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Current approaches to development of real-time qPCR test-kits

M. I. Doronin, D. V. Mikhalishin, A. V. Sprygin, A. Mazloum, T. V. Zhbanova, K. N. Gruzdev, E. V. Chernyshova
FGBI "Federal Centre for Animal Health" (FGBI "ARRIAH"), Vladimir, Russia

SUMMARY

Currently fluorescent quantitative real-time polymerase chain reaction, which is a cutting-edge technology in genetic diagnosis, is used in different areas of molecular biology. Practical advantage of simplicity as well as combination of high speed, sensitivity and specificity made it possible to use this analysis for nucleic acid quantitation. The paper presents general information and recommended rules for the development of real-time qPCR. The publication is aimed to acquaint the researchers and reviewers with necessary requirements to be followed in order to ensure high accuracy, reliability and transparency of the experiments, correct interpretation and repeatability of the test results. Current approaches are described that allow obtaining reliable and consistent results by different operators, at different times and in different laboratories. Basic requirements for reagents used, nucleotide sequences and validation methods are given. In general, the publication gives the information needed to achieve three ultimate goals: to provide the authors with a broad range of tools and requirements for the development of real-time qPCR based-techniques; to give the possibility to the reviewers and editors of assessing the quality of articles and guidelines/instructions in accordance with the required criteria; to obtain consistent and reliable results of tests performed using this method.

Keywords: review, real-time polymerase chain reaction, reverse transcription, requirements for test-kits, validation, oligonucleotide primers and probes, performance of amplification reaction

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For correspondence: Maksim I. Doronin, Doctor of Science (Biology), Head of Sector, Laboratory for FMD Prevention, FGBI "ARRIAH", 600901, Russia, Vladimir, Yur'evets, *e-mail: doronin@arriah.ru.*

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Современные подходы к разработке тест-систем на основе количественной ПЦР в режиме реального времени

М. И. Доронин, Д. В. Михалишин, А. В. Спрыгин, А. Мазлум, Т. В. Жбанова, К. Н. Груздев, Е. В. Чернышова
ФГБУ «Федеральный центр охраны здоровья животных» (ФГБУ «ВНИИЗЖ»), г. Владимир, Россия

РЕЗЮМЕ

В настоящее время в различных областях молекулярной биологии применяется основанная на флуоресцентной детекции количественная полимеразная цепная реакция в режиме реального времени, которая является передовой технологией генной диагностики. Практическая простота, а также сочетание высокой скорости, чувствительности и специфичности сделали возможным использование данного анализа для количественного определения нуклеиновых кислот. В статье представлены общие сведения и отражены рекомендуемые правила для разработки методик количественного ПЦР-анализа в режиме реального времени. Материалы публикации нацелены на предоставление исследователям и рецензентам необходимых требований, которых следует придерживаться, чтобы обеспечить высокую точность, надежность и прозрачность экспериментов, правильную интерпретацию и повторяемость результатов анализа. Представлены современные подходы, которые позволяют получать надежные и достоверные результаты, проводимые разными операторами, в разное время и в разных лабораториях. Приведены основные требования, предъявляемые к применяемым реагентам, перечням нуклеотидных последовательностей и методам проведения валидационного анализа. В целом в представленной публикации отражена информация для достижения трех конечных целей работы: предоставить авторам широкий арсенал инструментов и требований для разработки методик на основе количественной полимеразной цепной реакции в режиме реального времени; дать возможность рецензентам и редакторам оценивать качество представленных материалов статей и методических рекомендаций/указаний в соответствии с требуемыми критериями; получать однородные, сопоставимые и надежные результаты исследований, выполненных с помощью данного метода.

Ключевые слова: обзор, полимеразная цепная реакция в режиме реального времени, обратная транскрипция, требования к тест-системам, валидация, олигонуклеотидные праймеры и зонды, эффективность реакции амплификации

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Для корреспонденции: Доронин Максим Игоревич, доктор биологических наук, заведующий сектором лаборатории профилактики ящура, ФГБУ «ВНИИЗЖ», 600901, Россия, г. Владимир, мкр. Юрьевец, *e-mail: doronin@arriah.ru*.

INTRODUCTION

The fluorescence-based quantitative real-time PCR (qPCR) is a cutting-edge technology in genetic diagnosis used in different areas of molecular biology [1–3]. Its practical simplicity, together with its combination of speed, sensitivity, and specificity have made it useful for nucleic acid quantitation [4–6]. In recent years, many qPCR-based diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, etc. [4–15].

Currently, there is a lack of consensus on how best to perform qPCR experiment development followed by data analysis. The problem is exacerbated by the lack of precise requirements for these developments to permit the investigator to critically evaluate the quality of the results presented [16–18]. Thus, some authors make a number of technical errors while developing qPCR-based test-kits. Some of them are presented in Table 1.

Consequently, there is the real danger of inadequate and conflicting results obtained by qPCR [19, 20]. In addition, information about sample acquisition and handling, RNA quality and integrity, reverse transcription details, PCR efficiencies, and analysis parameters is frequently omitted, whereas sample normalization is carried out against single reference genes without adequate justification [21]. The qPCR method standardization problem has been reported in many scientific publications [19–23], therefore, this task is currently relevant.

