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O.S. Kazakov-Kravchenko[1], S.A. Kravchenko[1, 2], S.M. Yarmoluk[1]
[1] Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143
[2] Kyiv Institute of the National Guard of Ukraine, MIA of Ukraine
7, Oborony Kyieva Str., Kyiv, Ukraine, 03179
kazakkrav44@gmail.com

EVALUATION OF THE MONOMETHINE CYANINE DYE FB128 IN REAL-TIME PCR APPLICATIONS

Aim. *To evaluate the suitability of the novel DNA-binding dye fb128 for real-time PCR applications, including amplification efficiency, fluorescence performance, PCR inhibition threshold, and melting curve analysis.* ***Methods.*** *UV-vis absorption and fluorescence spectroscopy, agarose gel electrophoresis, real-time PCR, melt curve analysis.* ***Results.*** *fb128 exhibited low intrinsic fluorescence and strong DNA-specific signal (ΔQ = 160.7). Optimal concentrations (0.2–1.6 μM) provided early Ct values without PCR inhibition, whereas the concentrations of ≥3 μM caused a delay in amplification or complete suppression. PCR efficiency with fb128 was 102.6%, within the optimal range for quantitative applications. Melting peaks with fb128 were stronger and appeared at 74.5 °C, 2.5 °C lower than with SYBR Green I, suggesting weaker dsDNA binding.* ***Conclusions.*** *fb128 demonstrates high amplification efficiency, strong fluorescent signal, broad concentration tolerance, and robust melting analysis performance. These properties establish fb128 as a competitive alternative to SYBR Green I for real-time PCR applications.*

Keywords: *monomethine cyanine dye, fluorescence intensity, real-time PCR, DNA binding, PCR efficiency, melt curve analysis.*

Introduction

Real-time polymerase chain reaction (PCR) is a comprehensive and powerful technique for detecting and quantifying nucleic acids, and it has become one of the leading technologies in molecular diagnostics, the biological sciences, agriculture and medicine [1—4]. The key principle of real-time PCR relies on the presence of a fluorescent molecule that enables real-time monitoring of target DNA amplification. This fluorescent reporter can be a fluorescently labelled oligonucleotide probe or a simple DNA-binding dye [5].

Probe-based methods such as TaqMan and Light-up have become widely used in clinical and biological research due to their high sensitivity

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and specificity, which is required for demanding applications such as medical diagnostics [6]. However, these methods have several limitations: they are less suitable for analysing highly polymorphic genomic regions (e.g. rapidly mutating SNPs or hypervariable loci), and probe synthesis is often expensive and time-consuming [7]. As an alternative, simple fluorescent DNA-binding dyes have been widely adopted for real-time PCR to monitor the accumulation of the target DNA fragment during amplification [8]. Currently, numerous fluorescent dyes with high DNA-binding affinity have been developed, including intercalating and groove-binding molecules such as BEBO, SYBR Green I, SYTO9 and EvaGreen [8—12]. Nevertheless, SYBR Green I remains the most widely used cyanine dye in PCR applications. However, its limitations, including limited dye stability, PCR inhibition at higher concentrations, preferential binding to specific DNA sequences and reduced accuracy during melting curve analysis, have motivated the search for improved intercalating dyes for real-time PCR.

