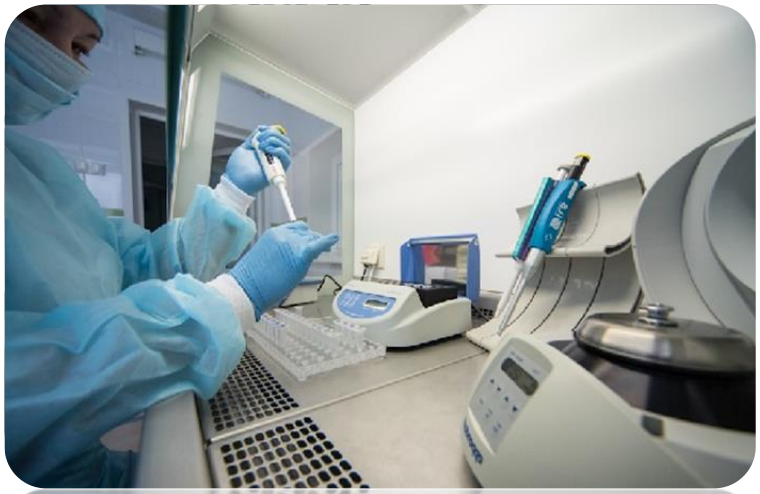
The role of modern molecular genetic methods in conducting scientific research and in practical medicine



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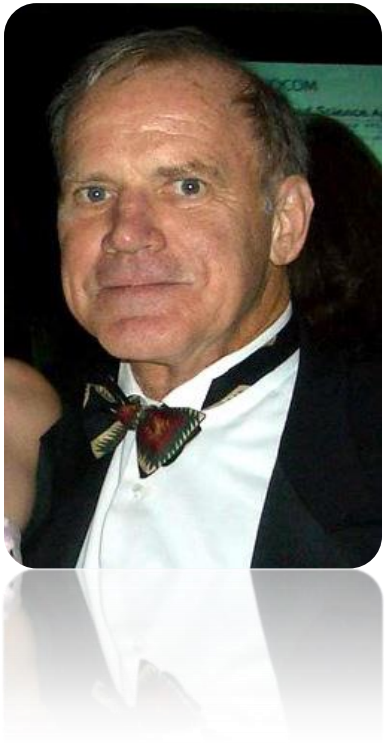
Polymerase chain reaction (PCR) is an experimental method in molecular biology, a way to significantly increase small concentrations of desired DNA fragments in biological material

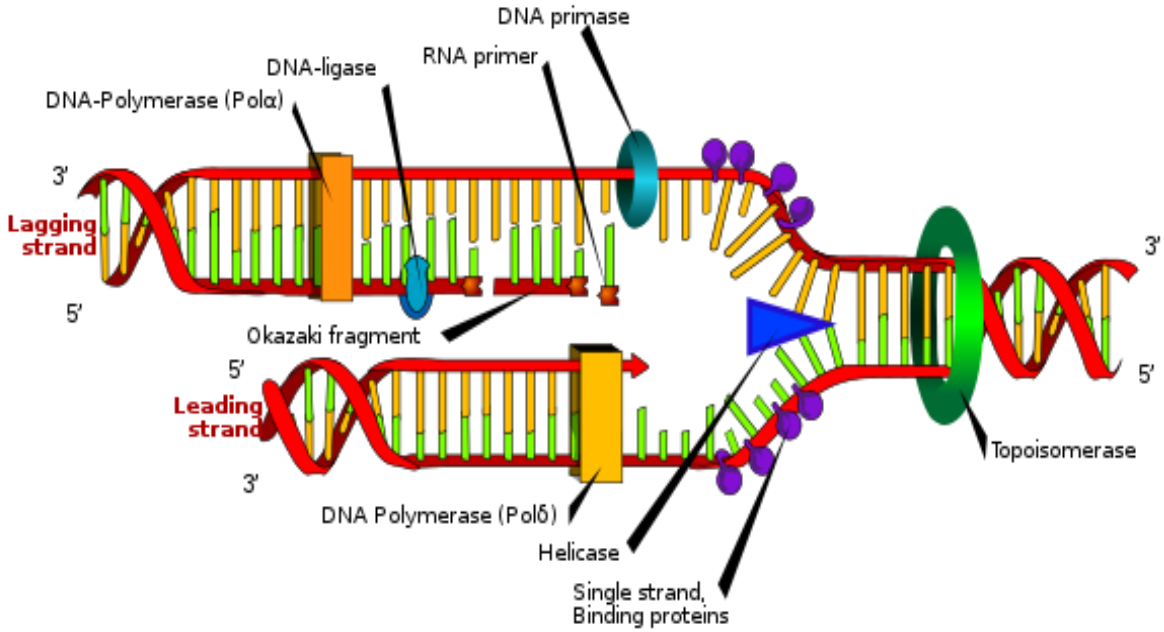
In addition to simply increasing the number of DNA copies - amplification, PCR allows manipulation of genetic material (introduction of mutations, splicing of DNA fragments), and is widely used in biological and medical practice for:

- gene cloning
- introduction of mutations
- selection of new genes
- sequencing
- creation and definition of genetically modified organisms
- diagnosis of hereditary and infectious diseases
- identification of small amounts of DNA
- establishing paternity

- The polymerase chain reaction (PCR) was invented in 1983 by the American scientist Kary Mullis
- For this discovery, he and Michael Smith were awarded the Nobel Prize in Chemistry in 1993
- The principle of the method consists in the **doubling (amplification)** of the DNA region limited by the primers with the help of the DNA polymerase enzyme
- For each subsequent cycle of amplification, the original DNA section and newly synthesized fragments (amplificates) double
- As a result, the number of fragments increases in geometric progression (chain reaction)
- After 30 - 40 cycles, their number exceeds several billion, which makes it possible to detect them



- **Amplification (lat. amplificatio - amplification, increase)** in molecular biology - an increase in the number of DNA copies
- In the cell, amplification occurs as a result of DNA replication, under artificial conditions, an increase in the number of DNA copies is achieved using the polymerase chain reaction
- A **primer -** is a short oligo- or polynucleotide sequence with a free OH group, complementary to single-stranded DNA or RNA
- from its 3'-end, DNA polymerase begins to build a polydeoxyribonucleotide chain



https://www.linkedin.com/pulse/pcr-real-time-polymerase-chain-reaction-basic-jean-essam-pmp-psm