Table 1
Possible technical errors that affect real-time qPCR assay performance and their negative consequences

No.	Possible technical errors	Negative consequences
1	Inadequate sample storage, preparation, and nucleic acid quality	Yielding highly variable results
2	Poor choice of oligonucleotide primers and probes for the PCR	Inefficient and less-than-robust assay performance;
3	Inappropriate statistical analyses	The results obtained may be misleading

There are a number of publications, in particular guidelines for qPCR method development, which reflect the basic requirements for the development of such techniques [24–26].

Applications of qPCR technology are currently used for research and diagnostic purposes [23]. Research applications usually analyze a wide range of targets with a fairly low throughput and many different sample types [25]. The main parameters that need to be addressed relate to assay analytical sensitivity and specificity [20, 26].

Diagnostic applications usually analyze a limited number of targets, but require high throughput protocols that are targeted at only a few sample types [22]. The test characteristics include information on analytical sensitivity and specificity that in this context refers to how often the assay returns a positive result when a target is present and how often it is negative in the absence of the target [19, 24]. In addition, tests for accuracy, repeatability and reproducibility of the analysis are required (Table 2).

The aim of this document is to provide authors and reviewers general current requirements that should be followed when developing qPCR-based methods in order to ensure high accuracy and reliability, experimental transparency, correct interpretation and repeatability of the analysis results.

STANDARDIZATION OF SOME TERMS USED IN THE DEVELOPMENT OF QPCR-BASED METHODS

In accordance with the proposals available in Real-time PCR Data Markup Language (RDML) resource[1] [27], it is required to make some edits to the use of generally accepted terms used in the development of PCR-based techniques, in particular:

– TaqMan probes should be referred to as hydrolysis probes;

– the term FRET probe (fluorescence resonance energy transfer probe) refers to a generic mechanism in which emission/quenching relies on the interaction between the electron-excitation states of two fluorescent dye molecules;

– LightCycler probes should be referred dual hybridization probes [25];

[1] Real-time PCR Data Markup Language. Available at: https://rdml.org.

– the nomenclature describing the fractional PCR cycle used for quantification is inconsistent, with threshold cycle (C_t), crossing point (C_p), and take-off point (TOP) currently used in the literature. To unify the nomenclature, many authors use the term "quantification cycle" (C_q) [28, 29].

GENERAL REQUIREMENTS FOR DIFFERENT ANALYTICAL STAGES

Sampling of biological material and sample storage. Sampling can be responsible for the experimental variability due to fragile nature of RNA. According to P. Micke et al. [30], fresh biological material can be kept on ice without any major influence on RNA quality, but this approach cannot be applied everywhere. It is essential to record the entire history of sampling and transportation of biological samples in PCR protocols [31].

Nucleic acid extraction. Nucleic acid extraction is also a critical step in operations the qPCR setup [32, 33]. Extraction efficiency depends on adequate homogenization, the type of sample (e.g., tissue homogenate, culture suspension, saliva, blood, etc.), target density, physiological status (e.g., healthy, cancerous, or necrotic), genetic complexity, and the amount of biomass processed [27, 34–36].

Therefore, it necessary that details of the nucleic acid extraction method be provided and that the methods used for measuring nucleic acid concentration and assessing its quality be described. Such details are particularly important for RNA extracted from fresh frozen biological samples, because variations in tissue-preparation procedures have a substantial effect on both RNA concentration and quality [37–39].

Quality control of RNA extracts

Quantification of RNA in extracts. Quantification of RNA in the extracted samples is important for the correct analysis of nucleic acids at the stage of reverse transcription and amplification reactions.

There are several groups of RNA quantification methods, namely:

1) spectrophotometry using various spectrophotometers;

2) microfluidic analysis, for example, using bioanalyzer systems by Agilent Technologies, Inc. (USA), Bio-Rad Laboratories, Inc. (USA), etc.;

3) capillary gel electrophoresis, for example, using the QIAxcel instrument by Qiagen (Germany), etc.;

4) fluorescent dye detection (Ambion, RiboGreen, Thermo Fisher Scientific, Inc., USA, etc.) [14, 23, 40].

It is worth remembering that RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental factors [41, 42]. Moreover, even high-quality RNA samples can show differential degradation of individual molecules, which is difficult for a researcher to control.

