STRUCTURAL-FUNCTIONAL ANALYSIS OF BIOPOLYMERS AND THEIR COMPLEXES

SUBSTRATE BEHAVIOR OF DIFFERENT Cy5-DEOXYPYRIMIDINE NUCLEOTIDES IN PCR WITH DNA MATRICES OF DIFFERENT GC-COMPOSITION

© 2025 P. M. Monakova*, V. E. Shershov, V. E. Kuznetsova, A. V. Chudinov, S. A. Lapa

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

**e-mail: polina.monakova02@gmail.com*

Received April 16, 2024

Revised June 21, 2024

Accepted June 25, 2024

Abstract. The substrate properties of six pairs of fluorescently labeled deoxyuridine and deoxycytidine triphosphates (Cy5-dUTPs and Cy5-dCTPs) in PCR with Taq polymerase were compared. In each pair, the modified dU and dC contained identical fluorescently labeled Cy5 substituents; for different pairs, the substituent structures differed in the length of the linker between the nitrogenous base and the fluorophore, the length of the linker between the quaternary ammonium group and the second heterocycle of the fluorophore, as well as the structure of the fluorophore itself. DNA fragments of *Staphylococcus aureus* (AT-rich template) and *Mycobacterium tuberculosis* (GC-rich template) were used as matrices. With both templates, deoxycytidine derivatives showed slightly higher amplification efficiency (*E*). The influence of the fluorophore structure and the GC-composition of the template on the kinetics of the reaction was insignificant. At the same time, a high incorporation efficiency was observed on the AT-rich matrix for uridine derivatives, and on the GC-rich matrix for cytidine derivatives (and in both cases - for substituents with a longer linker length). Nevertheless, the specific incorporation density, which takes into account the number of similar nucleotides in the DNA chain, was in all cases higher for dU derivatives. It was found that in pairs with similar fluorophore modifications, uridine derivatives, compared with cytidine, are characterized by a higher incorporation density, regardless of the composition of the template, but at the same time they have a greater inhibitory effect. The results obtained will increase the sensitivity of fluorescence analysis using the immobilized phase (microarray analysis).

Keywords: *indodicarbocyanine dyes, fluorescently labeled nucleoside triphosphates, substrate efficiency of modified dNTPs, GC composition, PCR kinetics*
DOI: 10.31857/S00268984250109e8

INTRODUCTION

Deoxyribonucleoside triphosphates (dNTPs) labeled with Cy5 cyanine fluorescent dyes are capable of being incorporated into the growing DNA chain during polymerase chain reaction (PCR) while preserving the properties of the fluorophore when using different temperature regimes (the signal decreases with increasing temperature, and when the temperature is lowered to the initial value, it increases again to the original value) [1-3]. Fluorescent labeling of DNA is currently widely used in medical diagnostics, biology, and biotechnology.

The effectiveness of incorporation of fluorescent labels depends on the used DNA polymerase, GC-content of the template, and the structure of the fluorophore [4]. The absence of 3′-5′-(proofreading) exonuclease activity in polymerases (e.g., Taq) provides an advantage for the incorporation of labeled dNTP analogs into the DNA chain [4]; the structure of the linker and label, and the charge of the fluorophore can significantly affect the incorporation of nucleotides into the growing DNA chain using Taq polymerase [5].

To date, the influence of the structure of incorporated fragments, template composition, and the ability of DNA polymerases to accept labeled triphosphates as substrates on the reaction efficiency has been studied [6-8]. The incorporation of nucleotides into the growing DNA chain was also analyzed using a library of different dU and dC pairs with similar substituents on templates with a pronounced predominance of AT and GC content.

In this work, we investigated the influence of the chemical nature of labeled pyrimidine triphosphates (deoxyuridine, deoxycytidine) on substrate compatibility with Taq polymerase on templates *Staphylococcus aureus (* 28.9% AT) and *Mycobacterium tuberculosis* (68% GC) of the same length (278 bp). For the convenience of studying the effect of linker length and lateral substituents of the fluorophore, we used a specially created library of dU and dC analogs (eight derivatives in total) with similar fluorophore structure. Two more pairs of dU and dC were used as reference standards.

