].

In the Data in Brief (DiB) a literature based overview is given of the absorption, excitation and emission wavelengths at which maxima occur for the various dyes, as free dye in solution and as dye/dsDNA complex [22].

The reported linearity of each dye obtained with various detection methods, is shown in Table 1. Since some groups report the linear range in concentration units [2,12,15] whereas others present the range in absolute amounts of dsDNA [11,14,18], comparison is difficult. When reported as concentration range, either the final concentration in the detection volume can be given or the added concentration of dsDNA to the dye. Similar, reports in absolute amounts of dsDNA can give the total amount in the detection volume or the added amount of dsDNA to the dye. Therefore, if it is mentioned in literature, the linear range is converted to absolute amounts of dsDNA (when reported in concentration units) or the end concentration (upon reporting in absolute amounts) and the volume of dsDNA in the total volume is given in Table 1.

From the review in the DiB, and in particular the data in Table 1 and Table 1.1 (DiB) [22], it becomes clear that for each dye the reported spectral data and linear ranges vary significantly. The most striking differences in terms of linearity are found at low dsDNA concentrations, which is likely due to the use of different experimental setups. Therefore the six selected dyes are restudied under exactly similar conditions, in one and the same instrument (a microplate reader), with a focus on the pg-ng range of dsDNA. For AccuClear and AccuBlue NextGen, this will be the first report in an academic journal. Note that in this paper no statements are made about the use and linearity of the dyes in the context of real-time PCR, which is another well-established method to quantify dsDNA. Here only the direct fluorescent quantification is discussed.

2. Materials and methods

Materials. Deoxyribonucleic acid sodium salt from salmon testes (D1626) was obtained from Sigma-Aldrich. SYBR® Green I (10.000X concentrate in DMSO) and Quant-iT™ PicoGreen® (400X concentrate) in DMSO were purchased from Life Technologies, as well as

Table 1
Linearity of dsDNA dyes according to literature for the given dye concentration, detection volume and detection method. If no information on the exact wavelength of excitation and/or emission was reported (e.g. because a LED was used for excitation), mentioned excitation and emission values are maxima of spectra. # = on assumption that the end concentration or final amount of dsDNA has been reported, * = information from the manufacturer of the dye.

	Linear range (DNA)	Concentration (dye)	Volume	Detection method	Incubation	Ref.
EG	5–250 pg/μL 10 μL (1–50 ng)	0.63 μM (0.5X)	200 μL	Microplate reader (485/530 nm)	30 min @ RT	[5]
	10–500 ng 95 μL (0.1–5 ng/μL)#	1.0X (1.33 μM)	100 μL	Spectrofluorometer (503/527 nm)	5 min @ 72 °C	[11]
	0–10 ng/μL n/a (0–2000 ng)#	11.15 μM	200 μL	Microplate reader (490/530 nm)	30 min @ 50 °C	[12]
	2.4–60 pg/μL n/a (n/a)	0.1X (0.133 μM)	~10 nL	CE & LIF (488/520 nm)	Measuring @ RT	[13]
	1–100 ng 23.5 μL (0.04–4 ng/μL)#	1.0X (1.33 μM)	25 μL	Real-time PCR (490/520 nm)	Measuring @ 25 °C	[14]
SG	2.4–30 pg/μL n/a (n/a)	Not known (1/40,000 dilution)	~10 nL	CE & LIF (488/520 nm)	Measuring @ RT	[13]
	0–2 pg/μL n/a (n/a)	1.0X (0.68 μM)	n/a	Spectrofluorometer (480/520 nm)	5 min @ RT	[15]
	0.5–50 ng n/a (n/a)	1.0X (0.68 μM)	n/a	Agarose gel (460/560–700 nm)	not given	[16]
PG	0.025–1000 pg/μL 1 mL (0.05–2000 ng)	1.0X (0.8 μM)	2 mL	Spectrofluorometer (480/520 nm)	2–5 min @ RT	[2], [9],*
	3–450 pg/μL n/a (n/a)	Not given	Not given	Spectrofluorometer (480/520 nm)	not given	[17]
	0–2 pg/μL n/a (n/a)	1.0X (0.8 μM)	n/a	Spectrofluorometer (480/520 nm)	5 min @ RT	[15]
	0.25–150 ng 10 μL (1.67–1000 pg/μL)	Not known (1/200 dilution)	150 μL	Spectrofluorometer (485/520 nm)	not given	[18]
AC	0.03–250 ng 10 μL (0.14–1190 pg/μL)	1.0X	210 μL	Microplate reader (468/507 nm)	5 min @ RT	[19]*
AB	1–3000 pg 10 μL (0.005–14.29 pg/μL)	1.0X	210 μL	Microplate reader (468/507 nm)	5 min @ RT	[20]*
YO	0.5–100 pg/μL n/a (1–200 ng)#	200 nM	2 mL	Fluorometer (470/510 nm)	not given	[21]