RNA sample purification quality assessment. To assess the purity of the prepared samples, a spectrum analysis is performed. The sample absorbance spectrum is measured at a wavelength of 205–325 nm and a temperature of 20–22 °C. In RNA samples, the content of residual phospholipids, polysaccharides and guanidine isothiocyanate, carbolic acid, polypeptides and suspended particulate matter is evaluated by determining the optical density values (OD) at 205, 235, 270, 280 and 320 nm, respectively. The greatest RNA absorbance should be observed at 260 nm [40]. RNA eluate is considered free from protein and carbolic acid contamination if OD_{260}/OD_{280} (extinction coefficient R_1) is within 1.8–2.2 and is approximately 2.0. Lower readings of R_1 suggest the presence of DNA, protein and residual phenolic compounds in the sample. Higher R_1 readings are indicative of RNA degradation and the presence of free ribonucleotides. The RNA molecules are considered free of polysaccharides if OD_{260}/OD_{235} (extinction coefficient R_2) is close to 2.000 [40, 43, 44]. If 1% of RNA is replaced with polysaccharide components, R_2 decreases by 0.002. R_2 readings greater than 2.000 may indicate degradation of RNA molecules. The absence of suspended particulate matter in the sample is confirmed if the optical density at 320 nm is close to zero.

Table 2
Parameters required for the validation of real-time qPCR-based methods

Application	Analysis characteristics	Major validation parameters
Research	1. Low throughput 2. A great number of different types of samples	1. Analytical sensitivity 2. Analytical specificity
Diagnostics	1. Limited number of targets 2. High throughput protocols targeted at only a few sample types	1. Analytical sensitivity 2. Analytical specificity 3. Accuracy 4. Repeatability 5. Reproducibility

It is important to test for the level of RNA eluate contamination by genomic DNA, as well as to record the cut-off criteria for admissible levels of such contamination in the test protocol. It is required to record information on whether the RNA sample was treated with DNase, as well as to record the results of comparing the C_q obtained with positive control and no reverse transcriptase control for each target nucleic acid [20].

Assessment of RNA integrity in the eluate. To assess the integrity of RNA and the absence of DNA contamination, horizontal gel electrophoresis of denatured RNA is performed, which gives a clearly distinguishable band of nucleic acid molecules without apparent extraneous polynucleotide fragments [45]. Electrophoresis is performed in a thin 0.5% agarose gel prepared using agarose E and 1× RNase-free running buffer, in a voltage gradient of 1–2 V/cm of gel for 45 min. For RNA color-coding after electrophoresis, the gel is dyed with a dye solution with ethidium bromide concentration of 0.4 μg/mL in 25 mM tris-HCl (pH 9.0) for 50–60 minutes. RNA bands are observed using an ultraviolet transilluminator [45, 46].

DNA sample assessment. It is important to assess the extent of DNA degradation for forensic applications, i.e., in cases in which harsh environmental conditions at scenes of crimes may have degraded the chemical structure of DNA. It should be noted that DNA molecules are more stable and less susceptible to degradation than RNA. For this reason, the DNA purity and integrity is assessed much less often. At the same time, there is a general rule for DNA samples, to routinely use dilutions of nucleic acids to demonstrate that observed decreases in qualification cycles or copy numbers are consistent with the anticipated result [47].

Requirements for the reverse transcription reaction. When handling RNA samples, a reverse transcription step

introduces substantial variation into a qPCR assay [48, 49]. Hence the following information should be given in detail in the test protocol:

– reagent composition;
– temperature-time parameters of the reverse transcription step;
– RNA amount in the collected sample;
– priming strategy;
– enzyme type;
– reaction volume.

It is recommended that the reverse transcription step be carried out in duplicate or triplicate and that the total RNA concentration be the same in every sample [49].

Requirements for qPCR. To develop a method based on quantitative PCR, the author must know the following information:

– database accession numbers of each target and reference genes (for example, GenBank, WIPO Sequence and others);
– the exon locations of each primer and any probe;
– the sequences and concentrations of each oligonucleotide, positions of dyes and/or modified bases used in the probe;
– published sequences of primers and probes (since the amplification efficiency largely depends on the oligonucleotides used);
– the concentration and identity of the polymerase;
– the amount of template (DNA or cDNA) in each reaction;
– the Mg^{2+} concentration;
– the exact chemical composition of the buffer (salts, additives, hydrogen ion concentration);
– total volume of the components for one reaction;
– PCR machine calibration certificate;
– data on thermal cycling time and temperature conditions;
– information about the degree of transparency of the plasticware used and the material from which they are made (because different plastics exhibit substantial differences in fluorescence reflection and sensitivity) [50, 51].

Requirements for the calculation of oligonucleotide primers and probes. The structure of the target nucleic acid (for example, stem and loop secondary RNA) has a significant impact on the efficiency of reverse transcription and the PCR [52]. Therefore, the positions of primers, probes and PCR amplicons must take the folding of RNA templates into consideration.

To develop qPCR-based methods it is useful to use such tools for oligonucleotide specificity assessment *in silico*, as BLAST[2], etc. Any appreciable homology to pseudogenes or other unexpected targets should be documented and provided as aligned sequences for review.

Another important requirement is that specificity must be validated empirically with direct experimental evidence (electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion) [53, 54].

