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METROLOGICAL SUPPORT FOR REAL-TIME POLYMERASE CHAIN REACTION AMPLIFIERS

Yu. A. Kudeyarov,[1] E. V. Kulyabina,[1]
A. V. Mardanov,[2] O. N. Melkova,[1]
and M. V. Sukhacheva[2]

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We present a test procedure and test results for purposes of approval of type for real-time polymerase chain reaction (PCR) amplifiers.

Keywords: *polymerase chain reaction, deoxyribonucleic acid, primers, real-time PCR amplifiers, calibration curve.*

The polymerase chain reaction (PCR) became widely used in molecular biology starting in the 1980s, as an experimental method that can be used to increase low concentrations of certain fragments of deoxyribonucleic acid (DNA) in samples. Today, the PCR method is one of the most widely used methods in molecular biology, applicable in medical diagnosis, forensics, paternity testing, etc.

The PCR method is based on repeated selective copying under artificial conditions (*in vitro*) of a certain section of DNA, using enzymes. The reaction is based on the mechanism realized in nature for intracellular duplication (replication) of DNA molecules by the enzyme DNA polymerase. In contrast to DNA replication in living organisms, relatively short sections of DNA are amplified (repeatedly copied) using PCR under artificial conditions. In conventional PCR, the length of the sections of DNA to be copied is several thousand base pairs. Using a mixture of different polymerases under certain conditions and with certain additives, the length of the fragment to be amplified can be as long as 20–40 thousand nucleotide pairs, while the human genome, for example, consists of approximately 3 billion base pairs.

The specificity of PCR is based on the formation of complementary complexes between the DNA template and the primers (short synthetic oligonucleotides of length 18–30 bases). The oligonucleotide is a short fragment of DNA or RNA (ribonucleic acid) used as a probe or primer, obtained by chemical synthesis or cleavage of longer polynucleotides. Each primer is complementary to one of the strands of the double-stranded template and delimits the beginning and the end of the section to be amplified.

The amplification process is rather complicated, and consists of several steps. Let us consider the steps and features of this process.

The first step is denaturation. During denaturation, the double-stranded DNA molecule (the template) is heated up to 94–96°C (when using an especially heat-stable polymerase, up to 98°C) for 0.5–2 min, so that the DNA strands are separated. During such heating, the hydrogen bonds between the two DNA strands are broken, and a mixture of single-stranded DNA molecules and primers is formed in the reaction zone.

The second step is annealing. After cleavage of the strands of the DNA molecules, the temperature is lowered so that the primers can bind to the single-stranded template. The annealing temperature depends on the composition of the primers, and

[1] All-Russia Research Institute of Metrological Service (VNIIMS), Moscow, Russia; e-mail: kudeyarov@vniims.ru.
[2] Bioengineering Center, Russian Academy of Sciences, Moscow, Russia.

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it is selected to be equal to their melting point. In an annealing time of 30 sec, the polymerase has already been able to synthesize several hundred nucleotides, and therefore it is recommended that primers be selected with a melting point above 60°C.

The last step is elongation. During this period, the DNA polymerase replicates the template strand, using the primer as the initiator. For any DNA strand, there is a natural chemical orientation, i.e., each nucleotide is bonded to its neighbors in two positions: at the phosphate addition site (the 5 carbon atom of the ring) and at the OH radical addition site (where the 3 carbon is found). Therefore, we can talk about the 5 (5′) or 3 (3′) end of the entire strand. The polymerase begins synthesis of the second strand from the 3′ end of the primer, having bonded with the template, and moves along the template, synthesizing the new strand in the direction from 5′ to 3′.

The elongation temperature depends on the polymerase. The commonly used polymerases *Taq* and *Pfu* are most active at 72°C. The elongation time is due to the type of DNA polymerase and the length of the fragment to be amplified. The elongation time is assumed to be equal to one minute for every thousand base pairs. After all the cycles are finished, an additional final elongation step is performed so that all the single-stranded fragments are completed. This step can take 7–10 min.

A certain set of reagents is required to run PCR [1], consisting of: two primers complementary to opposite ends of different strands of the required DNA fragment; a heat-stable DNA polymerase (an enzyme catalyzing the DNA polymerization reaction and maintaining its activity at high temperature for a long time); deoxyribonucleoside triphosphates (*dATP*, *dGTP*, *dCTP*, *dTTP*) and Mg^{2+} ions, needed for functioning of the polymerase; a buffer solution providing the needed reaction conditions (pH, ionic strength of the solution, etc.); the DNA template, containing the section needing to be amplified.