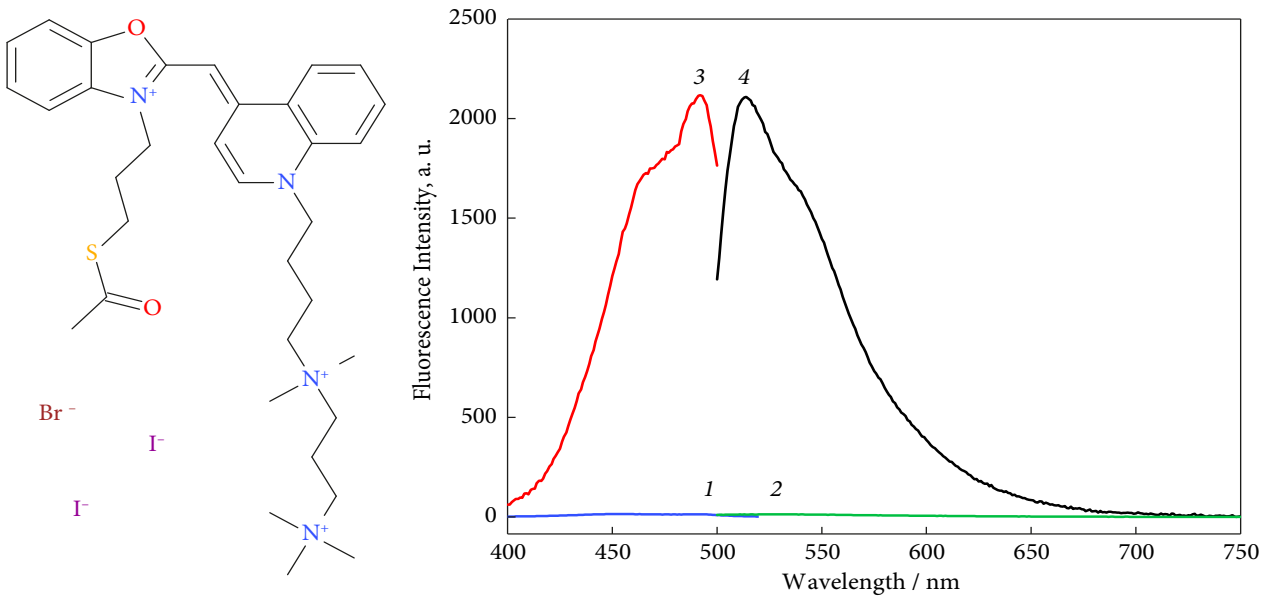
In a recent study, we investigated the impact of DNA, RNA and HSA on the spectral and luminescent properties of eight monomethine cyanine dyes with different structural features [14]. Of these, the fb128 dye, which contains an oxazole yellow chromophore, exhibited the highest fluorescence intensity in the presence of nucleic acids. Furthermore, this dye exhibited the largest increase in the fluorescence intensity ratio upon the addition of DNA/RNA to the dye solution in TRIS-HCl buffer. It is well established that binding of dye molecules to DNA is critical for monitoring the amplification process. However, dyes with excessively high affinity for DNA can inhibit the polymerisation reaction by stabilising the DNA duplex and increasing its melting temperature by up to 10 °C [10]. In addition to inhibiting polymerisation, strong DNA binding can distort melting curves and affect PCR efficiency and specificity. The aim of this study was therefore to evaluate the FB128 dye in terms of fluorescence signal intensity, potential reaction inhibition, amplification efficiency and amplicon melting behaviour, in order to assess its suitability for real-time PCR applications.

Materials and Methods

Spectral analysis of the dye. The monomethine cyanine dye FB128 (see Fig. 1*a*) was kindly provided by Todor Deligeorgiev of Sofia University "St. Kliment Ohridski". The structure of FB128 was confirmed by ^{1}H NMR and elemental analysis. Dimethyl sulfoxide (DMSO) and a 0.05 M Tris-HCl buffer solution at pH 8.0 were used as solvents. A stock solution was prepared in DMSO at a concentration of 2 mM. Stock solutions of DNA were prepared in a 50 mM Tris-HCl buffer solution (pH 8.0). Working solutions of the free dye were prepared by diluting the dye stock solution in 50 mM Tris-HCl buffer solution (pH 8.0). Working solutions of the dye in the presence of nucleic acids were prepared by adding an aliquot of the stock solutions of the dye and nucleic acid to 50 mM Tris-HCl buffer solution (pH 8.0).