An important primer characteristic is their size, responsible for the reaction specificity. As a rule, the length of primers is from 17 to 35 b.p., but there may be exceptions (a larger length is acceptable). The length of the nucleotide chain affects the melting temperature. Melting temperature (hybridization, dissociation, T_m) is the temperature at which half of the oligonucleotides in solution are in the double-stranded state and half single-stranded. T_m depends on the hydrogen bonding between the nucleotides of the primer and the DNA molecule it is hybridized with. There are different approaches to this indicator assessment. Traditionally the melting temperature is defined as the inflection point on the hybridization graph, which corresponds to the maximum modulus of the first derivative. Less often, the temperature at which 50% of the fluorescent signal is reached, is considered to be primer dissociation temperature [17, 54–56]. It should be noted that there are calculated and true values of the melting temperature. The calculated value is obtained using a number of formulas, and this value is theoretical, the true value is obtained by molecular biological experiment. In practice, various formulas or bioinformatic tools are used to solve this problem [57, 58].

Thermodynamic calculations in the context of the base energy are performed as described by K. J. Breslauer et al. [59], but using the values published by N. Sugimoto et al. [60]. RNA thermodynamic properties are taken from the publication of T. Xia et al. [61]. Melting temperature calculations are based on the thermodynamic relationship between entropy, enthalpy, free energy and temperature: $\Delta H = \Delta G + T\Delta S$, where ΔH is enthalpy; ΔG is Gibbs energy; T is absolute temperature (K); ΔS is entropy.

The change in entropy (order or a measure of the randomness of the oligonucleotide) and enthalpy (heat released or absorbed by the oligonucleotide) are directly calculated by summing the values for nucleotide pairs obtained by N. Sugimoto et al. [60]. The relationship between the free energy and the concentration of reactants and products at equilibrium is given by the following formula:

$$\Delta G = RT\ln\left\{\frac{[\text{DNA}\times\text{primer}]}{[\text{DNA}]\,[\text{primer}]}\right\},$$

where R is the gas constant (8.31 [J/mol × K]); T is the absolute temperature (K); ln is the natural logarithm; [DNA × primer] is the concentration of the bound DNA × primer complex; [DNA] is the concentration of unbound DNA target sequence; [primer] is the concentration of unbound primer.

Substituting for ΔG gives:

$$\Delta H = T\Delta S + RT\ln\left\{\frac{[\text{DNA}\times\text{primer}]}{[\text{DNA}]\,[\text{primer}]}\right\}.$$

The absolute temperature is expressed using the following equation:

$$T = \frac{\Delta H}{\Delta S + R\ln\left\{\frac{[\text{DNA}\times\text{primer}]}{[\text{DNA}]\,[\text{primer}]}\right\}}.$$

We can assume that the concentration of DNA and the concentration of the DNA × primer complex are equal (that is, the concentration of primer is in excess of the target DNA and the melting temperature is where the concentration of bound and unbound DNA are at equilibrium), so this simplifies the equation considerably. It has been determined empirically that there is a 5 kcal free energy change (according to N. Sugimoto et al. [60]) during the transition from single stranded to B-form DNA. This

[2] Basic Local Alignment Search Tool (BLAST). Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi .

represents the helix initiation energy. Finally, adding an adjustment for salt gives the equation:

$$T = \frac{\Delta H - 3{,}4\ \frac{\text{kcal}}{^{\circ}\text{K mole}}}{\Delta S + R \ln\left\{\frac{1}{[\text{primer}]}\right\}} + 16{,}6 \log_{10}([Na^+]),$$

where T – absolute temperature (K); ΔH – enthalpy; kcal – energy; mole – amount of substance; ΔS – entropy; R – gas constant (8.31 [J/mol × K]); ln – natural logarithm; [primer] – concentration of unbound primer; $\log_{10}$ – decimal logarithm (lg); $[Na^+]$ is the concentration of sodium cations.

An adjustment constant for salt concentration is not needed, since the various parameters were determined at 1 M NaCl, and the log of 1 is zero. The thermodynamic calculations assume that the annealing occurs at pH 7.0. The T_m calculations assume the sequences are not symmetric. The oligonucleotide sequence should be at least 8 bases long to give reasonable T_m. The accuracy of the calculation decreases after 20–25 nucleotides since the equations and parameters were defined with oligonucleotides in the size range of 14–20 nucleotides. Monovalent cation concentrations (either Na^+ or K^+) should be between 0.01 and 1.00 M. The melting temperature of oligonucleotides can also be calculated using bioinformatics software, for example Primer3plus[3] [50].

If a theoretically calculated melting temperature differs significantly from the true one, this is not critical, since the annealing temperature (T_a) and the ratio of oligonucleotide T_m to each other and their correspondence to this temperature in the thermocycler program are more important for the test system. In this context, the investigators test several conditions for the amplification reaction, namely the annealing temperature of primers and select the optimal one [15, 48, 50].

Many different algorithms are used to theoretically determine the hybridization temperature, but none of them gives 100% confidence in obtaining the true value. The recommended melting temperature for oligonucleotides is from 55 to 75 °C. The hybridization temperatures of the forward and reverse primers should differ by no more than 5 °C. It should be noted that the greater the G+C content and the length of the oligonucleotide, the higher the T_m [22].