Stages of PCR research

1. Isolation of nucleic acids

At the first stage, all DNA is isolated (for DNA-containing microorganisms) or RNA (for the NASBA method or RNA viruses) from the test material

2. PCR or amplification itself DNA isolated at the first stage is added to a solution containing a mixture of nucleotides, PCR buffer, polymerase and primers

PCR is carried out as follows: the reaction mixture is heated to a temperature of 90-94° C, causing DNA denaturation, then the temperature is lowered to 50-70° C, depending on the nucleotide sequence of the primers, so that annealing occurs in strictly complementary regions, and the mixture is set to a temperature optimal for works of **DNA polymerase**

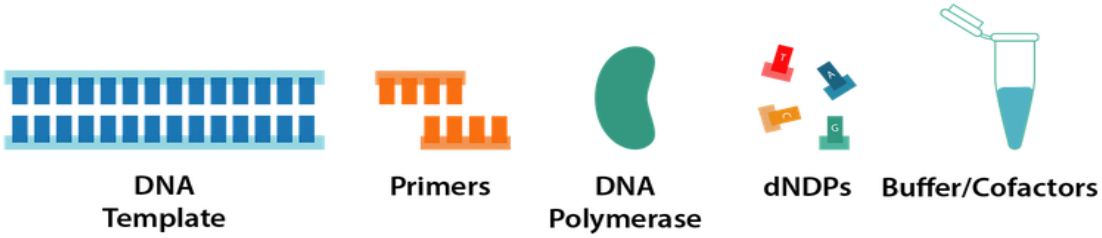
When these cycles are repeated, the number of copies of the DNA section located between the primer landing sites increases in a geometric progression

3. Accounting of results

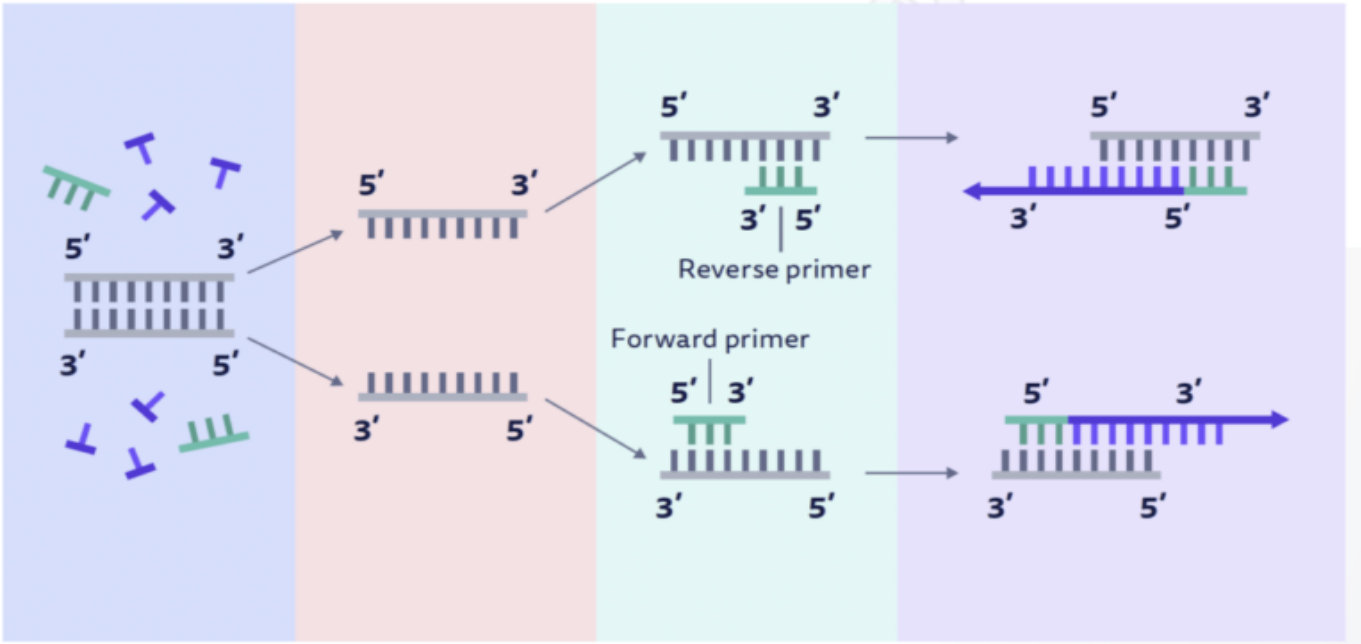
The resulting amplification products (a large number of DNA copies between the primer landing sites) can be detected by gel electrophoresis, when using fluorescently labeled probes, it is possible to record the results of the change in fluorescence relative to the negative control

NASBA (Nucleic Acids Sequence-Based Amplification) - unlike conventional PCR, the target for NASBA (Nucleic Acids Sequence-Based Amplification) is the RNA molecules of the ribosomes of microorganisms, which provides a number of advantages

Components of the reaction mixture



- A DNA template containing the DNA region to be amplified
- Two primers complementary to opposite ends of different strands of the required DNA fragment
- Thermostable DNA polymerase is an enzyme that catalyzes the DNA polymerization reaction
- Polymerase for use in PCR must retain activity at high temperature for a long time, so enzymes isolated from thermophiles are used:
  - Thermus aquaticus (Taq polymerase)
  - Pyrococcus furiosus (Pfu polymerase)
  - Pyrococcus woesei (Pwo polymerase)
- Deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP)
- Mg2+ ions, necessary for polymerase operation
- A buffer solution that provides the necessary reaction conditions - pH, ionic strength of the solution, contains salts, bovine serum albumin

