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The principle and advantages of Real-time PCR

The article is devoted to the modern methods of molecular diagnostics — polymerase chain reaction in real time. The components, stages of this reaction, the amplification products visualization techniques and its advantages are also described. The main components of the Real-time PCR are DNA polymerase enzyme, primers, DNA template, fluorescent dye, nucleotides. The advantages of Real-time PCR include direct determination of the presence of pathogens, a high specificity, high sensitivity, universality of the various procedures to identify DNA and RNA, high speed of analysis, the possibility of the diagnostics of acute and latent infections, the possibility of pre-clinical and retrospective diagnostics, the ability to conduct analysis with minimal sample, the possibility of simultaneous diagnosis of several pathogens or abnormal genes in one, the possibility of research results examining; exclusion of the possibility to the personnel infection. The information about additives and enhancing agents can be included in PCR amplifications to increase yield, specificity and consistency are also given in the article.

Key words: Real-time PCR, amplification, nucleic acids, SYBR Green dye, primer, probe, DNA polymerase, melting curve.

Polymerase chain reaction (PCR) was discovered by Kary B.Mullis in 1983, for which he was awarded the Nobel Prize in 1993 [1].

PCR is often described as the method by which scientists can find a needle in a haystack, and then build a stack of these needles. «Needle» is a tiny fragment of genetic material and the PCR not only accurately detect the fragment, and then, using a natural property of DNA replication (reproduction), it makes a copy of itself [2].

The PCR-based method is a unique characteristic of both DNA and RNA, the property of nucleic acids — the ability of self-reproduction, which is reproduced artificially *in vitro*; it is synthesized only when strictly specific nucleic acid fragments presents [3].

The following components are required for the implementation of PCR:

– a mixture of primers,
– deoxyribonucleotides
– a thermostable DNA polymerase (an enzyme of thermophilic bacteria *Termus aquaticus*),
– matrix — the test sample nucleic acid is isolated from the biological material in the sample preparation step,
– Mg^{2+} ions are required for polymerase;
– a buffer solution which provides the necessary reaction conditions — pH, ionic strength of the solution. It contains salts, bovine serum albumin [4–6].

The above reaction mixture was subjected to repeated cycles of heating — cooling certain number of times: heating for denaturation and cooling for nucleic acid hybridization or annealing of the primers to synthesize new nucleic acids [4].

The PCR method has a number of advantages compared to many other types of laboratory diagnostic techniques:

– direct determination of the presence of pathogens;
– a high specificity (about 100 %);
– high sensitivity (detection of a few hundred copies in the sample);
– versatility of the various procedures to identify DNA and RNA [7];
– high speed of analysis;
– the possibility of the diagnostics of acute and latent infections;
– the possibility of pre-clinical and retrospective diagnostics;
– the ability to conduct analysis with minimal sample under study;

– the possibility of simultaneous diagnosis of several pathogens or abnormal genes in one;
– the possibility of research results examining (electrophoresis photos and reports on the Real-Time PCR results are stored in a computer database);
– exclusion of the infection possibility to the personnel who is carrying out the PCR process because material is disinfected by lyses and high temperature [8, 9].

Despite of the advantages PCR method has drawbacks and which, however, are offset by the improvement of analysis methods.

Since the sensitivity of PCR can reach mathematically possible limit (1 copy of template DNA), there is a high risk of false-positive results due to transfer through the objects and agents of both the template DNA (less often), and the amplicons (very often) produced in large quantities in many test tubes during daily operations. Therefore special planning requirements and mode of operation laboratory PCR are developed [10].

Factors causes of false-positive results are the following:
– Cross-contamination from sample to sample (in the processing of clinical samples or digging up of the reaction mixture), which leads to sporadic appearance of false-positive results;
– Contamination with recombinant plasmids containing the cloned gene sequence to be detected;
– Contamination of amplification products (amplicons) are the most frequent cause of false-positive results, as in the process of PCR diagnostic amplicons accumulate in large quantities and is very easy to carry with aerosols and through the instruments, hence, detection of PCR products should be conducted in an isolated room by employee which is not generating processing of clinical samples and prepares reagents for PCR [11].