Table 2
Excitation and emission wavelengths used for the various dyes.

Dye	Excitation	Emission
EG	500 nm	530 nm
SG	497 nm	520 nm
PG	502 nm	523 nm
AC	468 nm	507 nm
AB	468 nm	507 nm
YO	491 nm	509 nm

YOYO-1™ Iodide (1 mM) in DMSO. The AccuClear™ Ultra High Sensitivity kit (100X concentrate) in DMSO and the AccuBlue™ NextGen dsDNA quantitation Kit[1] (400X concentrate) in DMSO were obtained from Biotium, as well as EvaGreen® (20X concentrate) in MilliQ. For all experiments TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was used as solvent, as recommended in all protocols of the manufacturers of the dyes.

Absorption spectra. For EvaGreen, SYBR Green and PicoGreen 100 μL of various DNA solutions (0–50 pg/μL and 0–50 ng/μL) were added to 100 μL 2.0X concentrate dye solutions, to end up with an end concentration of 1.0X. For YOYO-1 100 μL of various DNA solutions and 100 μL of 200 nM dye solution was used (to end up with an end concentration of 100 nM, as is recommended in the protocol of the manufacturer). Thus the total volume of the solution in each well of a Corning 96 flat bottom black polystyrene microplate was 200 μL. In case of AccuClear and AccuBlue NextGen the manufacturers' protocols were followed by adding 10 μL of various DNA solutions to 200 μL working solution (AccuClear dye in the provided buffer or AccuBlue NextGen dye and enhancer (100X) in the provided buffer), yielding 210 μL.

Absorption spectra were taken in duplo (i.e. two separate 96 well plates) with a Tecan M200 PRO multimode reader, operated by Tecan I-control software. All spectra were recorded at room temperature, after incubation in the dark for about 20 min. 20 min is chosen as incubation time to obtain a steady state for all dyes, and to exclude intensity differences possibly present in the first minutes, since filling and mixing of a complete microplate takes some time.

Excitation and emission spectra. Excitation and emission spectra were taken of the 1.0X free dye solutions (note: 100 nM for YOYO-1). The dsDNA/dye complex consists of 1.0X dye and 50 ng/μL (note: 4.76 ng/μL for AccuClear and AccuBlue NextGen) dsDNA solution, according to the above described protocol.

The spectra were taken with a Perkin Elmer LS55 fluorescence spectrometer with an 8 μL 1 $\times$ 1 mm flow through precision cell from Hellma Analytics. Excitation spectra were recorded by monitoring the emission wavelength given in Table 2 and emission spectra were recorded by exciting at the wavelength given in Table 2. The excitation and emission slit were set at 2.5 nm and 3.5 nm, respectively. All spectra were recorded at room temperature, directly after adding the DNA to the dye.