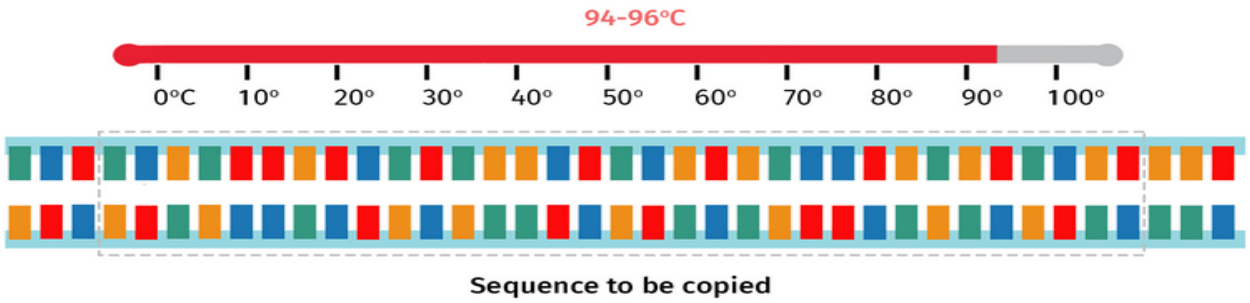


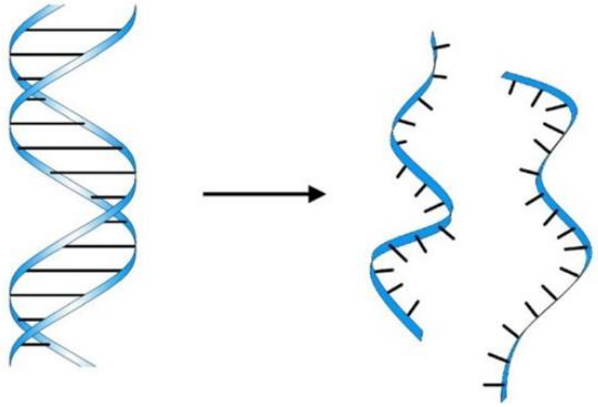
- In order to detect and multiply exactly the DNA sequence that is needed in the available sample, it is necessary to know the nucleotide sequences of its ends in order to create complementary short (18-30 nucleotide) fragments

- primers

- **forward and reverse**

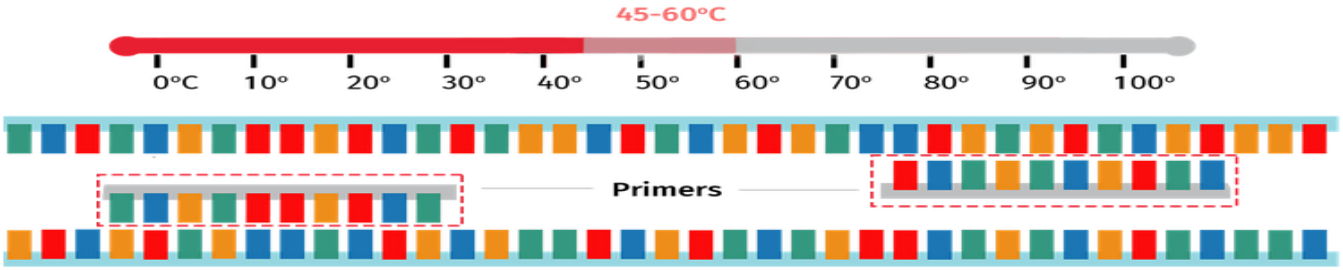
The first stage is denaturation





The double-stranded DNA matrix is heated to 94-96 °C
(or up to 98 °C, if a particularly thermostable polymerase is used)
for 0.5-2 min so that the DNA strands separate

The second stage is annealing

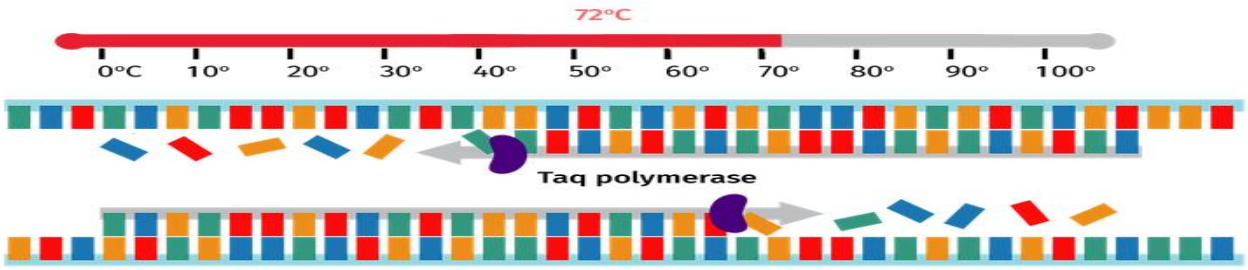


- When the strands have separated, the temperature is lowered to allow the primers to bind to the single-stranded template with complementary DNA regions

This stage is **annealing**

- The annealing temperature is 40-60 ° C, depends on the composition of the primers and is usually chosen 4-5 ° C lower than their melting point
- duration - 0.5-2 min
- Incorrect choice of annealing temperature leads to poor binding of primers to the matrix (at elevated temperature),
- or to binding in the wrong place and the appearance of non-specific products (at low temperature)

The third stage is elongation



Commonly used Taq and Pfu polymerases are most active at 72 °C

- **Polymerase** is an enzyme that completes the second strand of DNA
- To start work, it needs a double-stranded fragment of DNA - in this capacity, the place of interaction of DNA with the primer acts
- The polymerase always completes the chain from the **5' to the 3'** end
- these names are related to the orientation of the deoxyribose sugar in the chain;
- in ordinary double-stranded DNA, the chains are oriented opposite to each other:

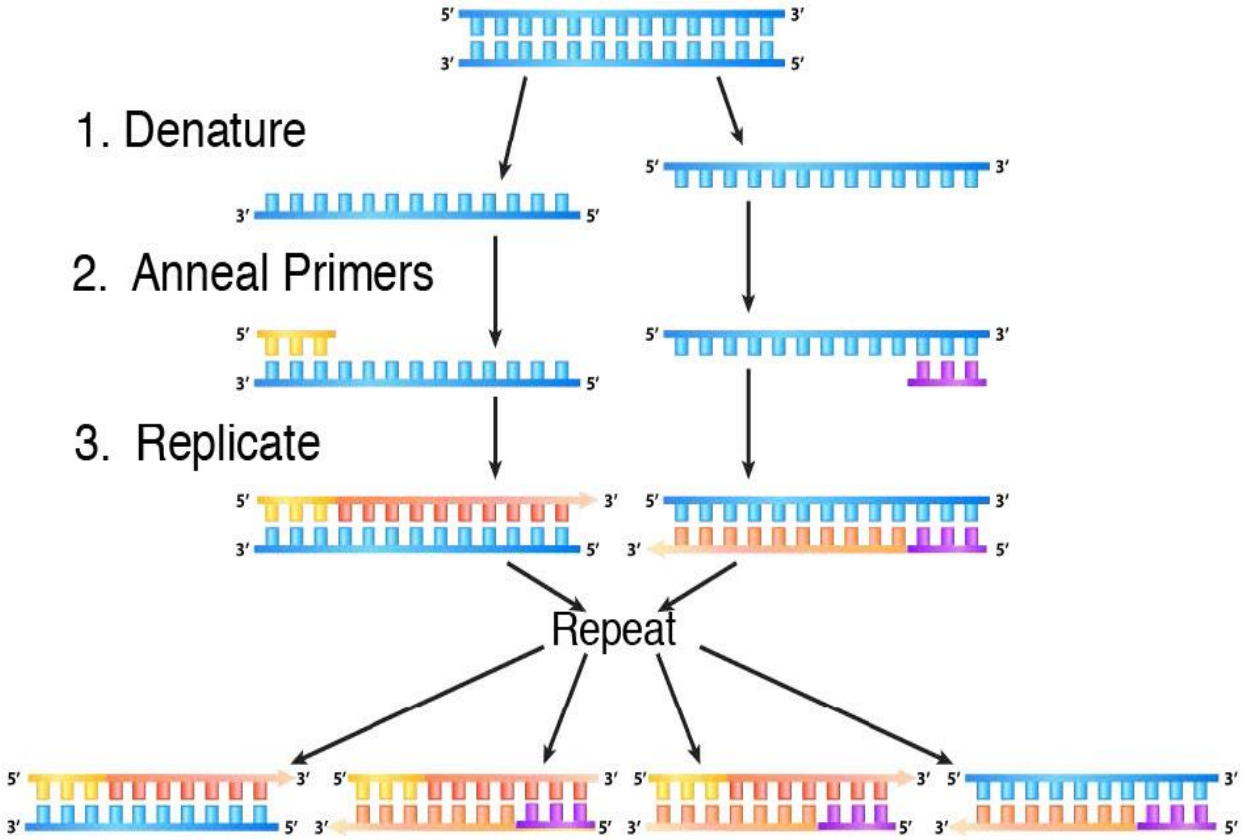
  5'_____3'

  3'_____5‘

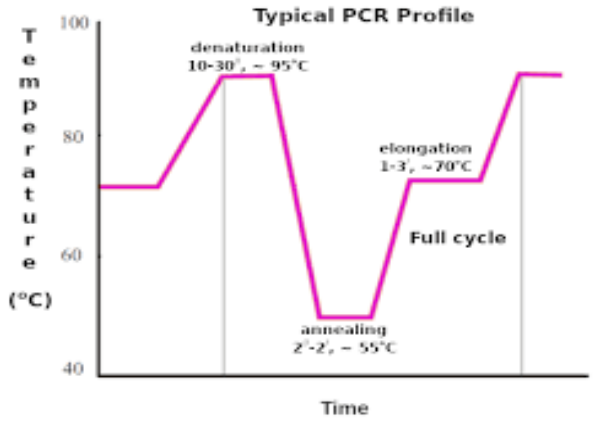
illustrations https://www.snapgene.com/guides/polymerase-chain-reaction