The transcripts of most genes in multicellular organisms are alternatively spliced, and these splicing variants specify alternative protein isoforms. It is known that there are differences in splicing patterns in different tissues or at different developmental stages. Consequently, single exon-based RT-qPCR assays may detect a number of splice variants, whereas intron-spanning primers may be more selective but may miss some splice variants altogether [45].

In this context the development of qPCR-based methods requires adherence to the following rules (and exceptions) when determining target genes for mRNA:

1) the use of an qPCR assay that simply targets one or at most two exons of an mRNA is no longer sufficient to describe the expression level of a particular gene;

2) sequence information for primers must be provided together with an assessment of their specificity with respect to known splice variants and single-nucleotide polymorphism positions [54]. For primer sets selected from the RTprimerDB database [56], this is easily done by consulting the web-site[4], that contains all the relevant information. It is not recommended to provide results that have been confirmed only *in silico*;

3) it must be remembered that detection of the presence of an mRNA provides no information on whether that mRNA will be translated into a protein or, indeed, whether a functional protein is translated at all [36].

Requirements for qPCR controls and calibrators. In addition to controls performed at the stages of nucleic acid extraction and reverse transcription reaction, additional controls and/or quantitative calibrators are also required for the development of quantification techniques [27, 36].

No template control. It is recommended to use a no template control (NTC), which allows screening for contamination and can also distinguish unintended amplification products (e.g., primer dimers) from the intended PCR products [48].

The NTC use is based on the possibility of performing many separate PCR reactions all together in one reaction for different DNA fragments (multiplex PCR) [62, 63]. For example, to control the amplification performance, two PCRs can be carried out simultaneously in one test tube. In one of these reactions, the target DNA (or cDNA) fragment is accumulated, and in the other, specially included DNA is amplified (usually a plasmid DNA fragment). Inclusion of NTC into the sample before the nucleic acid extraction, makes it possible to monitor the efficiency of all analytical stages.

For reverse transcription PCR, it is recommended to use NTC. This is a specially designed RNA product added to each tested sample at the stage of sample preparation (exogenous internal control), which goes through all stages of polymerase chain reaction. At the PCR detection stage, NTC allows us to judge the quality of the amplification result in general. It is added immediately before the nucleic acid extraction. If during PCR analysis a signal is detected in the NTC, this means the result of the PCR is reliable, otherwise the PCR result will be invalid [2, 52].

NTCs should be included on each plate or batch of samples, and conditions for data rejection be established. For example, NTCs with quantification cycle values 40 could be ignored if the C_q for the lowest concentration is 35 [9].

For optimal PCR results, physically separated working places for template preparation before PCR and setting up PCR reactions are recommended [17].

Positive controls in the form of nucleic acids extracted from experimental samples are useful for monitoring assay variation over time and are essential when calibration curves are not performed in each run.

Quantification calibrators may be the following:

– purified target molecules, such as synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon;

– plasmid DNA constructs;

– cDNA cloned into plasmids;

– RNA transcribed *in vitro*;

– reference RNA pools;

– RNA or DNA from specific biological samples or internationally recognized biological standards.

[3] Primer3Plus. Available at: https://www.primer3plus.com.

[4] RTprimerDB. Available at: http://www.rtprimerdb.org.

Suspension dilutions should be carried out into defined concentrations. Serial dilutions of a particular template can be prepared as stock solutions that resist several freeze-thaw cycles. A fresh batch should be prepared when a C_q shift of 0.5–1.0 is detected. Alternatively, solutions for calibration curves can be stored for not longer than a week at (2 ± 1) °C [12].

For diagnostic assays, the qPCR should include an independently verified calibrator, if available, that lies within the linear interval of the assay.

PCR negative control. In addition to positive control at the stage of the amplification, it is absolutely necessary to use negative control, which most often is deionized water, not contaminated by extraneous nucleic acids, enzymes, microorganisms [35].

Nucleic acid extraction controls. Positive and negative controls to be used during RNA/DNA extraction are also recommended [55].

Assay performance. The following assay performance characteristics must be determined when qPCR method is developed: PCR efficiency, linear dynamic range, limit of detection, and precision.

PCR efficiency. Robust and precise qPCR assays are usually correlated with high qPCR efficiency. qPCR efficiency is particularly important when reporting mRNA concentrations for target genes relative to those of reference genes.

The C_q ($\Delta\Delta C_q$) method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in C_q values (ΔC_q) between the target gene and the reference gene is calculated, and the C_qs of the different samples are compared directly. The 2 genes must be amplified with comparable efficiencies for this comparison to be accurate [21, 43]. As an example, the figure shows the fluorescence accumulation curves for FMDV cDNA suspensions with different template concentrations (from 10 fg to 300 ng/mL).

PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Amplification efficiency should be determined from the slope of the log-linear portion of the calibration curve using the following formula: $E = 10^{-1/k} - 1$, where k is the slope of the dependency graph between the logarithm of the initial template concentration (the independent variable), plotted on the x-axis and C_q (the dependent variable), plotted on the y-axis.