One of the most effective ways to combat contamination caused by the hit of the amplicons in the sample, a gradual transition to fluorescent detection methods of amplification products that eliminates the need to have the reaction tube opening and extraction [12].

Advances fluorometric technology, as well as the development of the instrument, which measures the concentration of amplicons directly during the reaction, led to the development of PCR method in real-time [13,14].

Real-time PCR method includes both amplification and detection. It uses the general principles of PCR [15]. There are all the reaction steps described above [16].

It is possible the quantification (measuring directly the number of copies, or copies measurement to the DNA or additional calibration genes) of specific DNA sequences in the sample [17]. This is particularly important for the anaerobic microorganisms, which is difficult in the case of using classical microbiological methods of anaerobic microorganisms cultivating [18–20].

Today, the method of quantitative determination of PCR products directly during amplification (RealTime) becomes one of the most popular methods of gene diagnostics in clinical and scientific research [21].

Real-time PCR using fluorescently labeled oligonucleotide probes for the detection of DNA during its amplification, and allowing a complete analysis of the sample for 20–60 minutes and it is theoretically capable of detecting even one molecule of DNA or RNA in a sample [22].

The most important feature of the method is to synchronize the check and amplification. This makes it possible to evaluate the kinetics of the process, which depends on the initial amount of testing hereditary material. If we compare the kinetics of the reaction in the test and standard samples, it can be concluded about the concentration of the pathogen in the test serum. Usually included software allows carrying out this operation automatically. It was shown that in comparison to other methods of quantitative PCR diagnostic real-time PCR is the most effective and least time-consuming procedure [23].

Conducting of real-time PCR requires cycler with special, distinctive feature of which is the ability to excite and detect fluorescence reflects the accumulation of amplicons on each amplification cycle.

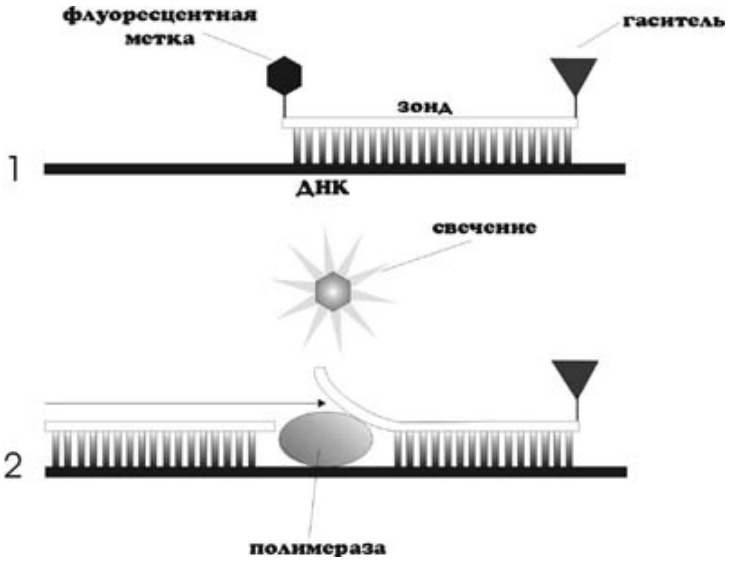
Detection of amplification products

To detect the amplification products in real-time it is using the following most common approach mode:

1. excision of 5' terminal tag (TaqManAssay).

This technique is based on the use of 5'-exonuclease activity of the polymerase. The DNA probes which include a fluorescent tag at the 5'-position and the fluorescence quencher in 3'-position and a phosphate group at the 3'-position was added to the reaction mixture. These probes have a landing site within the amplified region. The absorber absorbs the radiation emitted by a fluorescent label, a phosphate group at the 3'-position blocks polymerase [24].

In the initial stages the fluorescence is weak, because the product is not very much, so it is difficult to distinguish from the background. As the product is accumulating, the signal increases exponentially at first, and then reaches a plateau. The yield on the plateau is due to the lack of a reaction component — primers, nucleotides, tag. If the reaction product has accumulated too much, the limiting factor may be an enzyme, and then the dependence of the amount of the product from the cycle becomes linear. It should be noted that all samples of standard PCR reaction in real time will plateau and reached approximately the same signal level. Therefore, the endpoint will not say anything about the initial amount of the sample. On the other hand, in the exponential phase can be traced to differences in the growth rate of the product. Differences in the initial amount of the molecules affect the number of cycles required for the higher level concepts fluorescence noise [25]. Figure 1 shows a diagram of the PCR method with the use of end tags excision using fluorescence analysis in Real-time PCR [24].