Fluorescence intensity. For the fluorescence intensity, the same protocol as for the absorption measurements was followed with the optimal dye concentration and buffer as prescribed by the manufacturer. When a kit with control dsDNA was used, the fluorescence intensity of this DNA was also measured. Excitation and emission wavelengths given in Table 2 were used. Due to the small Stokes shift at optimal wavelengths (502/523 nm), for the experiments with PicoGreen excitation and emission wavelengths of 480 nm and 520 nm were used, respectively. All measurements were carried out in triplo (three measurements within the same 96 well plate) and in duplo (i.e. two separate 96 well plates) in a Corning 96 flat clear bottom black polystyrene microplate or a Greiner 96 flat dark bottom black polystyrene microplate at room temperature, after incubation in the dark for about 20 min.

The limit of detection (LOD) is defined as $LOD = x_{bl} + k\sigma_{bl}$, where x_{bl} is the mean of the blank measurements (only TE-buffer added to the dye and no DNA) and σ_{bl} the standard deviation of the blank measurements. k is numerical factor for the confidence level desired and is chosen to be 3, as recommended for spectroscopic analysis [23].

3. Results and discussion

Below the spectra and optimal wavelengths are discussed for each dye. All the maxima are also given in Table 3. In case the value differs from literature (±5 nm) the wavelength is italicized in the table. Values not given in literature are underlined. Moreover, the linear range of each dye is compared with literature and marked italic when the found values deviate from literature or values given by the manufacturer. These deviations are also discussed and explained, where possible, below.

Absorption spectra. For EvaGreen, SYBR Green, PicoGreen and AccuClear a bathochromic shift in the absorption spectrum can be observed between the free state and the dsDNA/dye complex (Fig. 2.1 DiB) [22].

The absorption spectrum of 1.0X EvaGreen with 1 ng salmon DNA shows a peak around 470 nm (Fig. 2.2 DiB) [22]. For increasing amounts of DNA the peak at 470 nm decreases and it turns into a minor peak, while a peak around 500 nm appears, which becomes the major peak. The peak of free 1.0X SYBR Green is relatively broad with two maxima at about 475 nm and 494 nm. A maximum can be seen at 495 nm upon binding of dsDNA (10 ng/μL, which corresponds to 100 ng) to the dye (Fig. 2.3 DiB) [22]. When the same amount of dsDNA is added to 1.0X PicoGreen the absorbance peak shift from 498 nm to 500 nm (Fig. 2.4 DiB) [22]. Upon addition of 10 ng/μL (which corresponds to 10 ng) to 1.0X AccuClear, a little shift from 465 nm to 468 nm can be seen (Fig. 2.5 DiB) [22]. With increasing amounts of DNA the spectra of EvaGreen, SYBR Green, PicoGreen and AccuClear become more distinct and in most cases the absorbance values increase (Fig. 2.2–2.5 DiB) [22]. In comparison with other dyes (except for AccuClear) AccuBlue NexGen shows a low absorption peak with a maximum around 472 nm for both the free dye and the dsDNA/dye complex (Fig. 2.6 DiB) [22]. No absorption peak could be observed in the spectra of YOYO-1 with 0–1000 ng of salmon DNA, while maxima around 460 nm and 490 nm are expected for the free dye and the dsDNA/dye complex, respectively (Fig. 2.7 DiB) [22]. However, in literature for this experiment a higher dye concentration than 100 nM was used, namely 1–10 μM [24].

For each dye the absorption maxima given in Table 3 are mostly (EvaGreen, SYBR Green, Picogreen, AccuClear and AccuBlue NextGen) in agreement with the values reported in literature (Table 1.1 DiB) [12,19,20,22,24,25].