EXPERIMENTAL PART

DNA templates. In this work, we used decontaminated genomic DNA of Staphylococcus aureus *S. aureus* and the causative agent of human tuberculosis *M. tuberculosis* .

Fluorescently labeled deoxynucleoside triphosphates. The structural formulas of the compounds used are shown in Fig. 1 (divided into three groups depending on the structure of fluorophores and linkers, Dye1-Dye3), chemical formulas of substituents are presented in Table 1.

Fig. 1. Structural formulas of fluorescent dyes Dye 1 (*a*), Dye 2 (*b*), Dye 3 (*c*), fluorescently labeled 5-allylamino-2′-deoxyuridine-5′-triphosphate (AAdUTP) (*d*) and 5-allylamino-2′-deoxycytidine-5′-triphosphate (AAdCTP) (*e*).

Table 1. Fluorescently labeled deoxyuridine and deoxycytidine triphosphates

Cy5-dNTP*	№ $_{lab}$ **	Dye 1	Dye 2	Dye 3
dU $_{c1}$	49	AAdUP	-	-
dC $_{c1}$	129	AAdCP	-	-
dU $_{c2}$	2	AAdUP	AAdUP	dC
dC $_{c2}$	128	AAdCP	AAdCP	dU
dU $_{ss}$	79	-	-	R = $(CH_2)_5$COAAdUTP *n* = 3
dC $_{ss}$	175	-	-	R = $(CH_2)_5$COAAdCTP *n* = 3
dU $_{sl}$	81	-	-	R = $(CH_2)_5$COAAdUTP *n* = 5
dC $_{sl}$	176	-	-	R = $(CH_2)_5$COAAdCTP *n* = 5
dU $_{ls}$	80	-	-	R = $(CH_2)_5$CONH$(CH_2)_5$COAAdUTP *n* = 3
dC $_{ls}$	177	-	-	R = $(CH_2)_5$CONH$(CH_2)_5$COAAdCTP *n* = 3
dU $_{ll}$	82	-	-	R = $(CH_2)_5$CONH$(CH_2)_5$COAAdUTP *n* = 5
dC $_{ll}$	178	-	-	R = $(CH_2)_5$CONH$(CH_2)_5$COAAdCTP *n* = 5

*"c1" – Control pair № 1; "c2" – control pair № 2; "s" or "l" in the first position of the subscript index – length of the linker between the nitrogenous base and the fluorophore, short or long respectively; "s" or "l" in the second position of the subscript index – length of the linker between the quaternary ammonium group and the second heterocycle.

**Internal laboratory number of the chemical compound (provided for the convenience of cross-references between publications in which these compounds appear).

Note. Synthesis schemes of dyes and triphosphates dU $_{c1}$, dU $_{ss}$, dU $_{sl}$, dU $_{ls}$, dU $_{ll}$ see in [9]; synthesis of dye and triphosphate dU $_{c2}$ – in [10]; synthesis of triphosphate dC $_{ss}$ – in [11]; triphosphates dC $_{c1}$, dC $_{c2}$, dC $_{sl}$, dC $_{ls}$ and dC $_{ll}$ were synthesized similarly to dC $_{ss}$.

Primers. DNA templates for amplification were obtained in two stages: a) from the initial full-genomic template, a product with the required sequence was generated using external primers, which, after purification from the components of the reaction mixture, was used as a template for studying substrate efficiency; b) substrate efficiency was analyzed with "nested" internal primers using the product obtained in the first stage as a template.

Primers were designed using the online resource www.idtdna.com (Integrated DNA Technologies, USA), their specificity was analyzed using the BLAST algorithm (NIH, USA).

Sequences and lengths for all primer pairs used are provided below.

Primers for obtaining the initial template from genomic DNA:

S. aureus , forward Sta-outer-f (5′-AGTTTTCTTGACCATTCACTGTATGTC-3′) – 27 nt.,
reverse Sta-outer-r (5′-CATTAGCGATATTTGGAGGCATGGC-3′) – 25 nt.
M. tuberculosis , forward Myc-outer-f (5′-GCACCAGCCAGCTGAGCCAATTC-3′) – 23 nt.
reverse Myc-outer-r (5′-ATCGATCGGCGAATTGGCCTGTG-3′) – 23 nt.