1 — The primers annealing; 2 — Elongation

Figure 1. The principle of real-time PCR [26]

As can be seen from Figure 1, main components of the Real-time PCR are:
– DNA polymerase enzyme
– DNA template
– probe
– quencher
– fluorescent tag
– phosphate group.

2. The use of probes with complementary terminal sequences (molecular beacons)

This method differs from that was described above in that the end of the probe sequences are mutually complementary region, so they collapse and form a hairpin at primer annealing temperature. The inner region of the probes has a nucleotide sequence complementary to the amplified region. Probes which are not joined to a DNA template at annealing of primers remain in «to collapse» condition, so that a fluorescence quenching. Those probes that anneal to the matrix, deployed, and a fluorescent tag and a quencher go in different directions. Thus, the light intensity increases.

3. The use of 2 probes with resonance energy transfer (Light Cycler assay)
– одновременно два зонда
– фосфатная группа
– гаситель
– углеводы
– ДНК-матрица
– процессы переноса энергии и свечения.

This method for detecting of the amplification products accumulation is shown in Figure 2, has high specificity, as the fluorescence increase occurs in binding to a complementary amplicons immediately 2 DNA probes. The principle of the method lies in the transfer of energy from one fluorophore located at the 3` end of the first probe to the second fluorophore, located on 5` end of the second probe, the distance between fluorophores is 1–3 nucleotides. The scheme of the Real-time PCR with the resonance energy transfer is shown in Figure 2. The following components are using:

– two simultaneous probe
– phosphate group
– quencher
– carbohydrates
– DNA template
– energy transport and fluorescence processes.

The scheme of the Real time PCR with the resonance energy transfer is shown in Figure 2.

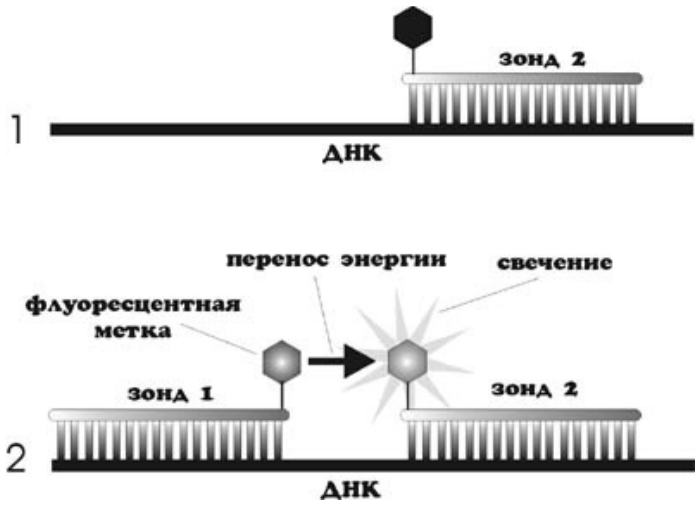


Figure 2. The scheme of the Real-time PCR with the resonance energy transfer [26]

When both probes simultaneously bind with the DNA template the radiation emitted by the first fluorophore is transferred to the second fluorophore, and its radiation is detected by the device. Thus, the specificity of the assay is increased [27].

4. The use of intercalating agents

This detection method is based on the fact that the fluorescence of ethidium bromide and SYBR Green I significantly increase when they are introduced in a double-stranded DNA molecule. Schematic representation of amplification process with using intercalating agents (SYBR Green I) is shown in Figure 3.