Excitation and emission spectra. For the free state of EvaGreen and PicoGreen no distinct peaks could be distinguished in the spectra. In literature the used dye concentrations to determine the maxima were higher than 1.0X, namely 11.42 μM and a ratio of 1:10 dye/dsDNA base pair for EvaGreen and PicoGreen, respectively [12,25]. The free dyes of SYBR Green and AccuClear showed maxima for excitation/emission at 496/518 nm and 486/511 nm, respectively (Fig. 2.8, 2.10 and 2.12 DiB) [22]. The emission of free SYBR Green in literature is claimed to be 530 nm, which is not in accordance with these results [25]. No data is reported regarding other maxima of these two dyes. For AccuBlue NextGen and YOYO-1 the maxima for excitation/emission are present at 479/514 nm and 484/511 nm, respectively. The emission maximum of YOYO-1 in free

[1] Biotium was so kind to provide a trial kit AccuBlue NextGen (200 assays).

Table 3
Absorption, excitation and emission wavelengths for dyes free in solution and dye/dsDNA complexes, Stokes shift of the dsDNA/dye complex and limit of detection with corresponding linear range. In case the value differs from literature (±5 nm) the wavelength is italicized. Values not given in literature are underlined. The linear range of each dye is compared with literature and marked italic when the found values deviate from literature or values given by the manufacturer. n.p. = not present.

Dye	Absorption		Excitation		Emission		Stokes shift		LOD	Linear range
	Free	Complex	Free	Complex	Free	Complex	Free	Complex		
EG	470 nm	500 nm	*n.p.*	503 nm	*n.p.*	525 nm	n.p.	22 nm	10 ng	*0.2 ng – 100 ng*
SG	494 nm	495 nm	496 nm	497 nm	*518 nm*	520 nm	22 nm	23 nm	n.p.	*n.p.*
PG	498 nm	500 nm	n.p.	500 nm	n.p.	521 nm	n.p.	21 nm	100 pg	*10 pg – 100 ng*
AC	465 nm	468 nm	486 nm	470 nm	511 nm	*498 nm*	25 nm	28 nm	100 pg	3 pg – 200 ng
AB	472 nm	472 nm	479 nm	*484 nm*	514 nm	507 nm	35 nm	23 nm	50 pg	*5 pg – 5 ng*
YO	*n.p.*	*n.p.*	484 nm	490 nm	*511 nm*	508 nm	27 nm	18 nm	n.p.	n.p.

state is not consistent with literature, but higher [24]. No data is reported regarding other maxima of these two dyes.

The excitation and emission maxima for the dsDNA/dye complex are for EvaGreen 503/525 nm, for SYBR Green 497/520 nm, for PicoGreen 500/521 nm, for AccuClear 470/498 nm, for AccuBlue NextGen 484/507 nm and for YOYO-1 490/508 nm (Fig. 2.9–2.15 DiB) [22]. All excitation and emission maxima are given in Table 3 and are in accordance with values reported in literature (Table 1.1 DiB) [10,12,19,20,22,25,26]. However, the emission maximum of AccuClear is lower and the excitation maximum of AccuBlue Next Gen is higher than values given by the manufacturer (486/507 nm for both dyes) [19,20]. For PicoGreen the maxima are in agreement with literature, although the manufacturer recommends to use 480/520 nm when this dye is used for (quantitative) fluorescence intensity measurements [2,9].

For the dsDNA/dye complex the fluorescence intensities increase significantly (in the order of 100 times, except for AccuBlue NextGen and YOYO-1) compared to the free state of the dye (Fig. 2.10–2.15 DiB) [22]. This might be due to H-dimer (side-to-side/sandwich aggregate) formation of the free dye, which reduces the background fluorescence resulting in a substantial increase in fluorescence intensity upon binding to dsDNA, possibly forming J-dimers (head-to-tail aggregate) resulting in high quantum yields characteristic for cyanine dyes [27,28]. H-dimer formation, which happens in aqueous solutions, is characterized by an absorption maximum at low wavelengths (hypsochromic shift) and a broader absorption band in combination with a large Stokes shift, compared to the monomer of the dye. Note that AccuClear and AccuBlue NextGen have significantly lower absorption and excitation maxima than the other investigated dyes. Another important mechanism of fluorescence intensity increase is the fixation of the dye conformation upon binding to DNA. This lowers the rate of non-radiative decay paths of the dye, and therewith enhances the quantum yield. Additionally, H-dimer formation lowers the toxicity of the dye, due to a high molecular weight, which reduces the permeation of the dye through the cell membrane [6,28,29].

