Lecturer: Senior Lecturer, Department of Molecular Biology and Genetics, PhD, Smekenov I.T.
Subject: Genetic engineering

(Lecture 1)

Analysis of the main methods used in “Genetic engineering”. Their goals and principles of work

Lecture Goal:
To study the main methods used in genetic engineering, their objectives, principles of operation, and areas of application.

Tasks:

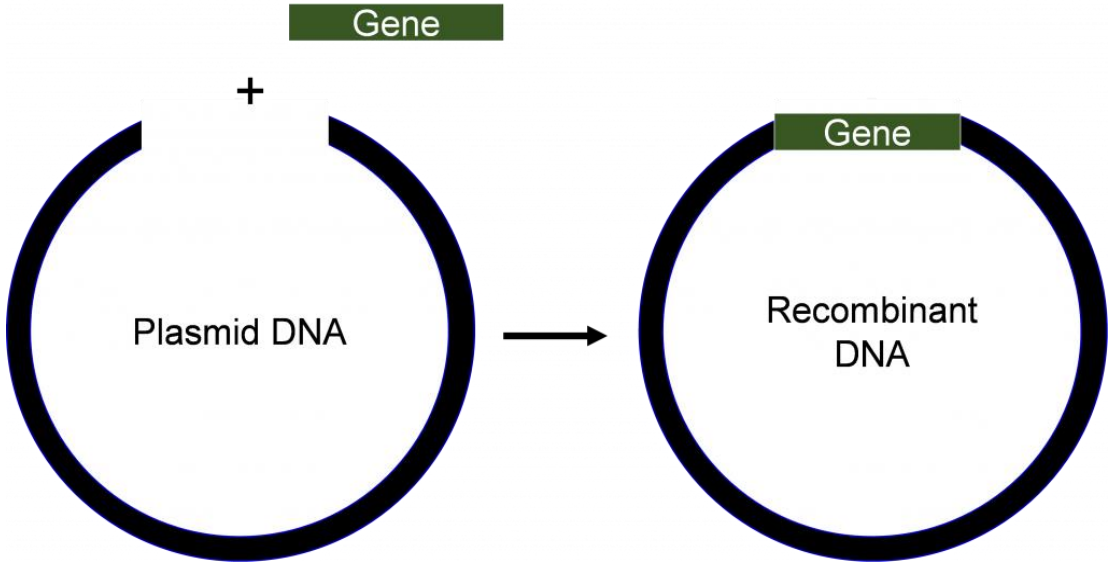
1. Characterize the restriction method, its role in genetic engineering, and its mechanisms.
2. Explore the ligation method, its principles, and how it is used to join DNA fragments.
3. Explain the principles of polymerase chain reaction (PCR) and its application for DNA amplification. Additionally, discuss reverse transcription PCR (RT-PCR) for RNA analysis.

Keywords: *Restriction endonucleases, DNA ligase, Polymerase chain reaction (PCR), Reverse transcription (RT-PCR), DNA amplification, Recombinant DNA, Vector and DNA fragmentation*

What is the "Genetic engineering"?

Genetic engineering, the artificial manipulation, modification, and recombination of DNA or other nucleic acid molecules in order to modify an organism or population of organisms.

- The term *genetic engineering* is generally used to refer to methods of recombinant DNA technology.
- **Recombinant DNA** technologies, developed in the latter half of the twentieth century, include the chemical splicing (recombination) of different strands of DNA generally using either bacteria (such as *Escherichia coli*) or bacteriophages (viruses that infect bacteria, such as λ phage), or by direct microinjection.

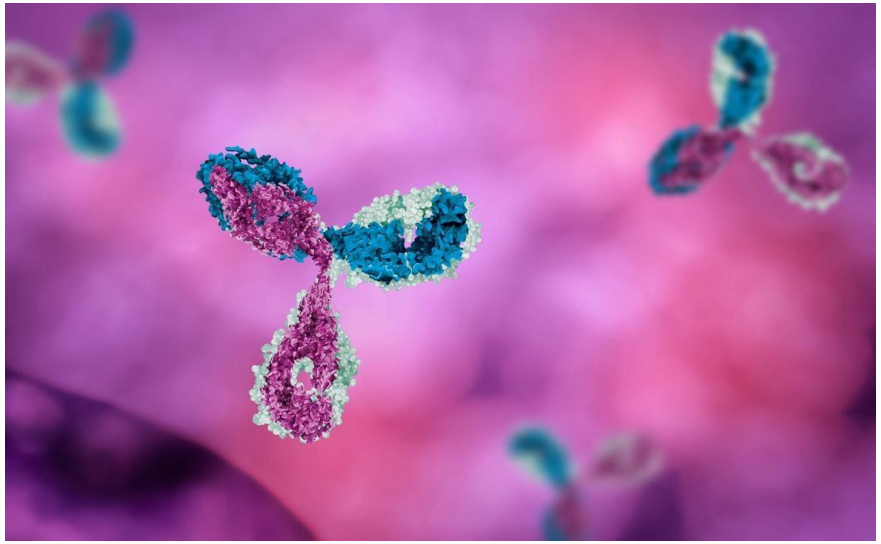


A simple illustration of recombinant DNA technology

https://www.britannica.com/science/genetic-engineering
https://www.sciencedirect.com/topics/neuroscience/genetic-engineering