All spectral measurements were performed immediately after preparing the corresponding working solutions. Absorption spectra were recorded using a Genesys 20 Visible Spectrophotometer (Thermo Fisher Scientific, USA). Fluorescence spectra were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). All measurements were performed in quartz cuvettes (10 × 10 mm) at room temperature. Wavelength accuracy of the Cary Eclipse fluorescence spectrophotometer is 1.5 nm. Fluorescence spectra were measured with excitation and emission slit widths equal to 5 nm.

PCR and gel electrophores conditions. A 210 bp DNA-fragment of human *SMN1* gene exon 7 was used as the target PCR template. The sequence of the PCR primers was as follows: SMN1_f: 5'-CCTTTTATTTTCCTTACAGGGTTTC-3' and SMN1_r: 5'-GATTGTTTTACATTAACCTT TCAACTTTT-3' [15]. The PCR was carried out in a final volume of 15 μl containing 1 x PCR (67 мМ TRIS-HCl (pH 8), 16 mM NH_4SO_4, 0.1% w/v Tween-20, 170 mcg/ml BSA), 2.5 mM $MgCl_2$,



a *b*

Fig. 1. Monomethine cyanine dyes fb128. *a* — Structures of dyes. *b* — Normalized excitation and emission spectra of fb128: *1* — excitation spectrum in buffer; *2* — emission spectrum in buffer; *3* — excitation spectrum in buffer in the presence of DNA; *4* — emission spectrum in buffer in the presence of DNA

0.2 mM each of dNTP, 500 nM each of forward and reverse primers, a human genomic DNA (15ng — 50 ng), 0.4 units of PhyScript DNA polymerase and fb128 dye at the indicated concentration. A sample of human genomic DNA obtained from the DNA collection of the Laboratory of Genetics of Hereditary Diseases at the Institute of Molecular Biology and Genetics of NASU, was used for the PCR analysis. The quality and quantity of the DNA sample were checked using an ND-1000 spectrophotometer (NanoDrop, USA). For further studies, the stock DNA sample was diluted in TE buffer to working concentrations. The fb128 stock solution was diluted immediately before use with the PCR buffer to the concentrations tested. The fb128 stock solution showed no signs of degradation after >9 months of storage in a refrigerator. The PCR temperature conditions were as follows: an initial denaturation step at 95 °C for 2 minutes, followed by 30 two-step cycles of denaturation at 95 °C for 15 seconds and annealing/elongation at 60 °C for 40 seconds. The PCR products were fractionated in a 1.6% agarose gel stained with ethidium bromide (0.5 μg/ml), and visualised by transillumination in ultraviolet light.

Real-time PCR and melting curve analysis. The qPCR reaction was carried out using the CFX96 real-time PCR system (Bio-Rad, USA). The fluorescent signal was measured at channel 1, corresponding to the FAM and SybrGreen I dyes, with the following parameters: excitation at 450—490 nm and emission at 510—530 nm. Fluorescent detection was performed at the end of each annealing/extension step, and qPCR values were evaluated using CFX Manager™ Software (Bio-Rad). The cycle threshold (Ct) value was defined as the minimum value in the exponential growth phase of fluorescence at which the PCR efficiency was closest to 100%. Melting curve analysis was performed with 0.5 °C increments and a 10-second hold at each step from 55 °C to 90 °C. PCR efficiencies (E) were determined from a DNA template dilution series and calculated according to the equation $E = (10^{-1}/m - 1) \times 100\%$, where m is the slope of the linear standard curve.