Fluorescence intensity. In Figs 2 and 3 the fluorescence intensity is shown for dsDNA amounts in the pg-range and the ng-range, respectively. For the range of 0–100 pg only AccuBlue NextGen exhibits a linear response, as can be seen in Fig. 2. Fig. 3 shows that EvaGreen, PicoGreen and AccuClear exhibit linearity for the range of 0–100 ng.

EvaGreen does not show linear behavior when dsDNA is added in the pg-range (Fig. 2.16 DiB) [22]. In the presence of 0.2–100 ng dsDNA EvaGreen is found to be linear (Fig. 2.17 DiB) [22]. For SYBR Green no linear range is found in the pg- or ng-range of added dsDNA (Fig. 2.18 and 2.19 DiB) [22]. PicoGreen is highly linear ($R^2 \geq 0.95$) in the range of 10 pg till 100 ng for the salmon dsDNA as well as for the control dsDNA from the kit (λ dsDNA standard) (Fig. 2.20 and 2.21 DiB) [22]. When 5–500 pg salmon dsDNA or 3–1000 pg control dsDNA (calf thymus dsDNA standard) is added to AccuClear a linear response is observed (Fig. 2.22 DiB) [22].

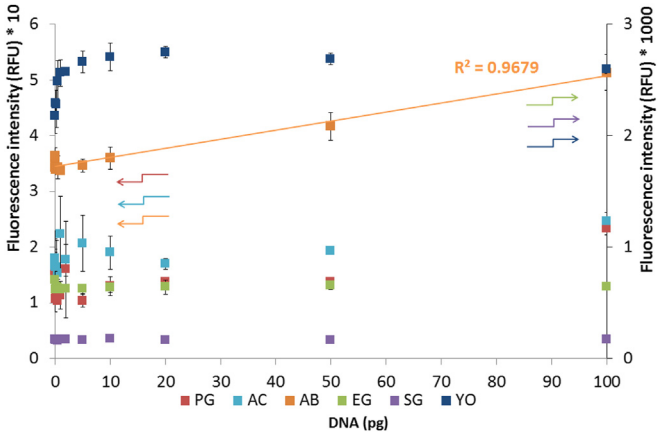

Fig. 2. Fluorescence intensities of the dyes in the presence of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 & 100 pg salmon dsDNA and 1.0X dye (100 nM for YOYO-1) in a volume of 200 μL (except for AccuClear and AccuBlue NextGen where 210 μL is used). Spectra were recorded with a M200 PRO multimode reader (Tecan). The error bars are ±1 standard deviation. PicoGreen, AccuClear and AccuBlue NextGen are depicted on the left y-axis, whereas EvaGreen, SYBR Green and YOYO-1 are depicted on the right x-axis, as is indicated by the coloured arrows in the graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