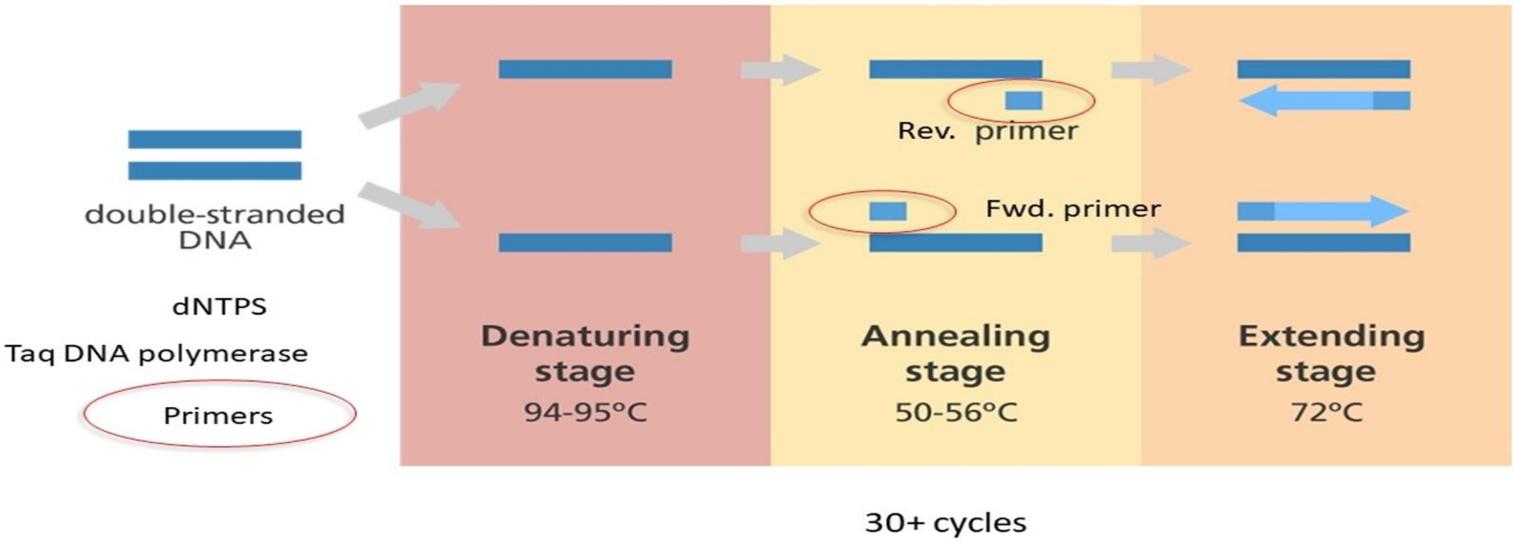
Stages of the polymerase chain reaction

Picture of Alley Kim

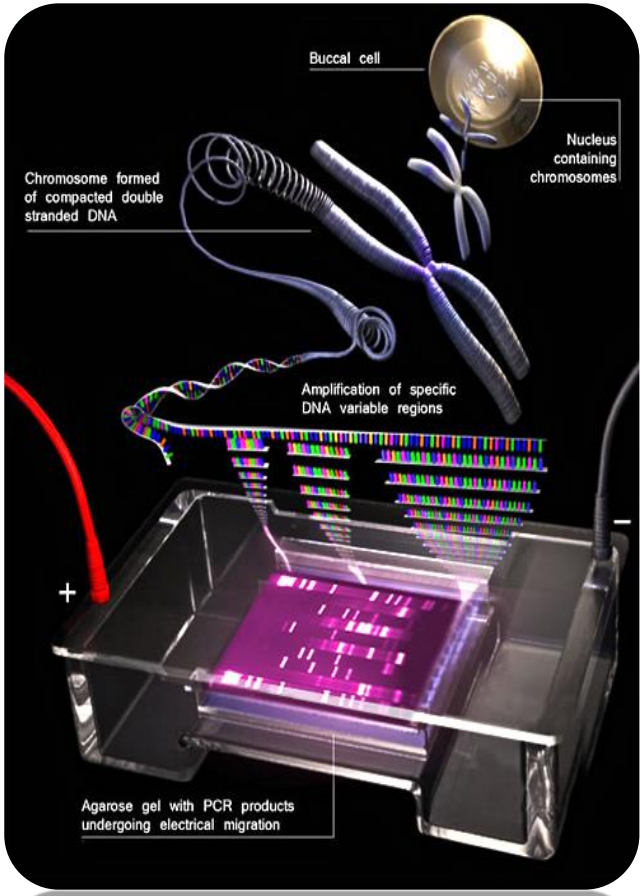


The main element of PCR is a multiple thermal cycle
in which the DNA sample is exposed to three different temperatures

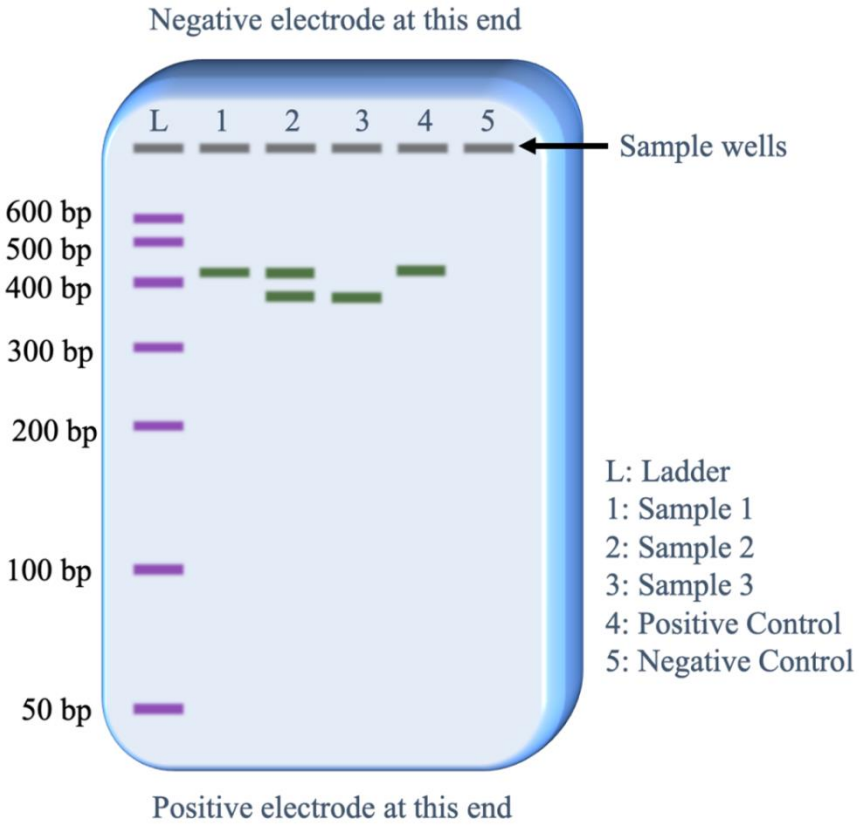




The fourth stage is detection



- At the end of the PCR, we have a solution with a large number of multiplied desired areas, DNA fragments
- The solution is applied to the gel, voltage is applied to the gel
- Over a certain time, fragments of DNA molecules (they have a charge) move in the gel at a distance proportional to their mass
- The gel is placed under ultraviolet light and a mass of identical DNA fragments begins to glow in the hole where they are located
- If the glow is registered, then there was a section corresponding to the primer in the DNA, the required «gene»
- If nothing lights up, a cloudy band without a clear section - the required section is not in the DNA

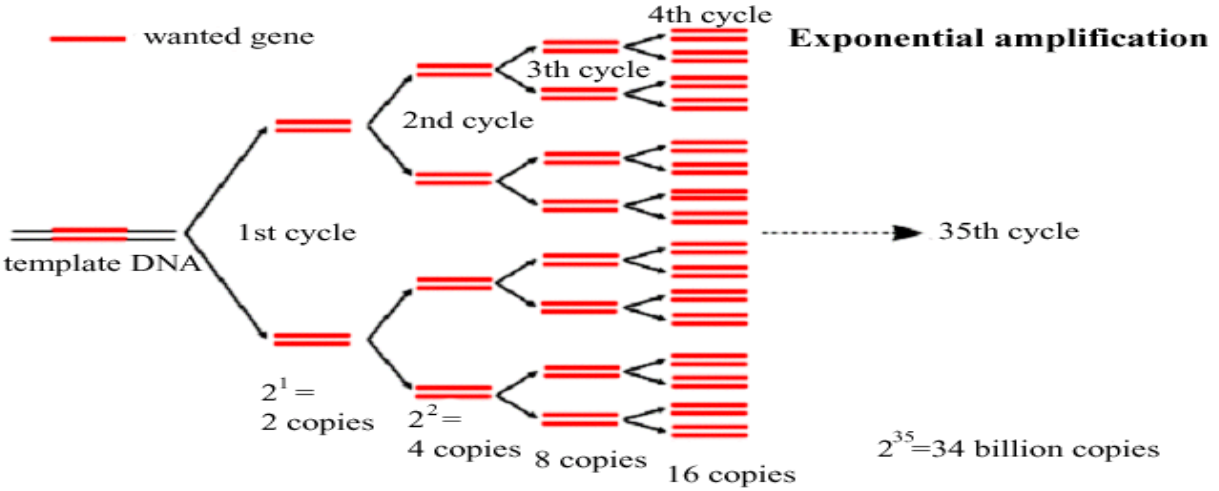


- Image of an electrophoresis gel with six sample wells that were loaded with either a DNA size ladder (well L) or a sample from a PCR run (1-5.)
- The gel was subjected to DNA dye staining