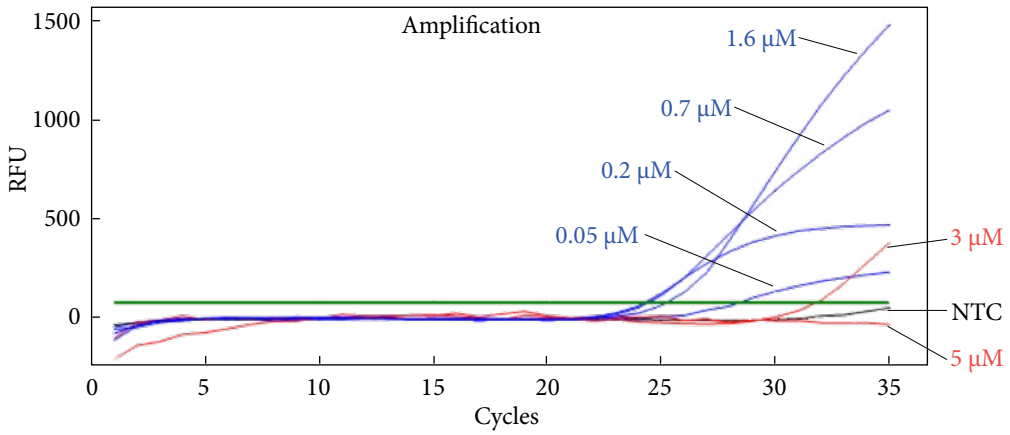


Fig. 2. Amplification plots of the 201 bp SMN1 fragment using fb128 as the DNA-binding dye for real-time fluorescence monitoring. The dye concentrations (μM) are indicated in the figure. NTC — no template control

Results and Discussion

Spectral-luminescent properties of dye fb128

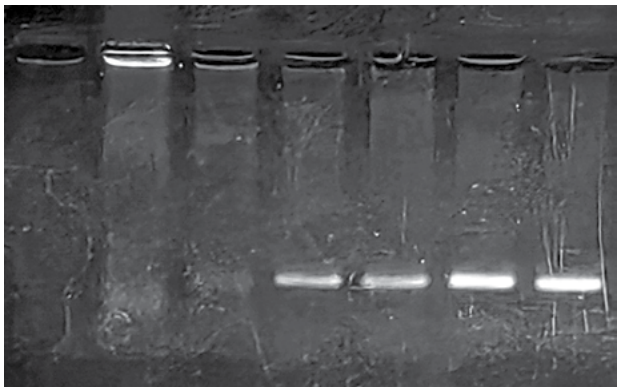
Our recent research has demonstrated that fb128 exhibits promising spectral-luminescent properties for potential application in real-time PCR [14]. Fig. 1*b* shows the fluorescence spectra of fb128 in aqueous medium (50 mM TRIS-HCl buffer, pH 8.0) in the absence (*1*, *2*) and in the presence (*3*, *4*) of dsDNA. The excitation (λ_{ex}) and emission (λ_{em}) maxima for fb128 bound to DNA were 491 nm and 513 nm, respectively, with the fluorescence intensity ($I_{DNA+buf}$) reaching 2090 arbitrary units (a.u.). By contrast, the fluorescence intensity of the free dye in the buffer (I_{buf}) was only 13 a.u. Thus the addition of DNA resulted in a 160.7-fold increase in fluorescence intensity ($\Delta Q = I_{DNA+buf}/I_{buf}$) of the dye in buffer.

Effect of the dye concentration on real-time PCR performance

It is well established that intercalating dyes can inhibit PCR if their concentration exceeds a critical threshold due to their strong affinity for double-stranded DNA (dsDNA). To determine whether the fb128 dye exhibits an inhibitory effect, the real-time PCR assays were conducted in which the amount of template DNA and the composition of the reaction mixture were held constant while the concentration of fb128 varied from 0.05 μM to 6 μM. Fig. 2 shows the amplification curves for the 201 bp SMN1 fragment at six different concentrations of fb128: 0.05, 0.2, 0.7, 1.6, 3 and 6 μM. The results revealed that fb128 at 0.7 μM and 0.2 μM produced comparable and relatively early Ct values. At 1.6 μM, a slight Ct delay was observed; increasing the dye concentration to 3 μM, however, caused substantial Ct delays. At 6 μM, the reaction failed to produce a detectable fluorescence signal (no Ct), indicating complete inhibition of amplification. The 0.05 μM concentration yielded a detectable product, but with delayed Ct values and low endpoint fluorescence.