Primers for obtaining the studied template region for kinetics and incorporation analysis (calculated product length 278 bp):

S. aureus , forward Sta-inner-f (5′-TTGACGTTGTTGTTGCTGTTGTTGA-3′) – 25 nt.,
reverse Sta-inner-r (5′-ACAAAAGAAAATAAAATCGCGAATAC-3′) – 26 nt.
M. tuberculosis , forward Myc-inner-f (5′-CCCGCTGTCGGGGTTGAC-3′) – 18 nt.,
reverse Myc-inner-r (5′-CCTCCTCGTCGGCGGTCA-3′) – 18 nt.

Real-time PCR. The reaction mixture (20 μl) contained natural dNTPs at a concentration of 200 μM; various labeled deoxynucleotide triphosphates (depending on the reaction) at a concentration of 16 μM; 5 units/μl of Taq DNA polymerase ("Thermo Scientific", USA); specific primers at a concentration of 5 pM/μl and one of two templates with different GC content. Amplification was performed on an IQ5 instrument ("Bio-Rad Lab., Inc.", USA) according to the following program: preliminary heating at 95 °C for 2 min; then 32 cycles: 94 °C, 10 s; 65 °C, 30 s; 72 °C, 30 s; final incubation at 72 °C for 2 min. EvaGreen dye ("Biotium", USA) was added to the reaction mixture for visualization of the process.

Purification of products. PCR products were purified on microcolumns from unreacted fluorescently labeled dNTPs (for subsequent spectrophotometric measurements) using the GeneJET PCR Purification Kit ("Thermo Scientific") according to the manufacturer's instructions.

Horizontal electrophoresis. The absence of by-products after amplification was monitored by electrophoresis. Separation was performed in 4% Agarose LE gel ("Helicon", Russia), and

ethidium bromide was used for staining. The result was visualized on a GenoSens Touch 2100/2200 gel documentation system ("Clinx Science Instruments", China).

Spectrophotometry. Measurements were taken at wavelengths of 260 and 647 nm on a NanoPhotometer NP80 spectrophotometer ("Implen GmbH", Germany).

Definitions introduced: incorporation density - the number of fluorescently modified nucleotides per one double-stranded molecule of PCR product dsDNA (this parameter depends on the GC-composition of the template);

specific incorporation density - the ratio of the number of modified nucleotides to the total number of identical nucleotides in the DNA strand of a double-stranded PCR product, in percentage (this parameter takes into account the number of potential "binding sites", i.e., identical nucleotides in the DNA strand for the incorporation of a fluorescently modified nucleotide); incorporation coefficient - the ratio of absorption of the fluorescently labeled DNA sample (purified PCR product) at a wavelength of 647 nm (fluorophore) to absorption at a wavelength of 260 nm (DNA). In the measurements, it was assumed that the density of label incorporation into the growing DNA product chain under the selected amplification conditions does not lead to signal quenching caused by energy transfer between adjacent fluorescently labeled nucleotides in the DNA chain.

RESULTS AND DISCUSSION

In this work, we studied the substrate behavior, namely, inhibition and efficiency of incorporation into the growing DNA chain during PCR of six different pairs of zwitterionic electroneutral deoxynucleoside triphosphates (dU and dC) with similar modifications on two DNA templates with distinctly different GC-composition. The reaction was carried out with incomplete substitution of natural triphosphates with fluorescently labeled analogs according to the previously established average optimum concentration (varies for different derivatives) [8]. Modifications introduced into heterocyclic bases differed in the length of linkers and the structure of the fluorophore. Also, for comparison, two control pairs were taken, differing in the structure of their Cy5 fluorophores from the four pairs under study. These pairs are laboratory standards as they are used as the main reagents for introducing labels into DNA in biological microchip technology [9].

The real-time PCR method with EvaGreen staining was used to exclude signals from the introduced fluorescent Cy5 labels. The averaged fluorescence signal accumulation curves from both templates appear as follows (Fig. 2). From the obtained amplification signal accumulation curves, the amplification efficiency *E* [12, 13], shown in Table 2, was determined.

Fig. 2. Rate of PCR product accumulation on AT-rich template *S. aureus* (*a*) and GC-rich template *M. tuberculosis* (*b*) using dU $_{ss}$ and dC $_{ss}$ as examples. Control – sample without modified deoxyribonucleoside triphosphates.