PCR is performed in special devices: PCR amplifiers (thermal cyclers) providing for periodic cooling and heating of reaction tubes accurate within at least 0.1°C. Modern PCR amplifiers make it possible to set complicated amplification programs, and also to subsequently store the amplified molecules at 4°C.

Most PCR amplifiers are not measuring instruments or objects for metrological support. An exception is PCR amplifiers running real-time PCR. Technically, such instruments are a conventional DNA amplifier additionally equipped with a fluorescence detector (a photoelectron multiplier, a photodiode, a CCD array), permitting measurements of the concentration of DNA molecules in the reaction volume directly during the reaction. Such devices have been called detecting PCR amplifiers. The external appearance of these instruments and their block diagrams are shown in [1].

Real-time PCR amplifiers are used to observe the change in concentration of a specific PCR product in each reaction cycle. In this method, fluorescent-labeled primers are used for accurate measurement of the amount of the reaction product as it accumulates.

From the operating principle of a real-time PCR amplifier, it follows that there are several physical quantities determining the operation of the PCR amplifier: temperature, range of fluorescence emission, fluorescence intensity, sensitivity of the radiation detector, concentration of the reagents. Only one measurable quantity appears at the output of a real-time PCR amplifier: the concentration of DNA molecules, the uncertainty in measurement of which determines the metrological properties of the PCR amplifier. This quantity also should be the object of metrological support for the real-time PCR amplifier. The concentration in thermal cyclers is determined with the help of a calibration curve, representing the functional relationship between the number of threshold cycles and the logarithm of the initial concentration of DNA molecules.

The basic principles of metrological support for biotechnologies were formulated in [2–4]. An important element of such support is testing measuring instruments for purposes of approval of type. In the tests, besides experimental determination of the metrological characteristics of the measuring instruments (measurement range, uncertainties in the measurements, calibration (check) interval), the calibration (check) procedure is developed and approved.

In this paper, we outline the methodology and present the test results for purposes of approval of type for the CFX96 measurement modules included in C1000 Touch thermal cyclers for nucleic acid amplification, manufactured by BIO-RAD Laboratories (USA). The tests conducted by staff at the All-Russia Research Institute of Metrological Service (VNIIMS) and the Bioengineering Center (Russian Academy of Sciences) are important because the measurement results obtained using the indicated modules have serious medical and social significance. Real-time PCR amplifiers fall within the scope of government regulation in the area of assurance of measurement uniformity, and should be entered into the State Registry of Measuring Instruments. The methodology of the tests is useful for developers, manufacturers, users, and testers of real-time

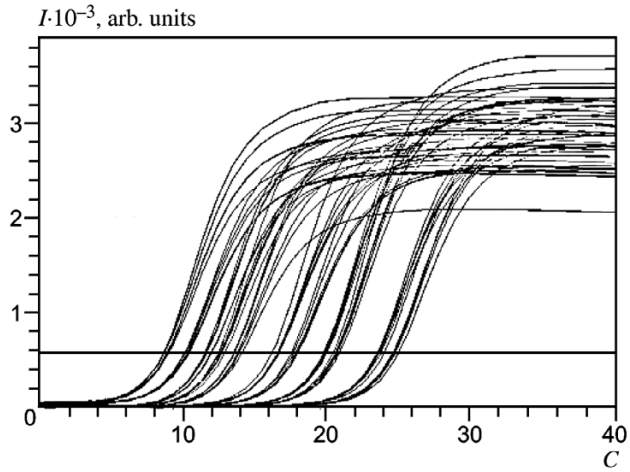

Fig. 1. Fluorescence signal intensity I vs. number of PCR cycles C, obtained on an actual instrument for different values of the initial concentration of the sample.

PCR amplifiers, and also for representatives of regulatory agencies. This methodology can be applied in tests of different types of PCR amplifiers, provided that their operating principle permits plotting and using calibration curves.

In order to consider questions about the methodology of the tests, experimental determination, and the meaning of the calibration curves, we will use the formula [1]:

$$I_n = \alpha N_0 E^n, \tag{1}$$

where I_n is the fluorescence intensity in the nth reaction cycle; α is the proportionality coefficient between the luminosity and the number of molecules, i.e., $I_n = \alpha N$; N_0 is the number of DNA molecules to be amplified at the beginning of the reaction (in the first cycle); and E is the PCR efficiency.

In the ideal case, the number of amplified molecules should be doubled after the first reaction cycle, i.e., as the reaction cycles increase, the number of amplified molecules should grow in a geometric progression with common ratio equal to 2. In fact, the number of fragments of the DNA molecule does not grow in a geometric progression, and this is taken into account by introducing the parameter E, showing by how many times the number of fragments of the DNA molecule increases per reaction cycle. In most cases, $1 < E < 2$.