To validate these observations, the PCR products from the reactions, shown in Fig. 2, were analysed using agarose gel electrophoresis (see Fig. 3). Specific products were detected at concentrations of fb128 between 0.05 and 1.6 μM, whereas no product was observed at 6 μM and only a trace of the PCR product was visible at 3 μM. These data suggest that concentrations of fb128 above 1.6 μM inhibit PCR under the conditions used here.

Interestingly, despite the Ct delay during real-time monitoring (Fig. 2 and Fig. 3, track 7), the



1 2 3 4 5 6 7

Fig. 3. 1.6% agarose gel electrophoresis of the PCR products: 1 — NTC; 2—7 — fb128 concentrations: 6 μM, 3 μM, 1.6 μM, 0.7 μM, 0.2 μM, and 0.05 μM respectively

reaction with a concentration of 0.05 μM produced a clear amplicon on the gel. This discrepancy suggests that, while low dye concentrations enable successful amplification, they can result in delayed signal accumulation, as indicated by the Ct value of 29 cycles compared to 24 cycles for concentrations of 0.2 μM and 0.7 μM, and 25 cycles for 1.6 μM. This is also evident from the lowest RFU values for the 0.05 μM concentration (see Fig. 2). It is important to note that the strength of the fluorescent signal in qPCR depends on the amount of amplified DNA and the dye concentration. Therefore, identification of the optimal dye concentration that provides the strongest fluorescent signal without inhibiting the reaction is a critical step in testing new fluorescent dyes for PCR applications. For fb128, the optimal working range under our experimental parameters was determined to be between 0.2 μM and 1.6 μM: a range that maximises the fluorescent signal while avoiding significant PCR inhibition.

PCR efficiency

The standard titration curve, which defines PCR efficiency, is affected by numerous factors, one of which is an intercalating dye, as it can alter polymerase activity and fluorescence output. To evaluate the impact of fb128 on amplification efficiency, a set of 1.5-fold serial dilutions of human genomic DNA (15—50 ng) was analysed by qPCR using 0.5 μM fb128. Fig. 4 shows the amplification curves for the SMN1 fragment across the dilution series. All reactions were performed in triplicate to ensure reproducibility. The reactions exhibited consistent efficiency over the studied concentration range, with the standard curve (shown as an inset in Fig. 4) indicating an amplification efficiency of 102.6%.

These results suggest that fb128 does not negatively impact amplification efficiency at the optimal concentration of 0.5 μM, supporting its suitability for quantitative applications in real-time PCR.

Melting curve analysis

Post-amplification melting curve analysis was performed to verify the specificity of the amplification and compare the melting profiles of the amplicons when intercalated with either fB128 or SYBR Green I. The derivative melting curves for both dyes are shown in Fig. 5.

At a concentration of 0.5 μM, fb128 produced a single, sharp melting peak at 74.5 °C, whereas SYBR Green I (1×) generated a peak at 77.0 °C. Therefore, the melting temperature of amplicons stained with fb128 was approximately 2.5 °C lower than that obtained with SYBR Green I, and no additional peaks were detected for either dye. This indicates the absence of non-specific amplification products or primer dimers. The melting peak for fb128 was significantly higher in amplitude than that of SYBR Green I, which is consistent with the higher final fluorescence intensity observed in qPCR amplification curves for fb128. This suggests that fb128 may be preferable for melting curve analysis due to its increased sensitivity.

Taken together, these results demonstrate that fb128 ensures high amplification specificity while producing a robust melting profile, making it a promising dye for both qPCR detection and post-PCR analysis.

Influence of reaction mixture on PCR performance

The composition of the reaction mixture (i.e. the PCR buffer) is also very important when testing new dyes, as it can significantly impact the efficiency and specificity of amplification [16]. We investigated the effect of different concentrations of Tris-HCl, $MgCl_2$, $(NH_4)_2SO_4$, KCl, Tween-20, DMSO, betaine and various DNA polymerases on the composition of the PCR reaction mixture, while keeping the primers, DNA template, dye and dNTPs constant (data not shown).