An E value of 1.00 (or 100%) indicates that the amount of product doubles with each cycle (theoretically).

The means of estimated PCR efficiencies and slope values should be recorded in the qPCR protocols. Differences in PCR efficiency will produce calibration curves with different slopes. As a consequence, differences between the C_q values of the targets and the references will not remain constant as template amounts are varied, and calculations of relative concentrations will be inaccurate, yielding misleading results [12, 35, 54].

Attention should be paid to the fact that if C_q values are close to 40, low efficiency is highly probable or template content is too low for analytical sensitivity [36].

qPCR linear dynamic range. The dynamic range of the quantitative analysis (the range of the template contents in the tested samples) over which a reaction is linear must be described [39]. The dynamic range should cover at least 3 orders of magnitude. The calibration curve's linear interval must include the interval for the target nucleic acids being quantified [14].

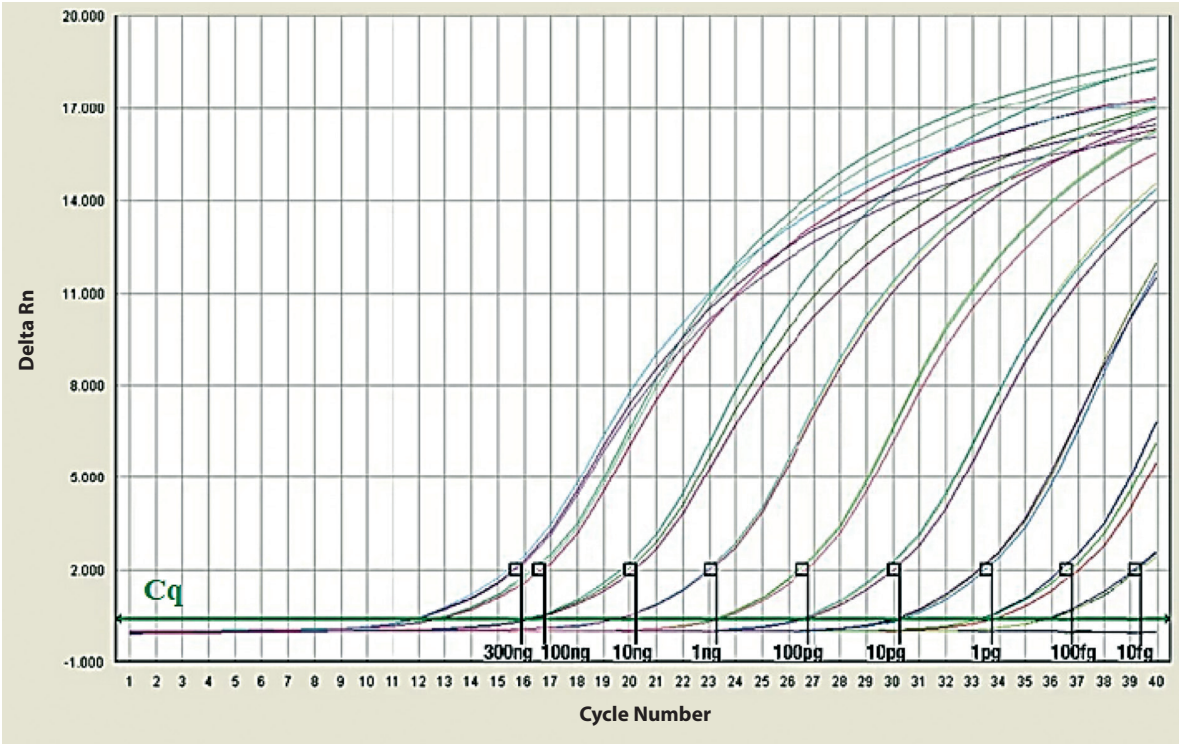

Fig. Fluorescence accumulation curves obtained by testing of FMDV cDNA suspensions at analyte concentrations within 10 fg to 300 ng/mL

Because lower limits of quantification are usually poorly defined, the variation at the lowest concentration claimed to be within the linear interval should be determined. Correlation coefficients (r^2 values) must be reported, and confidence intervals (CIs) should be provided through the entire linear dynamic range [64].

The limit of detection (LOD) is the minimum target concentration in a sample that can be detected with an acceptable level of confidence. Thus LOD is defined as the lowest concentration at which 95% of the positive samples are detected. In other words, within a group of replicates containing the target at concentrations at the LOD, no more than 5% failed reactions should occur. Low-copy qPCRs are stochastically limited, and LODs of 3 copies per PCR are not possible. If multiple reactions are performed, however, accurate quantification of lower concentrations can be obtained via digital PCR [18, 22].

The smallest target concentration is calculated by the formula: $LOD = 3.3 \times S_b/k$, where S_b is the standard deviation of the detected signal, which corresponds to the intercept standard deviation (b); k is the tangent of the slope [65, 66]. The intercept is calculated by analyzing a certain number of model samples with known target concentrations.