Let us take the logarithms in (1):

$$\log I_n = \log \alpha + \log N_0 + n \log E. \tag{2}$$

The threshold value of the number of molecules N_t, which is programmed, and the threshold fluorescence I_t correspond to the threshold value of the number of cycles C_t when the fluorescence signal is equal to the threshold (background). For the threshold, formula (2) takes on the form:

$$\log I_t = \log \alpha + \log N_0 + C_t \log E. \tag{3}$$

Let us rewrite (3) in the form

$$C_t = a - b \log N_0, \tag{4}$$

where

$$a = (\log I_t - \log \alpha)/\log E, \quad b = 1/\log E. \tag{5}$$

The graph (Fig. 1) of the fluorescence signal I vs. the number of amplification cycles (the reaction product accumulation curve) shows three stages of the reaction: initiation (signal baseline), exponential (product accumulation), and a saturation stage, when the graph goes to saturation (a "plateau") for various reasons.

TABLE 1

Component	Volume per reaction tube, μl
2x SsoAdvanced™ SYBR® Green Supermix	12.5
Primer F (20 pcmol/μl)	0.25
Primer R (20 pcmol/μl)	0.25
Deionized water	up to 25
DNA sample	5

Note that for the working exponential (linear) section of this curve, the coefficients of the linear dependence (4) remain constant, assuming that the constant α also does not change for different initial concentrations of the molecules. This means that if we use DNA from a reference material as the DNA template, then for its initial concentration N_{0r} we can write

$$y = a - bx, \tag{6}$$

where $y = C_{tr}$ is the value of the threshold cycle for the initial concentration of the reference material; $x = \log N_{0r}$.

Formula (6) is a mathematical expression of the calibration curve. Thus the calibration curve is linear only in the working concentration range, corresponding to the exponential (cumulative) stage of the polymerase reaction. When the number of cycles goes past this stage, the linearity of the calibration curve breaks down and it is no longer of interest for obtaining quantitative information.

In experimental determination of the calibration curve for different initial concentrations of the reference material, there is a series of values for the threshold cycles C_{tr}. The reaction product accumulation curves as a function of initial concentrations are a source of information for plotting the calibration curves (see Fig. 1). The family of curves shown is the display printout for the CFX96 measurement module of the C1000 Touch thermal cycler [5]. It is accompanied by a printout of tables which are the test protocol (not presented due to its large size), used for necessary quantitative estimates, in particular for determining the dependence of the values of the threshold cycles C_{tr} on $\log N_{0r}$. This dependence (the calibration curve) will be linear in the working concentration range. Its parameters are determined by the least-squares method using the built-in PCR amplifier program.

Formula (5) allows us, from measurement of the slope of the calibration curve, to estimate the PCR efficiency making the assumptions indicated above.

Likewise generally for the methodology for plotting the calibration curve of a real-time PCR amplifier, used in its tests for purposes of approval of type both for measuring the unknown concentration and for determining the uncertainties in measurements of this concentration.

For traceability of the measurement results for the concentration of DNA molecules and plotting the calibration curve, we used a state reference material GSO 9931–2011 developed by the Bioengineering Center together with the VNIIMIS, a fragment of plasmid *p*UC18 of length 271 nucleotides with DNA weight concentration 49 ng/μl and relative uncertainty limits ±11.0% for confidence level $P = 0.95$ [3].

The tests were performed in the laboratory facilities of the Bioengineering Center using a program developed by specialists at VNIIMS together with colleagues at the Center. The test results are presented with the corresponding protocols. Based on these protocols, we prepared documentation for issuing a certificate of approval of type for the CFX96 measurement modules of the C1000 Touch thermal cyclers and for their entry into the State Registry of Measuring Instruments.

Before beginning the tests, we prepared a reaction mixture including the components for running the amplification reaction. All the components of the reaction mixture were mixed in a single reaction tube, after which it was poured into 96-well plates in 20 μl portions. Then 5 μl of DNA was added to the wells. For the tests, we used ten dilutions of the refer-

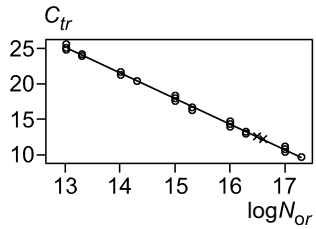

Fig. 2. Calibration curve for CFX96 measurement modules included in C1000 Touch thermal cyclers for nucleic acid amplification.

ence material with known molar concentration plus one analyte sample. For the negative control, instead of the DNA template we added the same volume of deionized water into the wells. All the measurements were repeated five times. In order to prepare the analyte sample with concentration n_x, the reference material was diluted to a concentration within the working range. The components of the reaction mixture are presented in Table 1.