As shown in Fig. 6, both the amplification efficiency and the fluorescence signal were strongly

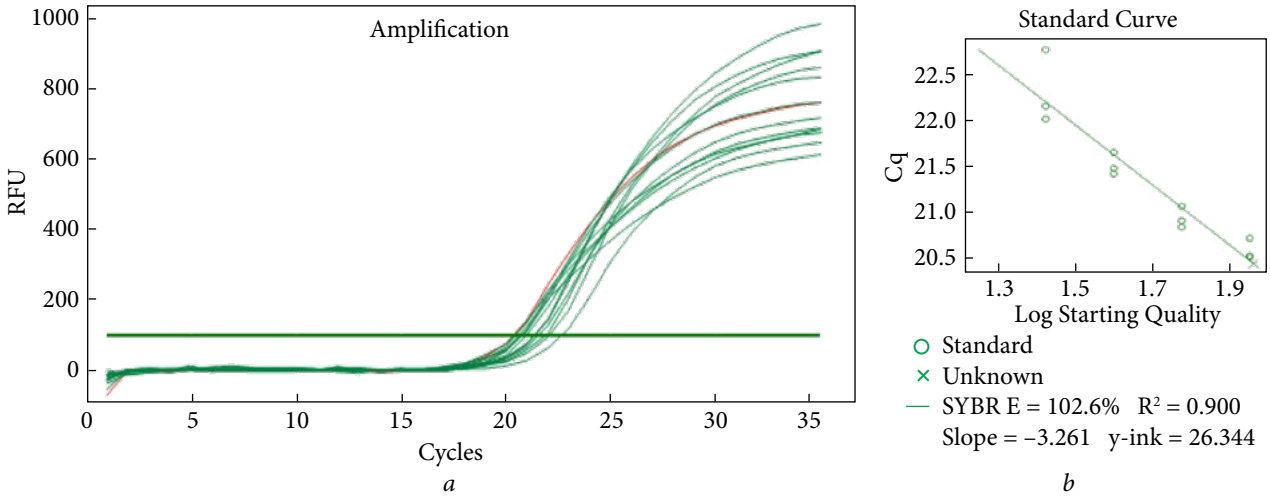


Fig. 4. *a* — Amplification plots of the SMN1 fragment from various DNA inputs within the range of 15—50 ng. All reactions were run in triplicates, RFU — Relative Fluorescence Units, *b* — Standard curve data