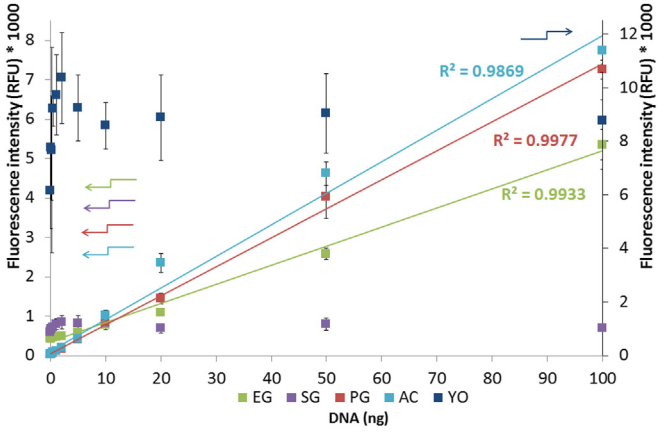

Fig. 3. Fluorescence intensities of the dyes in the presence of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 & 100 ng salmon dsDNA and 1.0X dye (100 nM for YOYO-1) 200 μL (except for AccuClear where 210 μL is used. Spectra were recorded with a M200 PRO multimode reader (Tecan). The error bars are ± 1 standard deviation. EvaGreen, SYBR Green, PicoGreen and AccuClear are depicted on the left y-axis, whereas YOYO-1 is depicted on the right x-axis, as is indicated by the coloured arrows in the graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AccuClear is also highly linear in the range of 0.01–200 ng salmon dsDNA and 0.003–100 ng control dsDNA, resulting in a linear range

of 3 pg up to 200 ng dsDNA (Fig. 2.23 DiB) [22]. The complete linear range of AccuBlue NextGen is 5–5000 pg (Fig. 2.24 DiB) [22]. For YOYO-1 no linear range was found in the pg- or ng-range of added dsDNA (Fig. 2.25 and 2.26 DiB) [22].

The limit of detection for the dyes can be found in Table 3. The linear range of the dyes is determined by an R^2 value of $\geq$0.95, whereas the limit of detection is based on the standard deviation of the blank (fluorescence intensity of free dye). The values obtained for the limit of detection can be higher than the lower limit of the linear range. Therefore, it would be more appropriate to take the limit of detection as lower value of the linear range.

4. Final remarks

When the Stokes shift (i.e. the difference in wavelength of the excitation and emission spectra) is small, as is the case for PicoGreen with a difference of about 20 nm, a lower excitation wavelength must be used for fluorescence intensity measurements. The sensitivity of an assay is limited by the Rayleigh-Tyndall scattering when the excitation and emission wavelengths are close to each other, whereby total separation is impossible. Since the emission spectrum is independent of the excitation wavelength a lower wavelength can be used for the excitation with only a lower fluorescence intensity as result [4].

The cyanine dyes are weakly fluorescent as free dye in solution (Fig. 2.10–2.15 DiB) [22]. However, this background fluorescence can still be significant relative to the fluorescence intensity when only a small amount of dsDNA is added, as is the case for YOYO-1 (Fig. 2.15 DiB) [22]. Therefore, the detection sensitivity is limited by the intrinsic fluorescence of the dye itself. The intrinsic background can be suppressed by the use of an anionic dye modifier. By adding such a dye modifier, for instance Coomassie Blue R-250 or Guinea Green B, the linear range of EvaGreen dye can be extended from 1 to 50 ng (5–250 ng/mL) to 0.2–100 ng (1–500 ng/mL). Increasing the dye concentration from 0.63 μM up to 1.25 μM results in a sigmoidal signal when no modifier is added. By adding the modifier the response becomes linear from 0.5 ng to 250 ng [5]. Most dyes become non-linear when only a small amount of dsDNA (pg-range) is added to the dye. Possibly the dye aggregates, which is initiated by the small amount of dsDNA. The dye molecules are that close to each other, that the emitted fluorescence is absorbed by a neighboring molecule via fluorescence resonance energy transfer. Therefore the complex formed might be even weaker fluorescent than the free dye, which can be observed in Fig. 2.16 and 2.18 (DiB). This effect can also be avoided by adding a dye modifier [22].

Another suggestion for the low fluorescence intensity at a low DNA base pair:dye ratio are the charges in the complex. Cyanine dyes, such as EvaGreen and YOYO-1, are positively charged, whereas DNA has a negative charge. Therefore, the complex might become neutral and even hydrophobic when the dye molecules are densely lined next to each other. This interaction results in a strong Van der Waals force, which results in further dye aggregation. Next to that, the complex can act as an inner filter, whereby the highly aggregated DNA-dye complex behaves as a super quencher, which captures the intrinsic fluorescence of the dye molecules in solution. By aggregation less dye is available in solution for dsDNA/dye complex formation, which reduces the overall fluorescence even further.