Image by Marjorie Hahnemann

Scheme of duplication of DNA fragments in PCR

(Andy Vierstraete, 2001)



- For the amplification process, it is necessary that the structure of the primers be **identical (complementary)** to the region of the original DNA
- If this is not present (no specific DNA), then there is no DNA duplication
- If the solution does not show a single DNA molecule with a region complementary to the introduced primers, the PCR reaction will not work, even though there are a million other DNA molecules floating in the solution
- This is due to the **high specificity** of the PCR method

Advantages of the PCR method

1. **Versatility.** PCR can be used to determine DNA in any biological samples. Moreover, this applies equally to the DNA of microorganisms and to the DNA of a person
2. **High specificity.** The specificity is determined by the fact that a unique region of the gene, characteristic only for this pathogen, is determined in PCR. To increase specificity, several different genes of the same microbe can be determined. So, for example, the determination of Ureaplasma urealyticum can detect both the 16S-RNA gene and the urease gene. And for the identification of Chlamydia trachomatis, in addition to the determination of chromosomal DNA and cryptic plasmid DNA, it became possible to detect ribosomal RNA (NASBA). This significantly increases the reliability of the study
3. **High sensitivity.** Polymerase chain reaction can detect single copies of DNA. On average, the sensitivity threshold of most modern test systems is from 10 to 100 DNA copies. This greatly exceeds the sensitivity of cultural research methods
4. **Minimum volume** of biological material. The analysis is possible in a minimal sample volume (up to several microliters), which is extremely important in pediatrics, neonatology, neurology, forensic medicine
5. **The possibility** of diagnosing not only acute, but also latent infections. The PCR method is particularly effective for the diagnosis of hard-to-cultivate, non-cultivable and persistent forms of microorganisms that are often encountered in latent and chronic infections

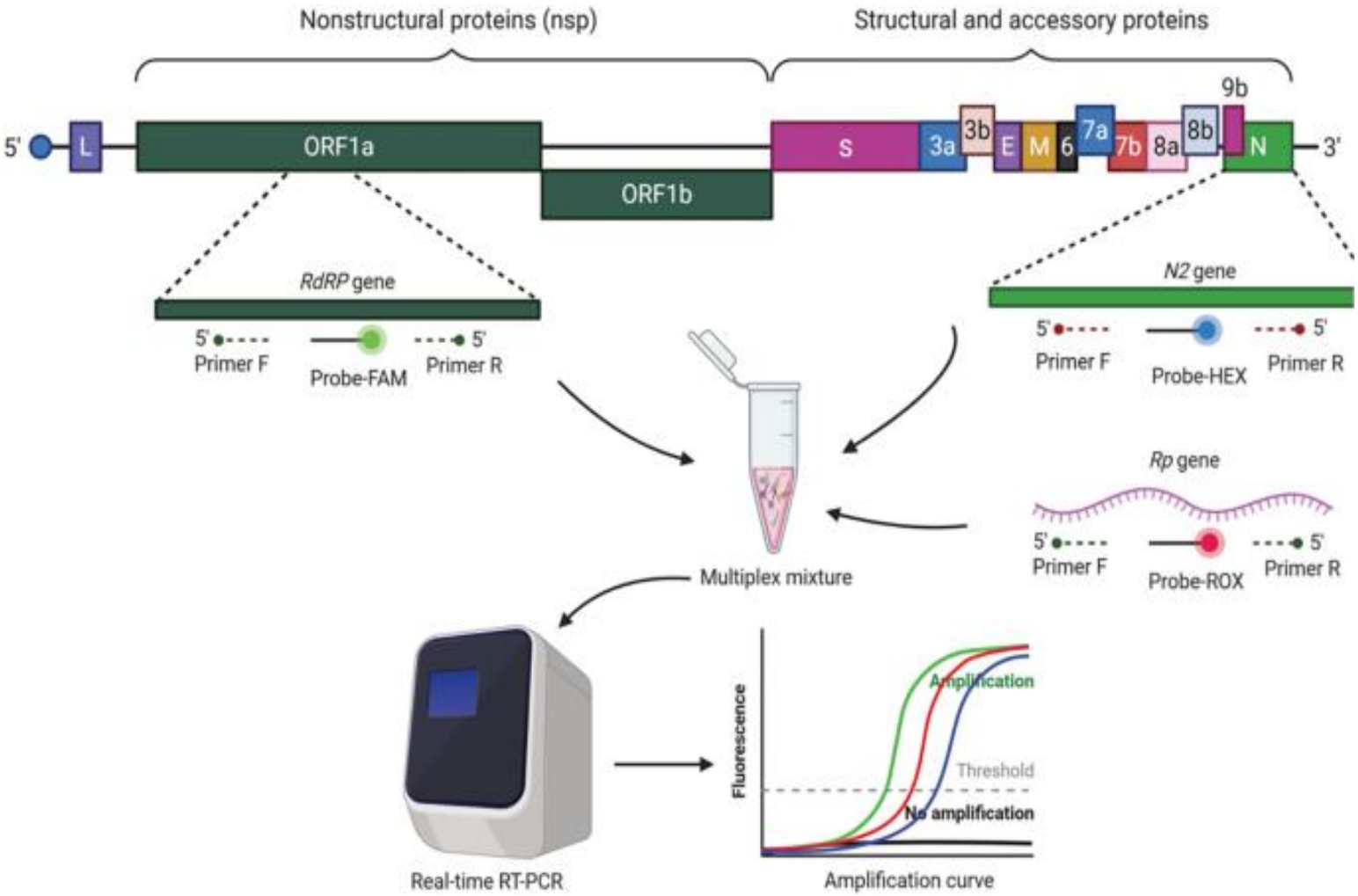
Disadvantages of the PCR method

1. The DNA of both living and dead microorganisms is amplified. This imposes certain requirements when using PCR to control the effectiveness of treatment. In this case, such control should be carried out after a period of time during which the pathogen is completely eliminated. However, the NASBA method detects the RNA of living microorganisms only and avoids these limitations
2. High sensitivity. A number of microorganisms (conditionally - pathogenic flora, UPF) can normally exist in a person in small quantities. Using the PCR method, even the smallest amounts of UPF are determined, even in the absence of pathology. However, this problem was solved with the advent of the method of quantitative determination of DNA (Real-time PCR)
3. Differences when using different test systems. Different regions of the pathogen's genome can be used for amplification. However, in the case of various mutations of microorganisms, a change or loss of genes is possible. This leads to different results when using test systems from different manufacturers