The limit of quantification (LOQ). The lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision of the validated technique which is calculated using the formula: $LOQ = 10 \times S_b/k$ [66]. The obtained LOQ value should be subsequently validated by direct experimental evidence using a suitable number of samples known to be near to the LOQ. The analysis should be performed in at least five replicates. The results of the test are considered reliable at $p < 0.05$ [66–69].

Linearity. The linear relationship between analyte concentration and response should be evaluated across the working range of the analytical procedure for ≥ 30 samples, using different analyte concentrations in at least three replicates. The obtained data are processed by calculation of a regression line by the method of least squares: $y = k \times x + b$, where k is the angular coefficient; b is the intercept. The reliability of the analysis results is confirmed by calculating the correlation coefficient (r^2), which should be ≥ 0.99 [69].

Accuracy. To determine the accuracy, samples with known analyte amounts are analyzed by quantitative PCR. The data are given as a linear equation for the experimental values (y) and the reference (x) values of the analyte: $y = k \times x + b$. For the resulting function, the hypotheses about the equality of the slope (k) to one and the equality of the intercept (b) to zero are verified. If these hypotheses are true with 0.05 reliability, the validated technique gives error-free results [64].

Precision. To assess the precision of the qPCR test kits being developed against repeatability and reproducibility, it is required to calculate absolute and relative measures of variation.

Absolute quantification. The range of variation (R) is defined as the difference between the maximum and minimum values of the quantification cycle: $R = Cq_{max} - Cq_{min}$. The individual linear deviation (d_i) is calculated using the following formula $d_i = |Cq_i - Cq_m|$. The mean linear deviation (d_m) is calculated as the arithmetic mean of individual linear deviations: $d_m = \Sigma|d_i| / N$, where d_i is the individual linear deviations of the quantification cycles; N is the population size. The dispersion (δ^2) of the values is estimated using the following formula $\delta^2 = (\Sigma d_i^2) / N$. To characterize C_q variations, the root-mean-square deviation (δ) is calculated using the following mathematical model $\delta = \sqrt{(\delta^2)}$ [64–66, 70].

Relative quantification. Relative variation coefficient (V_R) is calculated by the formula $V_R = R / C_{tm} \times 100$. The linear coefficient of variation (C_d) is calculated using a mathematical model $C_d = d_m / C_{tm} \times 100$. To assess the dispersion of individual C_p values, the coefficient of variation (C_δ) is determined by the formula: $C_\delta = \delta / C_{tm} \times 100$ [17]. The method is considered reliable at $C_\delta < 2\%$ for repeatability and at $C_\delta < 3\%$ for reproducibility [64–67].

The statistics of the diagnostic tests. The main statistical indices of the diagnostic tests are: diagnostic sensitivity (DSe), diagnostic specificity (DSp), k (Cohen's kappa coefficient), positive predictive value (PPV), negative predictive value (NPV). To measure them, the following test results are needed: a – true positive samples; b – false negative samples; c – false positive samples; d – true negative samples.

Diagnostic sensitivity and specificity are calculated by the formulas: $Dse = a / (a + b)$ and $DSp = d / (c + d)$, and expressed as a percentage.

The Cohen's kappa index value (k) is used to measure the of inter-rater agreement on any two tests. Cohen's kappa index value is calculated using the following formula: $k = (Pr(a) - Pr(e)) / (1 - Pr(e))$, where Pr (a) – relative observed agreement; Pr (e) – hypothetical probability of chance agreement.

The probability of the positive result in the test is calculated by the formula: $PPV = (Dse \times prevalence) / ((Dse \times prevalence) + (1 - DSp) \times (1 - prevalence))$, where prevalence is the number of events, in this variant, positive samples being detected from truly positive ones at a certain moment. This value should aim at 100%.

The probability of a negative test result when testing true negative samples is calculated by the formula: $NPV = DSp \times (1 - prevalence) / ((1 - DSe) \times prevalence + DSp \times (1 - prevalence))$. This value should aim at 100% [64–68, 70].

REQUIREMENTS FOR qPCR DATA ANALYSIS

General requirements. Data analysis includes an examination of the raw data, an evaluation of their quality and reliability, and the generation of reportable results [2, 71]. When developing qPCR, it is necessary to specify up-to-date information in the protocol:

– methods of data analysis and confidence estimation;
– specification of the software used;
– methods of identifying outliers;
– statistical and validation methods used to evaluate variances (e.g., 95% CIs) and presentation of the corresponding concentrations or C_q values for precision analysis for repeatability and reproducibility [67, 70].

Normalization of qPCR data. Normalization is an essential component of a reliable qPCR assay because this process controls for variations in extraction yield, reverse-transcription yield, and efficiency of amplification, thus enabling comparisons of mRNA concentrations across different samples.

The use of reference genes as internal controls is the most common method for normalizing qPCR data. Normalization involves reporting the ratios of the mRNA concentrations of the genes of interest to those of the reference genes. Reference gene mRNAs should be stably expressed, and their abundances should show strong correlation with the total amounts of mRNA present in the samples.