The techniques employed in genetic engineering have led to the production of:

- **Genetically Modified Crops:** Crops like corn, soybeans, and cotton have been genetically modified to be resistant to pests, herbicides, or harsh environmental conditions. This improves yield and reduces the need for chemical pesticides.
- **Insulin Production:** Genetically engineered bacteria are used to produce human insulin. This was one of the first major breakthroughs in biotechnology and revolutionized the treatment of diabetes.
- **Gene Therapy:** Genetic engineering techniques are used to treat diseases by correcting defective genes. For example, gene therapy is being used to treat genetic disorders like cystic fibrosis and muscular dystrophy.
- **Pharmaceuticals:** Many medicines, including monoclonal antibodies and vaccines, are produced using genetically engineered organisms. These biologics are critical for treating diseases like cancer, autoimmune disorders, and infectious diseases.



- **Transgenic Animals:** Animals such as mice, goats, and pigs are genetically modified for research purposes or to produce substances like proteins and antibodies. For example, goats have been engineered to produce milk that contains therapeutic proteins.

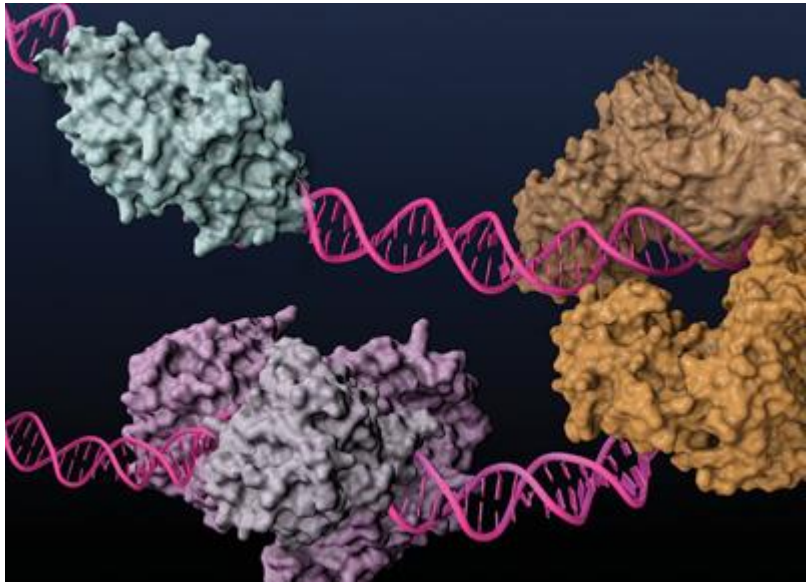
- **Golden Rice:** This genetically engineered rice is enriched with beta-carotene (a precursor of vitamin A) to help combat vitamin A deficiency, which is prevalent in many developing countries.

- **Biofuels:** Certain bacteria and algae have been genetically modified to produce biofuels like ethanol or biodiesel, offering a renewable energy source.

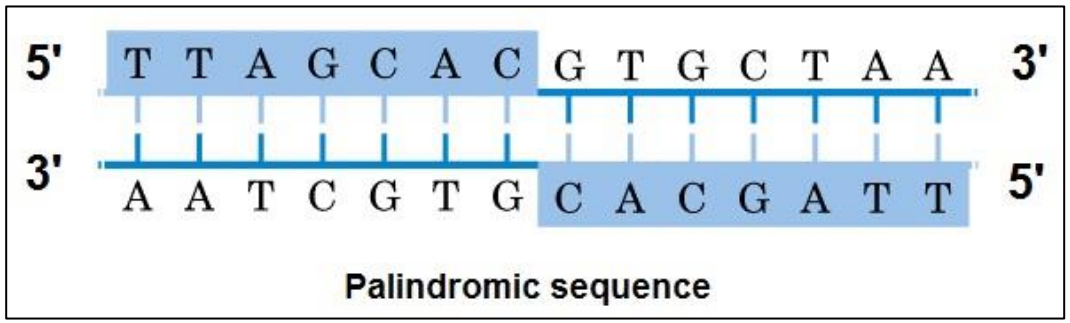
- **Lab-grown Meat:** Through genetic engineering, cells from animals are used to grow muscle tissue in the lab, providing a sustainable alternative to traditional livestock farming.