From the signal accumulation curves during PCR, it can be seen that on both templates, the amplification efficiency values of cytidine analogs exceed those of uridine analogs. On the AT-rich template, a significantly greater difference in the rate of signal accumulation can be observed.

Amplification products were purified using microcolumns to remove fluorescently labeled nucleotides that were not incorporated into the DNA chain and to avoid their influence on the total absorption in subsequent spectrophotometric measurements for calculating incorporation efficiency. Using electrophoretic analysis, the purified PCR products were checked for the absence of amplification by-products and compared with their theoretical (calculated) length. Then, the efficiency of label incorporation in the obtained purified product was determined spectrophotometrically by measuring the absorption of the PCR product solution at wavelengths of 260 and 647 nm (for total DNA and fluorophore absorption, respectively), and the values of incorporation coefficient, incorporation density, and specific incorporation density were calculated. Amplification followed by product purification and optical density measurements was performed in triplicate for each fluorescently labeled deoxyribonucleoside triphosphate.

The incorporation coefficient K_i was introduced as a quick way to assess the substrate efficiency of fluorescently labeled deoxyribonucleoside triphosphates and was calculated as a simple ratio of optical density at a wavelength of 647 nm to the optical density value at a wavelength of 260 nm for each sample. Incorporation density was calculated as the ratio of the molar amount of label to the molar yield of the DNA product. Specific incorporation density was calculated as the ratio of the molar amount of label in the sample to the molar amount of corresponding nucleotides in the resulting PCR product.

The average values obtained during the experiments, calculated from three replicates, are shown in Table 2.

From Table 2, it can be seen that on both matrices, in almost all cases, the amplification efficiency (*E*) in the presence of modified cytidine is greater than in the presence of uridine. But at the same time, it is noticeable that the difference in the obtained values is small within each pair; based on this, it can be concluded that the amplification efficiency depends weakly on the nature of the fluorescently labeled nitrogenous base (dU and dC) introduced into the reaction. This may be due to

incomplete substitution of natural triphosphates in the reaction mixture (16 μM labeled and 200 μM natural).

The incorporation coefficients K_i, in contrast to the amplification efficiency E, differ more significantly, they are larger for dU, and a greater difference can be seen when comparing different bases in one pair. On a GC-rich matrix, fluorescently labeled cytidines have more opportunities for incorporation into the chain due to the nucleotide composition of the matrix, i.e., a larger number of potential "landing sites." However, the greater efficiency of deoxyuridine incorporation leads to the fact that on a GC-rich matrix, the values of incorporation coefficients become similar for dU and dC. It is important to note that K_i should be used to compare the incorporation efficiency of the same nucleotides, as these coefficients depend on the GC composition of the matrix.

However, for a quick assessment of incorporation efficiency on matrices that are "polar" in GC composition, it is very useful to compare the dU/dC ratio (in the case of AT-rich matrix) and dC/dU (for GC-rich matrix). It can be seen that on an AT-rich matrix, the incorporation efficiency of uridine analogs is much higher than that of cytidine analogs, while on a GC-rich matrix, the superiority of the dC/dU incorporation coefficient ratio is neutralized by the higher incorporation efficiency of uridines.

The specific incorporation density indicator is suitable for comparing the substrate properties of modified dU and dC, as it takes into account the number of identical nucleotides in the target DNA. According to this indicator, it can also be concluded that fluorescently labeled uridine derivatives are better incorporated into the DNA chain, since in most pairs their values are greater than those of cytidine analogs.

Table 2. Amplification efficiency E *, incorporation coefficient K_i **, product yield and incorporation density on matrices *S. aureus* and *M. tuberculosis* and six pairs of deoxyribonucleoside triphosphate derivatives