In setting up the plate, we used the following components: prepared dilutions of the reference material with known molar concentration; the analyte sample, the weight concentration of which was previously measured using a G1600A capillary electrophoresis system; the negative control.

For the negative control, we used the reaction mixture to which, instead of the DNA template, we added the same volume of deionized water.

The working range of the measurements was determined with the help of a calibration curve plotted by the programming module of the PCR amplifier, using the results of running PCR on dilutions of the reference material. The calibration curve, taken using the reference material GSO 9931–2011, is shown in Fig. 2, where the number of cycles corresponding to the unknown concentration n_x is marked with a cross. On the graph, plotted along the y-axis is the number of threshold cycles C_{tr} with which the cumulative PCR stage began, and plotted along the x-axis is the logarithm of the ten working concentrations of the reference material.

The sequence of operations in the tests (recommended by the reagent manufacturer) is as follows:

- step 1 (denaturation) at 95°C for 30 sec;
- step 2 (annealing) at 95°C for 5 sec;
- step 3 (elongation) at 60°C for 20 sec;
- go to step 2, consisting of 35–40 repetitions.

The tests were run four times, as represented in the four test protocols indicated above. In order to determine the metrological characteristics, we used the measurement results with the greatest values of the mean square deviation. We found that the working range of the measurements is 10^{13}–10^{17} molecules per µl of solution.

The uncertainty limits for measurement of the molar concentration were calculated using the standard procedure specified in [6]. We took into account the fact that the major contribution to the residual systematic uncertainty comes from the uncertainty in measurement of the concentration of the reference material, which is ±11% for confidence level $P = 0.95$. The initial weight concentration of the reference material was 49 ng/µl. The following formula was used to convert the weight concentration (ng/µl) to the concentration measured as the number of molecules per µl of solution:

$$N = CN_A/(XM)\cdot 10^6, \tag{7}$$

where $C = 49\cdot 10^{-9}$ g/µl is the weight concentration of the reference material; $N_A = 6.022\cdot 10^{23}$ mol^{-1} is Avogadro's number; $X = 271$ nucleotides is the length of the reference material; and $M = 660$ g/mol is the average molecular weight.

In making the measurements, the original reference material was diluted to a concentration of 7 ng/µl, which corresponds to $2.5\cdot 10^{18}$ molecules per µl of solution. For the analysis, we used a sample with concentration $4\cdot 10^{16}$ molecules per µl of solution. The concentration of the analyte sample, obtained with the help of the calibration curve based on four indepen-

dent measurements, proved to be equal to $3.91 \cdot 10^{16}$ molecules per μl of solution, while the relative uncertainty in its measurement was ±34%.

Thus as a result of tests with the aim of approval of type for CFX96 measurement modules included in C1000 Touch thermal cyclers for nucleic acid amplification, we determined their metrological characteristics and have shown that they can be used to measure the concentration of DNA molecules. Development of a metrological support system for real-time PCR amplifiers is not complete with these tests. For a complete system, development and certification are needed for the corresponding measurement procedure, preferably a reference procedure, one of the basic tasks of which will be reducing the uncertainty in the reference materials used as a result of improving the technology by which they are prepared and as a result of improving the procedure for determination of their metrological characteristics.

REFERENCES

1. D. V. Rebrikov, *Real-Time PCR* [in Russian], 2nd ed., BINOM. Lab. Znanii, Moscow (2009).
2. S. S. Golubev et al., "Metrological support for fast medical diagnosis systems," *Metrologiya*, No. 10, 5–13 (2012).
3. S. S. Golubev et al., "Selection of a reference sequence for determining nucleotide sequences in DNA molecules," *Izmer. Tekhn.*, No. 12, 45–47 (2011); *Measur. Techn.*, **54**, No. 12, 1408–1411 (2011).
4. S. S. Golubev et al., "Metrological support for DNA sequencing," *Izmer. Tekhn.*, No. 3, 64–72 (2012); *Measur. Techn.*, **55**, No. 12, 345–350 (2012).
5. BIO-RAD, www.bio-rad.com/ru-ru/product/cfx96-touch-real-time-pcr-detection-system, accessed July 10, 2013.
6. GOST R 8.736–2011, *GSI. Multiple Direct Measurements. Methods for Processing Measurement Results. Basic Provisions*.