5. Conclusions and outlook

A total of six cyanine dsDNA are investigated: EvaGreen, SYBR Green, PicoGreen, AccuClear, AccuBlue NextGen and YOYO-1. The absorption, emission and excitation wavelengths are determined and compared with literature and information from the manufacturers. The obtained optimal wavelengths are used to analyse the linear range and limit of detection of these dyes. Whereas for SYBR Green and YOYO-1 no linear range nor limit of detection could be determined for low amounts of dsDNA, this is possible for the other dyes. EvaGreen has a linear range of 0.2–100 ng with a limit of detection of 10 ng. PicoGreen and AccuClear both have a detection limit of 100 pg with a linear range of 0.01–100 ng and 0.003–200 ng, respectively. AccuBlue NextGen has the lowest limit of detection of the investigated dyes with a value of 50 pg and a linear range of 0.005–5 ng. Therefore, AccuBlue NextGen exhibits the highest sensitivity of the tested dyes, whereas AccuClear has the widest linear range.

In this research all six dyes were studied under exactly the same conditions. Nevertheless, the linearity and the limit of detection might be influenced by changing other parameters, such as the type of DNA (e.g. different GC-content), the type and pH of the buffer solution, dye concentration or incubation time.

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References

[1] S. Lee, B. McCord, E. Buel, Advances in forensic DNA quantification: a review, Electrophoresis 35 (21–22) (2014) 3044–3052, http://dx.doi.org/10.1002/elps.201400187.
[2] V. Singer, L. Jones, S. Yue, R. Haugland, Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation, Anal. Biochem. 249 (2) (1997) 228–238, http://dx.doi.org/10.1006/abio.1997.2177.
[3] L. Kricka, Stains, labels and detection strategies for nucleic acids assays, Ann. Clin. Biochem. 39 (2) (2002) 114–129, http://dx.doi.org/10.1258/0004563021901865.
[4] Thermo Fisher Scientific, The Molecular Probes Handbook; a Guide to Fluorescent Probes and Labeling Technologies, Life Technologies Corporation, 2010.
[5] F. Mao, W.-Y. Leung, H. E. Hoover, Detection using a dye and a dye modifier, uS Patent 8,148,515 (Apr. 3 2012).
[6] F. Mao, W.-Y. Leung, T. Van, Methods of using dyes in association with nucleic acid staining or detection, patent US8877437 B1 (Nov. 4 2014).
[7] Biotium, EvaGreen Dye, 20X in Water, 2016. https://biotium.com/product/evagreen-dye-20x-in-water/.
[8] Thermo Fisher Scientific, SYBR Green I Nucleic Acid Gel Stain - 10,000X Concentrate in DMSO, 2015. https://www.thermofisher.com/order/catalog/product/S7563.
[9] Thermo Fisher Scientific, Quant-iT PicoGreen DsDNA Assay Kit, 2015. https://www.lifetechnologies.com/order/catalog/product/P7589.
[10] Thermo Fisher Scientific, YOYO-1 Iodide (491/509) - 1 MM Solution in DMSO, 2015. https://www.lifetechnologies.com/order/catalog/product/Y3601.
[11] J. Ihrig, R. Lill, U. Mühlenhoff, Application of the DNA-specific dye EvaGreen for the routine quantification of DNA in microplates, Anal. Biochem. 359 (2) (2006) 265–267, http://dx.doi.org/10.1016/j.ab.2006.07.043.
[12] F. Mao, W. Leung, X. Xin, Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications, BMC Biotechnol. 7 (76) (2007) 1–16, http://dx.doi.org/10.1186/1472-6750-7-76.
[13] F. Sang, J. Ren, Capillary electrophoresis of double-stranded DNA fragments using a new fluorescence intercalating dye EvaGreen, J. Sep. Sci. 29 (9) (2006) 1275–1280, http://dx.doi.org/10.1002/jssc.200600029.
[14] W. Wang, K. Chen, C. Xu, DNA quantification using EvaGreen and a real-time PCR instrument, Anal. Biochem. 356 (2) (2006) 303–305, http://dx.doi.org/10.1016/j.ab.2006.05.027.
[15] K. Rengarajan, S. Cristol, M. Mehta, J. Nickerson, Quantifying DNA concentrations using fluorometry: a comparison of fluorophores, Mol. Vis. 8 (2002) 416–421.
[16] A. Haines, S. Tobe, H. Kobus, A. Linacre, Properties of nucleic acid staining dyes used in gel electrophoresis, Electrophoresis 36 (6) (2015) 941–944, http://dx.doi.org/10.1002/elps.201400496.