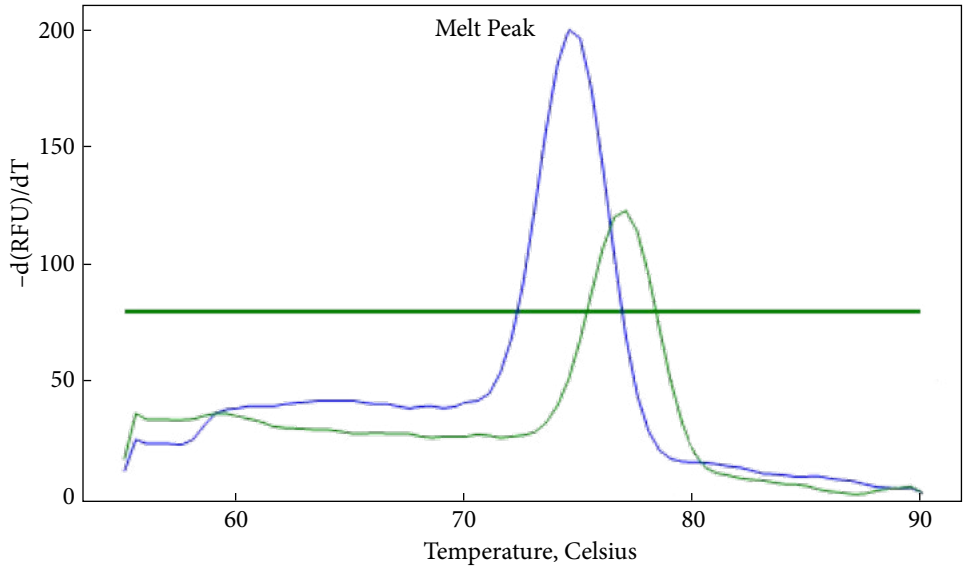


Fig. 5. Melt curve analysis of the SMN1 fragment with fb128 and SYBR Green I (SG) showing the first derivative of the fluorescence with respect to temperature (–dRFU/dT)

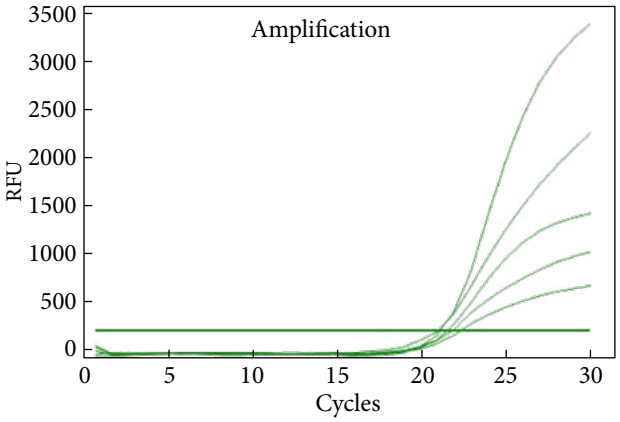


Fig. 6. Effect of reaction mixture composition (PCR buffer) on PCR performance at a 0.5 μM concentration of fb128

influenced by the composition of the buffer, while fb128 demonstrated high compatibility with multiple reaction conditions.

Discussion

In this study, we examined several important criteria that determine the suitability of the DNA-binding dye fb128 for qPCR applications. These criteria included fluorescent signal intensity, the effect of dye concentration on PCR performance, amplification efficiency and melt curve data. Fb128 has low intrinsic emission, which reduces its impact on amplification and makes it well suited to use in melt curve analysis. We found that fb128 has a low intrinsic emission level, which reduces its impact on amplification detection and melting curve analysis. At the same time, fb128 exhibits high fluorescence intensity (IDNA = 2090 a.u.) and high DNA-to-buffer intensity ratio (ΔQ = 160.7). The optimal concentration of fb128 was determined to be the concentration that provided the best combination of low PCR inhibition, high fluorescence and a low Ct. Under the conditions used in this study, this concentration ranged widely from 0.2 μM to 1.6 μM. For comparison, the optimal concentrations of other fluorescent dyes commonly used in PCR analysis are within the following ranges: 0.1—0.5 μM for BEBO dye [9], 0.34—0.64 μM for SYBR Green I and 0.67—1.33 μM for EvaGreen [11, 13]. It has been shown that SYBR Green I exhibits high affinity for single-stranded DNA (ssDNA) at high concentrations, and can bind to a single-stranded template at the stage of chain extension, thereby inhibiting PCR. We assume that fb128 predominantly binds to dsDNA and interacts weakly with ssDNA; therefore, it does not show an inhibitory effect. The ability to use the dye at high concentrations without PCR inhibition, as demonstrated with fb128, offers significant advantages for high-resolution melting analysis (HRM), particularly when the amplification curve is of poor quality and shows Ct > 30 [17].

Quantitative real-time PCR is widely used to determine gene copy number. The accuracy of the results critically depends on consistently high amplification efficiency and the use of a suitable reference gene with comparable amplification characteristics. Some quantitative analysis models assume that genes are optimally amplified with an efficiency close to 100% [18]. Therefore, amplification efficiency between 93% and 105% is considered high and sufficient [19]. Ensuring these two parameters allows for reliable normalisation and accurate calculation of relative gene copy numbers [20]. Given the promising performance of fb128 demonstrated in our study (PCR efficiency of 102.6%), further evaluation of its applicability in copy number quantification assays could provide additional insights. Such experiments require both the studied and referent genes to be amplified efficiently.