Cy5-dNTP	*Staphylococcus aureus*						*Mycobacterium tuberculosis*					
	E *	K_i **	product yield, pmol	incorporation density	specific incorporation density***, %	dU/dC****	*E* *	K_i **	product yield, pmol	incorporation density	specific incorporation density***, %	dC/dU****
Control*****	1.83	-	-	-	-	1.86	1.86	-	-	-	-	dU
dU_{c1}	1.43	0.47	1.36	0.42	1.05	1.59	1.75	0.15	1.50	0.27	0.61	2.16
dC_{c1}	1.63	0.30	1.69	0.27	0.28		1.78	0.33	1.64	0.37	0.39	
dU_{c2}	1.57	0.52	1.99	0.44	1.09	1.62	1.76	0.10	0.95	0.23	0.51	2.47
dC_{c2}	1.75	0.32	1.53	0.32	0.33		1.76	0.25	1.09	0.35	0.37	
dU_{ss}	1.72	0.18	2.10	0.19	0.48	1.73	1.75	0.18	1.04	0.21	0.47	0.91
dC_{ss}	1.77	0.10	1.42	0.29	0.29		1.77	0.16	1.20	0.25	0.26	
dU_{sl}	1.78	0.13	3.22	0.16	0.40	1.63	1.78	0.25	1.01	0.35	0.79	1.12
dC_{sl}	1.72	0.08	2.53	0.14	0.14		1.80	0.28	1.14	0.31	0.33	
dU_{ls}	1.66	0.47	2.86	0.50	1.23	2.33	1.80	0.20	0.93	0.41	0.93	1.21
dC_{ls}	1.72	0.20	2.40	0.18	0.18		1.74	0.25	1.01	0.38	0.40	
dU_{ll}	1.75	0.34	3.62	0.34	0.84	4.08	1.74	0.27	1.17	0.28	0.63	1.10
dC_{ll}	1.77	0.08	3.19	0.09	0.10		1.69	0.30	1.06	0.38	0.41	

*Amplification efficiency *E* was calculated using the formula $E = 10^{tg\alpha}$ [8].

**Incorporation coefficient K_i was calculated as the ratio of optical density at λ = 647 nm to its value at λ = 260 nm.

***Specific incorporation density was calculated using the formula $\frac{\nu_{\text{метки}}}{\nu_{\text{ДНК}} \times 278 \times X} \times 100\%$, where ν is the molar yield (pmol), 278 is the product length, X is the number of identical nucleotides.

****dU/dC and dC/dC were calculated as the ratio of the incorporation coefficient of the uridine analog to the value for the cytidine analog in a pair on the AT-rich template and similarly on the GC-rich template.

*****Amplification of the control sample was carried out without the use of modified deoxyribonucleoside triphosphates. Note. The determination of incorporation density and specific incorporation density can be found in the subsection Introduced definitions of the "Experimental part" section.

When analyzing the incorporation coefficient, one can see the influence of the triphosphate structure and template composition. Higher values were obtained on the *S. aureus* template for the control samples dU_{c1} and dU_{c2}, which are currently used by us as the main labels in microchip analysis. It can be noted that in the four studied pairs, better incorporation into the chain is observed on both templates for samples with a long linker between the fluorophore and the nitrogenous base.

In the case of the AT-rich template, the values of the incorporation coefficient in pairs are significantly higher than those of uridine analogs. On the GC-rich template, the advantage of incorporated cytidines is visible in terms of efficiency values and, in most cases, in terms of incorporation coefficients, but the difference in values is small.

Based on the obtained data, we can conclude that uridine derivatives are better substrates for matrices of different GC-content due to significantly higher incorporation efficiency compared to cytidines. The linker length between the fluorophore and nitrogenous base increases the incorporation efficiency of both deoxynucleosides. Higher incorporation density is accompanied by greater inhibition of the reaction, which apparently indicates the involvement of the modified substrate in a complex with polymerase with some complications and, consequently, slowing down the amplification kinetics. Conversely, less involvement in the reaction as a substrate leads to less inhibition of the reaction by such derivatives.

CONCLUSION

We have studied the substrate behavior of different pairs of fluorescently labeled nucleotides with similar electroneutral substituents on matrices of different GC-content during PCR. Based on the

values of the incorporation coefficient we introduced, it can be concluded that structures with a long linker R are better incorporated into the DNA chain. When considering the incorporation in pairs of dU and dC, better incorporation of deoxyuridines was noted. When comparing the composition of matrices, higher values of the incorporation coefficient are observed in the case of AT-rich matrix in control samples. The influence of the substituent structure, the type of nucleotide in the pair, and the matrix composition on the reaction kinetics (values of amplification efficiency E) was insignificant. Based on the research results, it can be concluded that uridine derivatives are incorporated into the growing DNA chain better on both types of matrices than cytidine derivatives, but this difference on GC-rich matrix is leveled by a significantly higher percentage of GC.