[17] G. Schofield, PicoGmeter, a custom-made fluorometer for the quantification of dsDNA by PicoGreen fluorescence, BioTechniques 37 (5) (2004) 778–782.
[18] S. Ahn, J. Costa, J. Emanuel, PicoGreen quantitation of DNA: effective evaluation of samples pre-or post-PCR, Nucleic Acids Res. 24 (13) (1996) 2623–2625.
[19] Biotium, dsDNA quantitation kits, AccuBlue and AccuClear DsDNA Quantitation Kits for Fluorescence-based DsDNA Quantitation in Solution, 2015. https://biotium.com/product-category/applications/genomics/nucleic-acid-quantitation-in-solution/.
[20] Biotium, AccuBlue NextGen DsDNA Quantitation Kit, 2015. https://biotium.com/product/accublue-nextgen-dsdna-quantitation-kit/.
[21] H. Rye, J. Dabora, M. Quesada, R. Mathies, A. Glazer, Fluorometric assay using dimeric dyes for double-and single-stranded DNA and RNA with picogram sensitivity, Anal. Biochem. 208 (1) (1993) 144–150, http://dx.doi.org/10.1006/abio.1993.1020.
[22] B. Bruijns, R. Tiggelaar, H. Gardeniers, Absorption, emission and excitation spectra and fluorescence intensity graphs of fluorescent cyanine dyes for the quantification of low amounts of dsDNA, Anal. Biochem. 350 (2016) submitted, DIB-D-16-00621.
[23] V. Thomsen, D. Schatzlein, D. Mercuro, Limits of detection in spectroscopy, Spectroscopy 18 (12) (2003) 112–114.
[24] A. Fürstenberg, M. Julliard, T. Deligeorgiev, N. Gadjev, A. Vasilev, E. Vauthey, Ultrafast excited-state dynamics of DNA fluorescent intercalators: new insight into the fluorescence enhancement mechanism, J. Am. Chem. Soc. 128 (23) (2006) 7661–7669, http://dx.doi.org/10.1021/ja0609001.
[25] G. Cosa, K. Focsaneanu, J. McLean, J. McNamee, J. Scaiano, Photophysical properties of fluorescent DNA-dyes bound to single-and double-stranded DNA in aqueous buffered solution, Photochem. Photobiol. 73 (6) (2001) 585–599, http://dx.doi.org/10.1562/0031-8655(2001)0730585PPOFDD2.0.CO2.
[26] A. Dragan, J. Casas-Finet, E. Bishop, R. Strouse, M. Schenerman, C. Geddes, Characterization of PicoGreen interaction with dsDNA and the origin of its fluorescence enhancement upon binding, Biophysical J. 99 (9) (2010) 3010–3019, http://dx.doi.org/10.1016/j.bpj.2010.09.012.
[27] M. Sauer, J. Hofkens, J. Enderlein, Handbook of Fluorescence Spectroscopy and Imaging, Wiley, 2011.
[28] B. Armitage, DNA Binders and Related Subjects – Chapter: Cyanine Dye–DNA Interactions: Intercalation, Groove Binding, and Aggregation, Springer, 2005.
[29] J. Lakowicz, Principles of Fluorescence Spectroscopy, Springer Science & Business Media, 2013.