Melting temperature (T_m) analysis is implemented in real-time PCR to confirm amplification specificity and for high-resolution melting (HRM) analysis, single nucleotide polymorphism (SNP) genotyping, methylation analysis, and pathogen identification [21, 22]. In our study, fb128 produced stronger melting peaks than SYBR Green I, despite displaying T_m values that were 2.5 °C lower. This suggests weaker dsDNA binding. We hypothesise that this moderate binding strength enables fb128 to produce stable, high-intensity melting signals without stabilising the DNA duplex

excessively. It should be noted that, to estimate the applicability of fb128 for amplicon differentiation by HMR analysis, several requirements must be met, primarily relating to the length and nucleotide sequence of the amplicons, as well as the composition of the other components. Thus, the melting analysis was evaluative and comparative in nature; however, its results suggest that research into the use of fb128 for HMR applications is promising.

Conclusions

Our study demonstrates that fb128 offers several advantages for real-time PCR applications, including low intrinsic fluorescence, a wide optimal concentration range, high amplification efficiency, reproducible quantification across DNA dilutions and strong, specific melting peaks. The moderate DNA-binding affinity of fb128 appears to be optimal for sensitive detection, avoiding both polymerase inhibition and excessive duplex stabilisation. The fluorescence properties and concentration flexibility of fb128 make it a competitive alternative to SYBR Green I, suggesting that further research into its use in HMR and qPCR is promising.

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Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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О.С. Казаков-Кравченко[1], С.А. Кравченко[1,2], С.М. Ярмолюк[1]
[1] Інститут молекулярної біології і генетики НАН України
вул. Академіка Заболотного, 150, Київ, Україна, 031432
[2] Київський інститут національної гвардії України, МВС України
вул. Оборони Києва, 7, Київ, Україна, 03179
kazakkrav44@gmail.com

ОЦІНЮВАННЯ МОНОМЕТИН-ЦІАНИНОВОГО БАРВНИКА FB128 ДЛЯ ЗАСТОСУВАННЯ В ПЛР В РЕАЛЬНОМУ ЧАСІ

Мета. Оцінити придатність нового барвника fb128, що проявляє афінні властивості до ДНК, для застосування в ПЛР в реальному часі, включаючи ефективність ампліфікації, флуоресцентні характеристики, поріг інгібування ПЛР та аналіз кривої плавлення. ***Методи.*** Спектроскопія поглинання в УФ-видимому діапазоні та флуоресцентна спектроскопія, електрофорез в агарозному гелі, ПЛР в реальному часі, аналіз кривої плавлення. ***Результати.*** fb128 продемонстрував низьку власну флуоресценцію та сильний ДНК-специфічний сигнал (ΔQ = 160,7). Оптимальні концентрації (0,2—1,6 мкМ) забезпечили ранні значення Ct без інгібування ПЛР, тоді як ≥ 3 мкМ спричиняли інгібування ампліфікації або повне пригнічення. Ефективність ПЛР з fb128 становила 102,6%, що знаходиться в оптимальному діапазоні для кількісних застосувань. Піки плавлення з fb128 були сильнішими і з'являлися при 74,5 °C, що на 2,5 °C нижче, ніж з SYBR Green I, що свідчить про слабше зв'язування з дволанцюговою ДНК. ***Висновки.*** fb128 демонструє високу ефективність ампліфікації, сильний флуоресцентний сигнал, широку толерантність до концентрації та надійну продуктивність аналізу плавлення. Ці властивості виділяють fb128 як конкурентоспроможну альтернативу SYBR Green I для застосувань ПЛР у реальному часі.

***Ключові слова*:** монометин-ціаніновий барвник, інтенсивність флуоресценції, ПЛР у реальному часі, ДНК-зв'язування, ефективність ПЛР, аналіз кривої плавлення**.**