FUNDING

The study was supported by a grant from the Russian Science Foundation (No. 22-14-00257).

ETHICS DECLARATION

This article does not contain any studies involving humans or animals as subjects.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

1. Salic A., Mitchison T.J. (2008) A chemical method for fast and sensitive detection of DNA synthesis *in vivo* . *Proc. Natl. Acad. Sci. USA.* **105** , 2415–2420.
2. Smith C.I.E., Zain R. (2019) Therapeutic oligonucleotides: state of the art. *Annu. Rev. Pharmacol. Toxicol.* **59** , 605–630.
3. Yu H., Chao J., Patek D., Mujumdar R., Mujumdar S., Waggoner A.S. (1994) Cyanine dye dUTP analogs for enzymatic labeling of DNA probes. *Nucl. Acids Res.* **22** , 3226–3232.
4. Lapa S.A., Chudinov A.V., Timofeev E.N. (2016) The toolbox for modified aptamers. *Mol. Biotechnol* . **58** , 79–92.
5. Shershov V.E., Kuznetsova V.E., Lysov Yu.P., Guseinov T.O., Barsky V.E., Spitsyn M.A., Zasedateleva O.A., Vasiliskov V.A., Surzhikov S.A., Zasedatelev A.S., Chudinov A.V. (2015) Effect of chromophore charge on the incorporation efficiency of fluorescently labeled nucleotides in DNA matrix synthesis by Taq polymerase. *Biophysics* . **60** , 1216–1218.

6. Lapa S.A., Guseinov T.O., Pavlov A.S., Shershov V.E., Kuznetsova V.E., Zasedatelev A.S., Chudinov A.V. (2020) Simultaneous use of Cy5-modified deoxyuridine and deoxycytidine derivatives in PCR. *Bioorg. Chem.* . **46** , 418–424.

7. Lisitsa T.S., Shershov V.E., Spitsyn M.A., Guseinov T.O., Ikonnikova A.Yu., Fesenko D.O., Lapa S.A., Zasedatelev A.S., Chudinov A.V., Nasedkina T.V. (2017) Kinetics of fluorescent DNA labeling using polymerase chain reaction depending on the chemical structure of modified nucleotides when using different Taq polymerases. *Biophysics* . **62** , 49–56.

8. Lapa S.A., Volkova O.S., Spitsyn M.A., Shershov V.E., Kuznetsova V.E., Guseinov T.O., Zasedatelev A.S., Chudinov A.V. (2019) Amplification efficiency and substrate properties of fluorescently labeled deoxyuridine triphosphates in PCR with DNA polymerases lacking 3′-5′ exonuclease activity. *Bioorg. Chem.* . **45** , 392–402.

9. Spitsyn M.A., Kuznetsova V.E., Shershov V.E., Emelyanova M.A., Guseinov T.O., Lapa S.A., Nasedkina T.V., Zasedatelev A.S., Chudinov A.V. (2017) Synthetic route to novel zwitterionic pentamethine indocyanine fluorophores with various substitutions. *Dyes Pigments* . **147** , 199–210.

10. Zasedateleva O.A., Vasiliskov V.A., Surzhikov S.A., Kuznetsova V.E., Shershov V.E., Guseinov T.O., Smirnov I.P., Yurasov R.A., Spitsyn M.A., Chudinov A.V. (2018) dUTPs conjugated with zwitterionic Cy3 or Cy5 fluorophore analogues are effective substrates for DNA amplification and labelling by Taq polymerase. *Nucl. Acids Res* . **46** , e73.

11. Shershov V.E., Lapa S.A., Levashova A.I., Shishkin I.Yu., Shtylev G.F., Shekalova E.Yu., Vasiliskov V.A., Zasedatelev A.S., Kuznetsova V.E., Chudinov A.V. (2023) Synthesis of fluorescently labeled nucleotides for labeling isothermal amplification products. *Bioorg. Khim.* . **49** , 649-656.

12. Ramakers C., Ruijter J.M., Deprez R.H., Moorman A.F. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339** , 62-66.

13. Peirson S.N., Butler J.N., Foster R.G. (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucl. Acids Res.* **31** , e73.