Real-time PCR

- The principle of the PCR method in real time (Real-Time PCR) is based on the detection of amplification products already in the reaction process and monitoring the kinetics of amplicon accumulation
- This means that the calculation of the PCR result (number of amplicons) occurs after each cycle of amplification, and not at the end, as in conventional PCR
- The more specific DNA was in the original sample, the earlier and more the number of specific fragments will increase
- Real-Time PCR allows quantitative assessment of the DNA content in the researched material

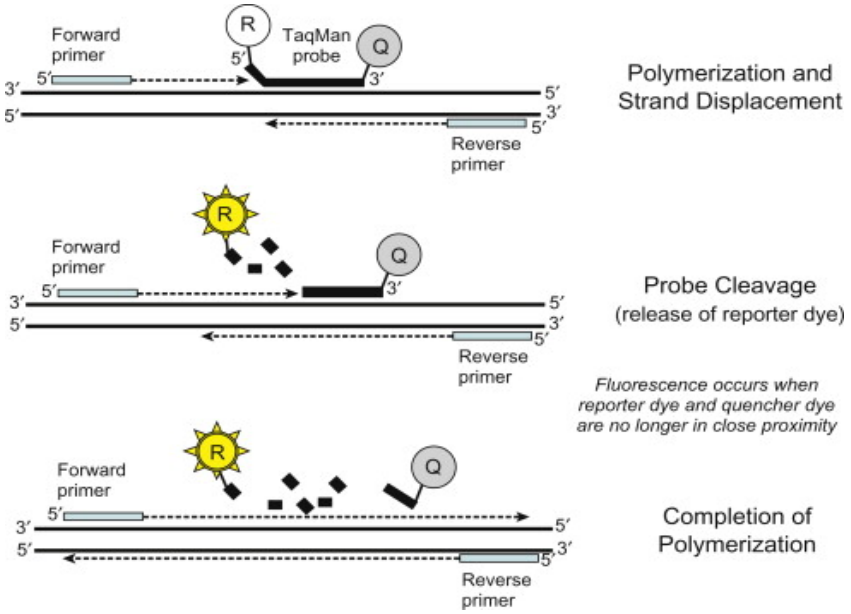
Multiplex real-time PCR



From: Multiplex real-time RT-PCR method for the diagnosis of SARS-CoV-2 by targeting viral *N*, *RdRP* and human *RP* genes

Detection of amplification products

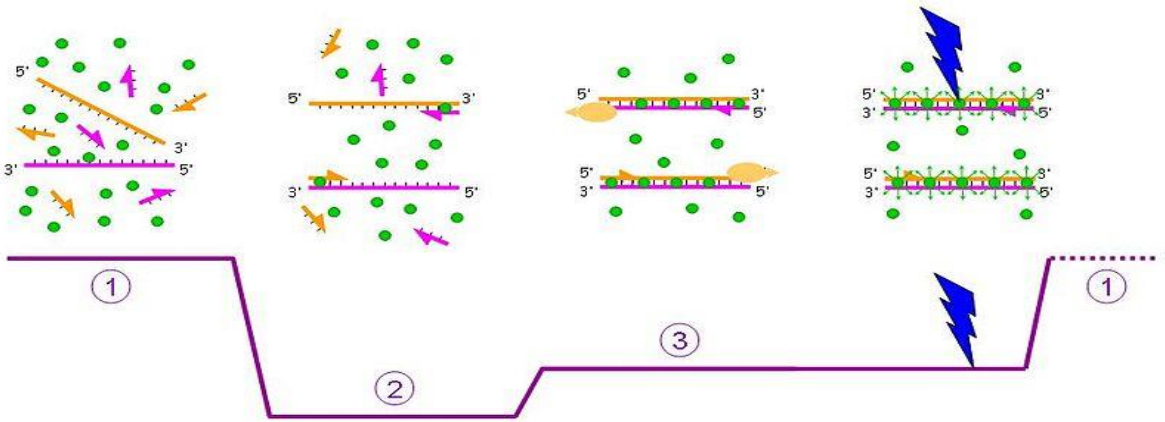
1. Extraction of the 5' end tag (TaqMan Assay). This technique is based on the use of the 5'-exonuclease activity of the polymerase
2. DNA probes are added to the reaction mixture, which include a fluorescent label in the 5'-position and a fluorescence quencher in the 3'-position, as well as a phosphate group in the 3'-position



- These probes have landing sites within the amplified region The quencher absorbs the radiation emitted by the fluorescent label, and the phosphate group in the 3'-position blocks the polymerase
- During PCR, during the primer annealing stage, the DNA probe is attached to the complementary DNA chain, and the more amplification products are formed during PCR, the more probe molecules will bind to the corresponding amplicons
- During the elongation stage, the polymerase synthesizes a complementary DNA chain and, upon reaching the probe, begins to cleave it due to the presence of 5'-exonuclease activity
- In this way, the separation of the fluorescent label and the quencher occurs, which leads to an increase in the detected glow
- It is obvious that the more amplicons that have been generated during PCR at this point in time, the more intense the glow will be