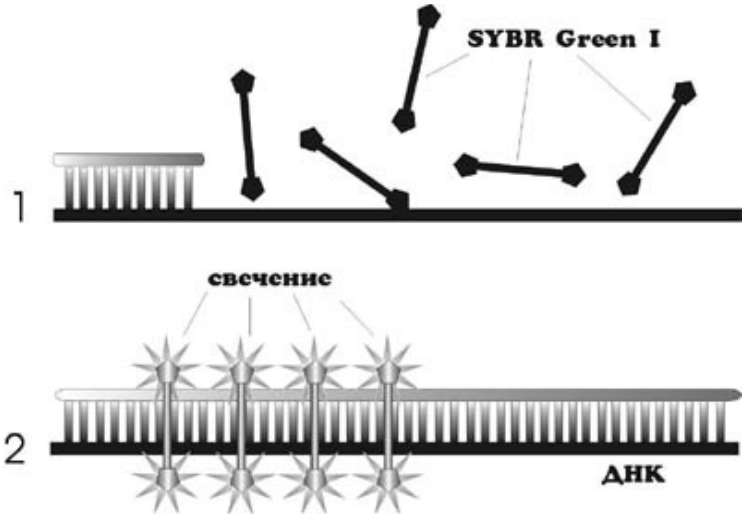


Figure 3. PCR using intercalating agents [26]

Thus, it is possible to observe the accumulation of amplification products [28].

It is important to note that the increase in fluorescence may be due to the accumulation of a specific product, and non-specific (primer-dimers, the downward smearing). To obtain correct results, it is necessary to study obtained additional amplicons by constructing so-called «melting curve».

Melting curves

To construct melting curve after the PCR reaction mixture is heated and continuously measure fluorescence. Upon reaching the melting temperature of amplification product fluorescence decreases dramatically. Figure 4 is a graph showing a melting curve as a function of temperature. The ordinate shows fluorescence.

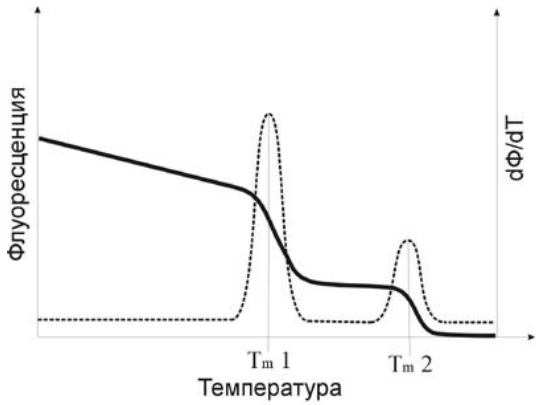


Figure 4. The melting curve [27]

Each sharp decrease in fluorescence on the graph corresponds to the number of bands obtained on electrophoresis, i.e. the number of different types of amplicons. To facilitate the handling of the information received to carry out differential melting curve analysis. This method of visualization of the data is much easier to understand and analysis [29].

The use of melting curves is not limited to only detection of amplification products using ethidium bromide and SYBR Green I.When melting curves are used in systems with a DNA probe (Taq-manassay, beacons) it is possible to distinguish point mutations located within the binding regions of the template DNA and the probe. The presence of such mutations can lead to a change of probe melting temperature and a change in the melting curve graph. Use melting curve does not require from thermocycler operator any additional manipulation with tubes and interpretation of the data is automated and formalized [30].

Dyes for Real-time PCR

In modern versions of PCR in real time sufficiently long time use multiple fluorescent probes labeled with different fluorescent dyes — the so-called «multiplex» variants real-time PCR (multiplexReal-TimePCR). This allows the detection in a single tube multiple PCR products. Which is very convenient in the case, for example, determining the level of gene expression, when it is possible to take two probes with different fluorescent dyes to determine the ratio of the gene expression for «housekeeping» gene, in the same reaction mixture for the same sample. In many modern devices for RT PCR detection of multiple variants of fluorescent dyes are provided simultaneously. For detection of the fluorescent signal of each dye comes within a certain range (channel) for it. This range is selected so as to detect the signal of only one dye, nor get into the neighboring region.

Channels are usually called by the name of the dye, the maximum of which they are detected. To date, five channels are well known:

1. Channel FAM / SybrGreen
2. Channel JOE / HEX
3. Channel TAMRA / Cy3
4. The channel ROX / SuperROX
5. Channel Cy5

The most common options of multiplex Real-Time PCR: two channels — FAM and HEX, on three channels — FAM, HEX and Cy5, on four channels — FAM, HEX, ROX and Cy5 [31].