It should be noted, that normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described [25]. The optimal number and choice of reference genes must be experimentally determined. This process is described in detail in the publications of J. Vandesompele et al. [72], C. L. Andersen et al. [73].

CONCLUSION

The analysis of publications and international protocols presents general requirements and recommended rules for the development of qPCR-based methods, compliance with which will allow reviewers to evaluate the work and other investigators to reproduce it.

In accordance with current approaches (in particular, with the MIQE guidelines – a database of checklists for developed qPCR techniques[5]), for the presentation of materials, a qPCR data markup language (RDML) is used, which is a structured and universal standard of results for the exchange of PCR quantitative data. According to these principles, the method should contain sufficient data to ensure correct interpretation and repeatability. The data standard is a flat text file in Extensible Markup Language (XML) and enables transparent exchange of annotated qPCR data between instrument software and third-party data analysis packages, between colleagues and collaborators, and between authors, peer reviewers, journals and readers.

In summary, the purpose of these guidelines is 3-fold:

1. To enable authors to design and report qPCR experiments that have greater inherent value.

2. To allow reviewers and editors to measure the technical quality of submitted manuscripts against an established yardstick.

3. To facilitate easier replication of experiments described in published studies that follow these guidelines. As a consequence, investigations that use this widely applied technology will produce data that are more uniform, more comparable, and, ultimately, more reliable.

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INFORMATION ABOUT THE AUTHORS / ИНФОРМАЦИЯ ОБ АВТОРАХ

Maksim I. Doronin, Doctor of Science (Biology), Head of Sector, Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0002-4682-6559, e-mail: doronin@arriah.ru.*

Dmitry V. Mikhalishin, Doctor of Science (Veterinary Medicine), Head of Laboratory for FMD Prevention, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0003-1718-1955, e-mail: mihalishindv@arriah.ru.*

Доронин Максим Игоревич, доктор биологических наук, заведующий сектором лаборатории профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия; *https://orcid.org/0000-0002-4682-6559, e-mail: doronin@arriah.ru.*

Михалишин Дмитрий Валерьевич, доктор ветеринарных наук, заведующий лабораторией профилактики ящура ФГБУ «ВНИИЗЖ», г. Владимир, Россия; *https://orcid.org/0000-0003-1718-1955, e-mail: mihalishindv@arriah.ru.*

Alexander V. Sprygin, Doctor of Science (Biology), Senior Researcher, Reference Laboratory for Bovine Diseases, FGBI "ARRIAH", Vladimir, Russia;
https://orcid.org/0000-0001-5982-3675,
e-mail: sprygin@arriah.ru.

Ali Mazloum, Candidate of Science (Biology), Senior Researcher, Reference Laboratory for African Swine Fever, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0002-5982-8393,*
e-mail: mazlum@arriah.ru.

Tatyana V. Zhbanova, Candidate of Science (Biology), Junior Researcher, Education and Scientific Support Department, Academic Secretary of Thesis Council, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0002-9857-5915,*
e-mail: zhbanova@arriah.ru.

Konstantin N. Gruzdev, Doctor of Science (Biology), Professor, Chief Researcher, Information and Analysis Centre, FGBI "ARRIAH", Vladimir, Russia; *https://orcid.org/0000-0003-3159-1969,*
e-mail: gruzdev@arriah.ru.

Elena V. Chernyshova, Candidate of Science (Veterinary Medicine), Junior Researcher, Reference Laboratory for Rabies and BSE, FGBI "ARRIAH", Vladimir, Russia;
https://orcid.org/0000-0003-4054-8532,
e-mail: chernishova@arriah.ru.

Спрыгин Александр Владимирович, доктор биологических наук, старший научный сотрудник референтной лаборатории болезней крупного рогатого скота ФГБУ «ВНИИЗЖ», г. Владимир, Россия; *https://orcid.org/0000-0001-5982-3675,*
e-mail: sprygin@arriah.ru.

Мазлум Али, кандидат биологических наук, старший научный сотрудник референтной лаборатории по африканской чуме свиней ФГБУ «ВНИИЗЖ», г. Владимир, Россия;
https://orcid.org/0000-0002-5982-8393, e-mail: mazlum@arriah.ru.

Жбанова Татьяна Валентиновна, кандидат биологических наук, младший научный сотрудник отдела образования и научной информации, ученый секретарь диссертационного совета ФГБУ «ВНИИЗЖ», г. Владимир, Россия;
https://orcid.org/0000-0002-9857-5915, e-mail: zhbanova@arriah.ru.

Груздев Константин Николаевич, доктор биологических наук, профессор, главный научный сотрудник информационно-аналитического центра ФГБУ «ВНИИЗЖ», г. Владимир, Россия;
https://orcid.org/0000-0003-3159-1969, e-mail: gruzdev@arriah.ru.

Чернышова Елена Владимировна, кандидат ветеринарных наук, младший научный сотрудник референтной лаборатории по бешенству и BSE ФГБУ «ВНИИЗЖ», г. Владимир, Россия;
https://orcid.org/0000-0003-4054-8532,
e-mail: chernishova@arriah.ru.