John M. Butler, in Advanced Topics in Forensic DNA Typing: Methodology, 2012

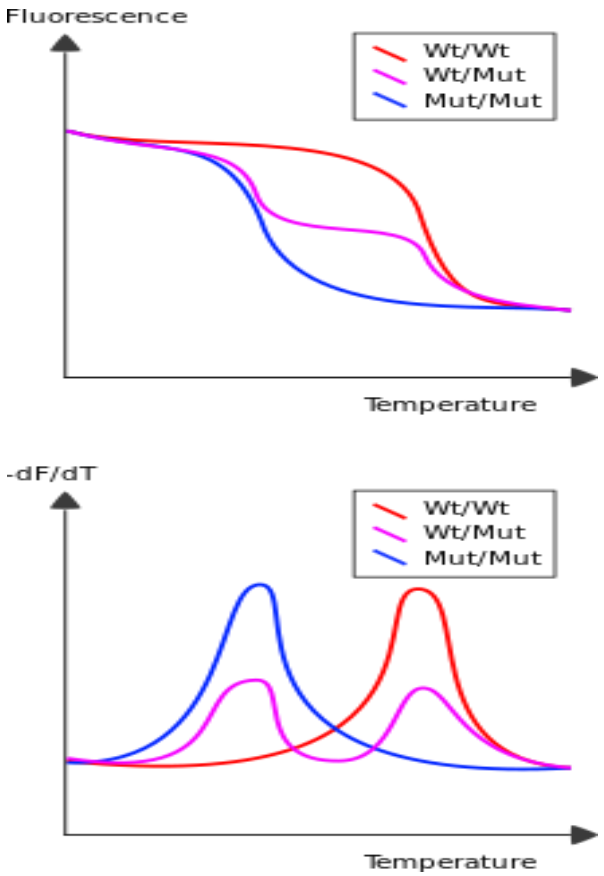
Use of intercalating agents



- This method of detection is based on the fact that the fluorescence of ethidium bromide and SYBR Green I increases significantly when they are introduced into DNA molecules
- In this way, it is possible to observe the accumulation of amplification products. An increase in fluorescence can be associated with both the accumulation of a specific product and a non-specific one - primers-dimers, scrams
- To obtain correct results, additional study of the amplicons obtained using the construction of melting curves is necessary

https://commons.wikimedia.org/wiki/File:PCR_with_SYBR_green.jpg

Melting curves



- After the end of PCR, the reaction mixture is heated and fluorescence is continuously measured
- After reaching the melting temperature of the amplification product, the fluorescence decreases sharply
- Each sharp decrease in fluorescence on the graph corresponds to the number of bands obtained on electrophoresis, that is, different types of amplicons

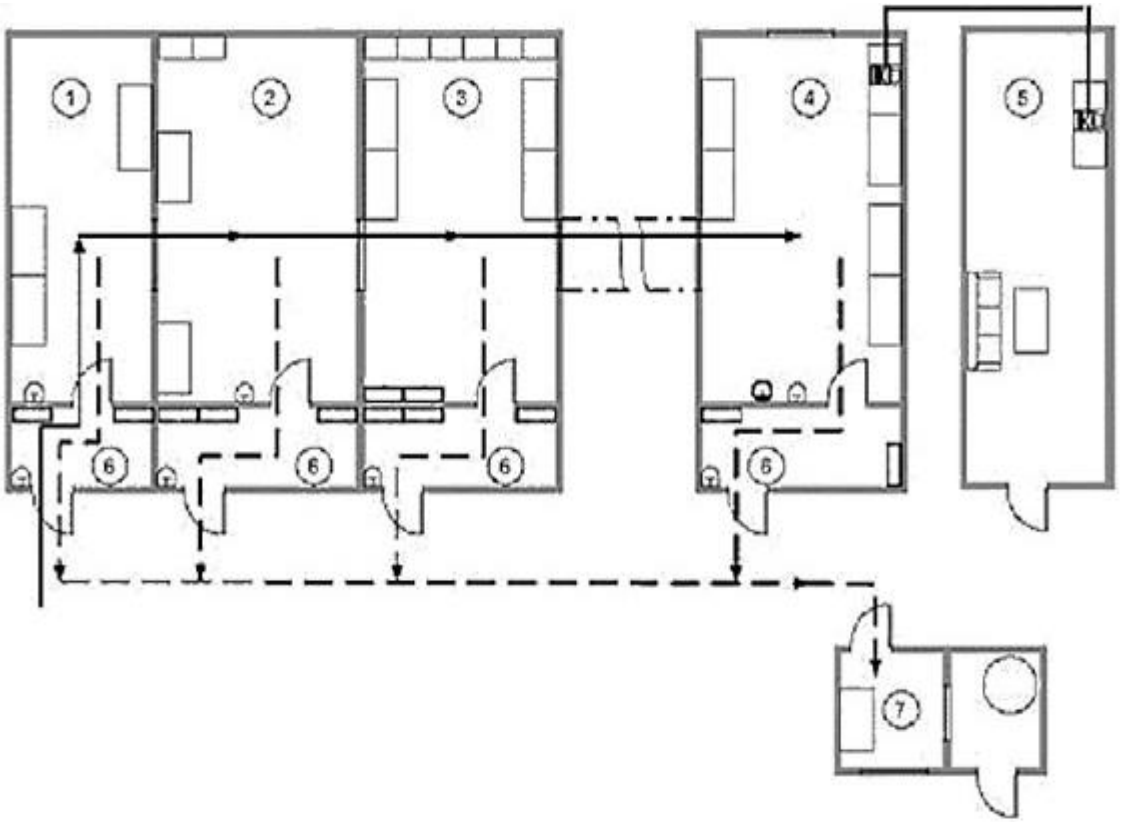
https://en.wikipedia.org/wiki/Melting_curve_analysis

Types of PCR

- 1. «Nested» PCR (Nested PCR) - there is a second pair of primers that amplify a piece of the resulting piece
- 2. «Inverted» PCR (Inverse PCR) - before carrying out PCR, using a series of enzymatic reactions, known fragments are glued to the end of the unknown, so that it can be amplified
- 3. PCR with reverse transcription (Reverse Transcription PCR, RT-PCR) - RNA is taken (a molecule that is an intermediate step between DNA and proteins in a living cell), and DNA is obtained from it with the help of a reverse transcriptase enzyme, from which PCR is already carried out .

  This is convenient, for example, to find out exactly which genes are expressed in this cell
- 4. Asymmetric PCR (Asymmetric PCR) - if amplification products of mainly one of the two DNA chains are required. An unequal number of primers is added
- 5. Quantitative Real-Time PCR (Quantitative Real-Time PCR) - fluorescently labeled reagents are used, and a special device examines the test tube in which the reaction is taking place and reports: "so much product is ready! and now there is twice as much!“
- 6. PCR of long fragments (Long-range PCR) - to amplify a long (more than 10 thousand base pairs) fragment, PCR with two polymerases is used: one of them, Taq polymerase, can synthesize a long chain, and the second, DNA polymerase with 3'5'-exonuclease activity, can correct mistakes made by the first
- 7. Multiplex PCR - add several pairs of primers and simultaneously amplify several fragments

Scheme of the PCR laboratory organization



Equipment

Laminar boxing
Protection class II

Detecting amplifier
(Real-time)

High-speed
centrifuge

Solid state
thermostat

Vortex


Thank You!