Figure 5 shows the fluorescence spectra of four dyes: FAM, HEX, ROX, and Cy5. As can be seen, by setting the device on detection of the fluorescence signal at the maximum of each dye, the other dyes in this peak will have very low fluorescence and can be neglected. Thus, in this case, we can get four independent channels for use in multiplex Real-Time PCR.

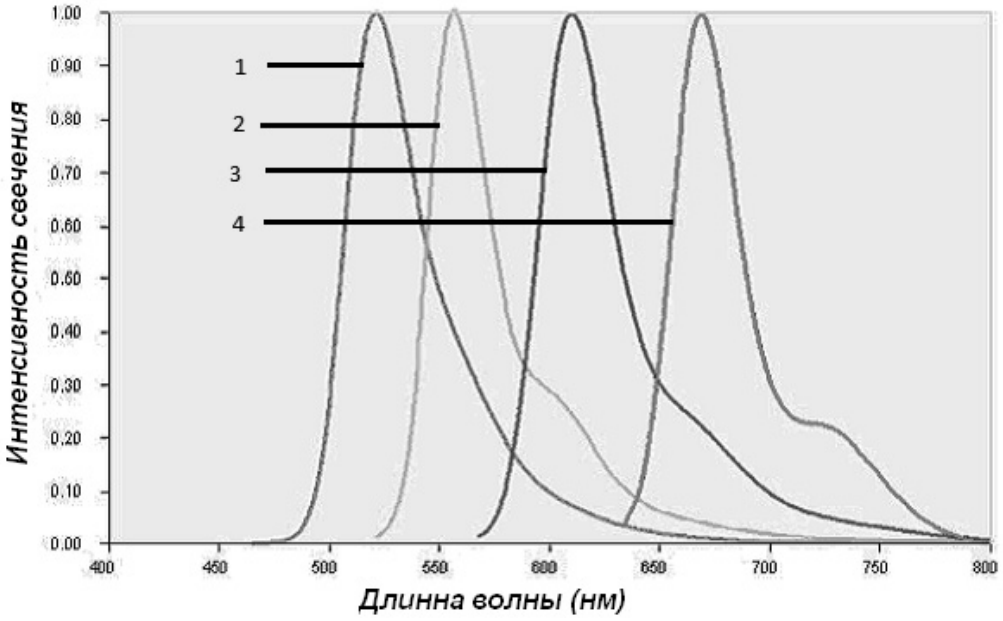


Figure 5. Fluorescence spectra for the four basic dyes: 1 FAM, 2 HEX, 3 ROX and 4 Cy5

It is not necessary that the name of the channel coincides with the name of the dye, the main thing to use dye spectrally matched channel response. For example, the channel can use probes JOE dye HEX or VIC. Select a suitable dye for use in PCR in real time is difficult, as it is necessary that it have good spectral properties, i.e. fluoresce in the desired range of its channel, and does not fluoresce in adjacent channels. It is also very important is its chemical and photostability and the possibility of use in the synthesis of oligonucleotides [31].

Absorbers for Real-time PCR

Today, there are already quite a few different quenchers, which are used for Real-time PCR. The main objective of such a quencher to do the initial (background) value of fluorescence of the probe as little as possible, in order to then as a result of PCR, the probe «broke» the best way possible. For this purpose extinguisher should have a substantial absorption capacity (molar extinction) in the range of fluorescence corresponding fluorescent dye it. For Taqman type probe, in which the dye and the quencher molecule are separated in space, effective quenching is achieved by appropriate selection of the quencher fluorescent dye spectrally [32].

PCR enhancers

A variety of additives and enhancing agents can be included in PCR amplifications to increase yield, specificity and consistency. Agents include: dimethyl sulfoxide (DMSO), N, N, N-trimethylglycine (betaine), formamide, glycerol, nonionic detergents, bovine serum albumin, polyethylene glycol and tetramethylammonium chloride. These additives have beneficial effects on some PCR amplifications; however, it is not possible to predict which agents might be useful for a particular target. PCR amplifications specificity was improved by formamide, but not DMSO (1), and reactions in which DMSO was more effective than formamide at increasing yield and specificity. Several agents that facilitate product formation in PCR amplifications are now commercially available. These agents alter the melting characteristics of DNA. Their identities, however, are not revealed by the respective suppliers [33].

Based on all the above facts it can be distinguished advantages of Real-time PCR:

– High sensitivity, specificity and universality;
– Guaranteed absence of contamination (contamination of samples) as well as the registration result of the analysis is carried out directly through the wall of the reaction tube;
– The possibility of simultaneous detection of multiple pathogens in a single test tube (multiplex reactions);
– The ability to analyze point mutations;
– Simplifying the process and reducing the analysis time;
– Reducing the requirements for the organization of PCR laboratory — the ability to accommodate all lab areas in the same room;
– Automation and standardization of the results registration;
– A measure of the initial DNA template;
– Reducing of the analysis time for 2–3 hours;
– Registration of data in electronic format.

Thus, thanks to the savings of production space, reducing the number of personnel and the demand for the quantitative determination of DNA / RNA, overcoming contamination of amplification products, this method in recent years have been used successfully in the largest sanitary-epidemiological, diagnostic and research centers in the developed countries of the world, replacing the PCR in its «classic» format.

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А.А. Коновалова, А.С. Машжан, А.В. Ходков, Г.П. Погосян, А.Г. Жумина, Р.Г. Оганесян

Нағыз уақыттағы полимеразалы тізбекті реакция принципі мен артықшылықтары

Мақала молекулалық диагностиканың заманауи әдісі — нағыз уақыттағы полимеразалы тізбекті реакцияға (ПТР) арналған. Мақалада оның компоненттері, кезеңдері, амплификация өнімдерінің визуализация әдістері және ПТР артықшылықтары қарастырылған. Нағыз уақыттағы ПТР-дың негізгі компоненттері болып ДНК-полимераза ферменті, праймерлер, ДНК-матрица, флуоресцентті боғыш, дезоксирибонуклеотидтер табылады. Осы әдістің артықшылықтарына патогеннің бар болуын тікелей анықтау, арнайлылығы, сезімталдылығы, ДНК мен РНК-ны анықтау іс-шараларының әмбебаптылығы, талдаудың жылдамдылығы, жедел және латентті инфекцияларды анықтау мүмкіндігі, клиникаға дейін және ретроспективті диагностика мүмкіндігі, үлгінің минималды көлемін талдау мен бір үлгіде бір уақытта бірнеше патоген немесе аномалды генді диагностикалау мүмкіндігі, талдау нәтижелерін зерттеу мүмкіндігі, персоналға жұғу мүмкіндігінің болмауы жатады. Сонымен қатар авторлармен реакцияның шығымы, арнайлылығы мен сәйкестілігін арттыру үшін қолдануға болатын қосылыстар мен агенттер туралы мәлімет ұсынылған.

А.А. Коновалова, А.С. Машжан, А.В. Ходков, Г.П. Погосян, А.Г. Жумина, Р.Г. Оганесян

Принцип и преимущества полимеразной цепной реакции в реальном времени

Статья посвящена современному методу молекулярной диагностики — полимеразной цепной реакции (ПЦР) в реальном времени. Описаны компоненты, этапы, методы визуализации продуктов амплификации и ее преимущества. Выделены основные компоненты ПЦР в реальном времени — фермент ДНК-полимераза, праймеры, ДНК-матрица, флуоресцентный краситель, дезоксирибонуклеотиды. Показаны преимущества ПЦР в реальном времени — прямое определение присутствия патогена, высокая специфичность, чувствительность, универсальность процедур определения ДНК и РНК, высокая скорость анализа, а также возможность диагностики острых и латентных инфекций, доклинической и ретроспективной диагностики, возможность анализа при минимальном количестве образца, одновременной диагностики нескольких патогенов или аномальных генов в одном образце, возможность изучения результатов анализа, исключение возможности инфицирования персонала. В статье также представлена информация о соединениях и агентах, которые могут быть использованы для того, чтобы увеличить выход, специфичность и согласованность реакции.

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