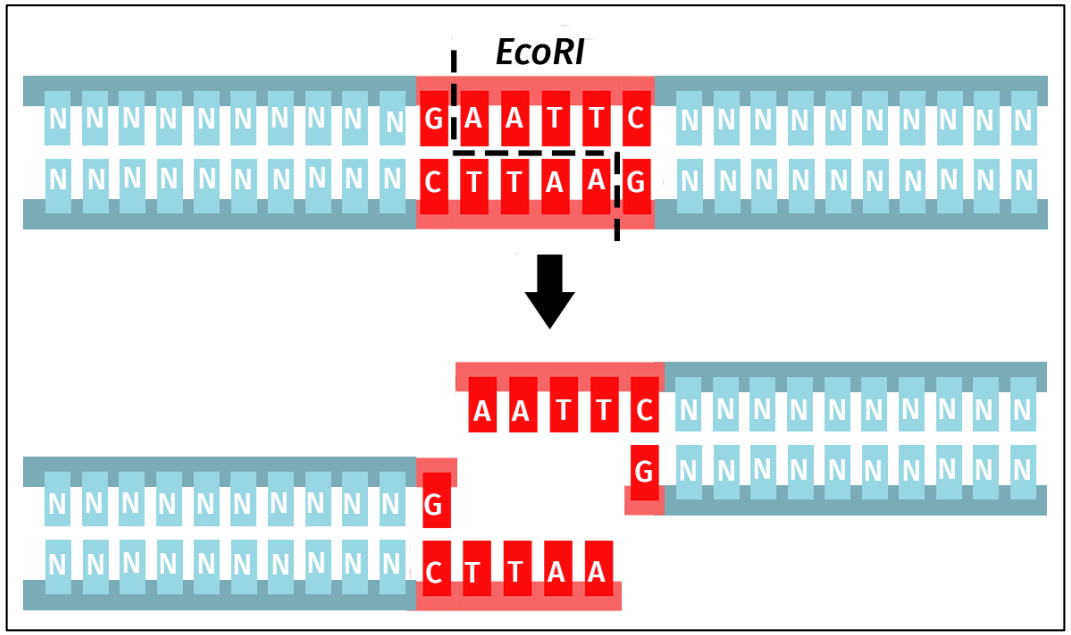
➢ Restriction methods

- ✓ The possibility for *recombinant DNA technology* emerged with the discovery of **restriction enzymes** in 1968 by Swiss microbiologist **Werner Arber**.
- ✓ The following year American microbiologist **Hamilton O. Smith** purified so-called **type II restriction** enzymes, which were found to be essential to genetic engineering for their ability to cleave a specific site within the DNA (as opposed to type I restriction enzymes, which cleave DNA at random sites).
- ✓ Drawing on Smith's work, American molecular biologist **Daniel Nathans** helped advance the technique of DNA recombination in 1970–1971 and demonstrated that type II enzymes could be useful in genetic studies.
- ✓ Genetic engineering based on recombination was pioneered in 1973 by American biochemists **Stanley N. Cohen** and **Herbert W. Boyer**, who were among the first to cut DNA into fragments, rejoin different fragments, and insert the new genes into *E. coli* bacteria, which then reproduced.

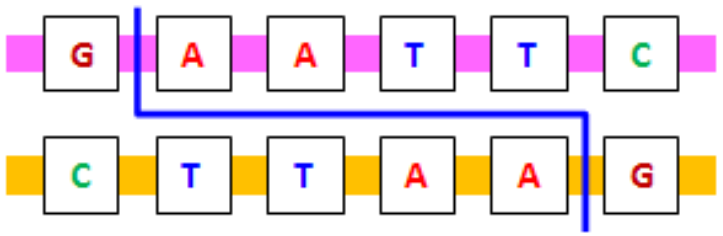


A restriction site (recognition site) - a short sequence of nucleotides in a DNA molecule that is recognized by the enzyme restriction-modification endonuclease (restriction enzyme).
The restriction enzyme binds to the DNA molecule at the location of the restriction site and cuts the chain of nucleotides within the site or in its immediate vicinity.

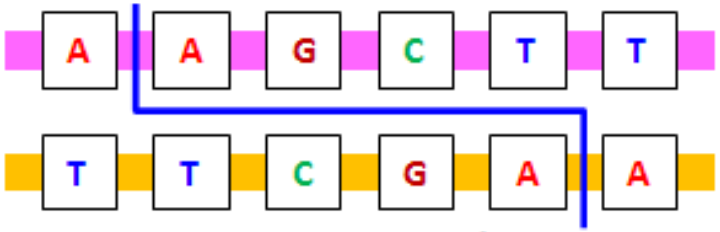




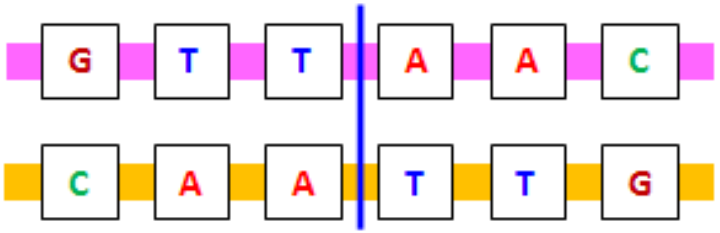
According to The Restriction Enzyme Database (REBASE), there are well over 5,000 known restriction enzymes!



Рестриктаза EcoR I
(бактерия *Escherichia coli*)



Рестриктаза Hind III
(бактерия *Haemophilus influenzae*)

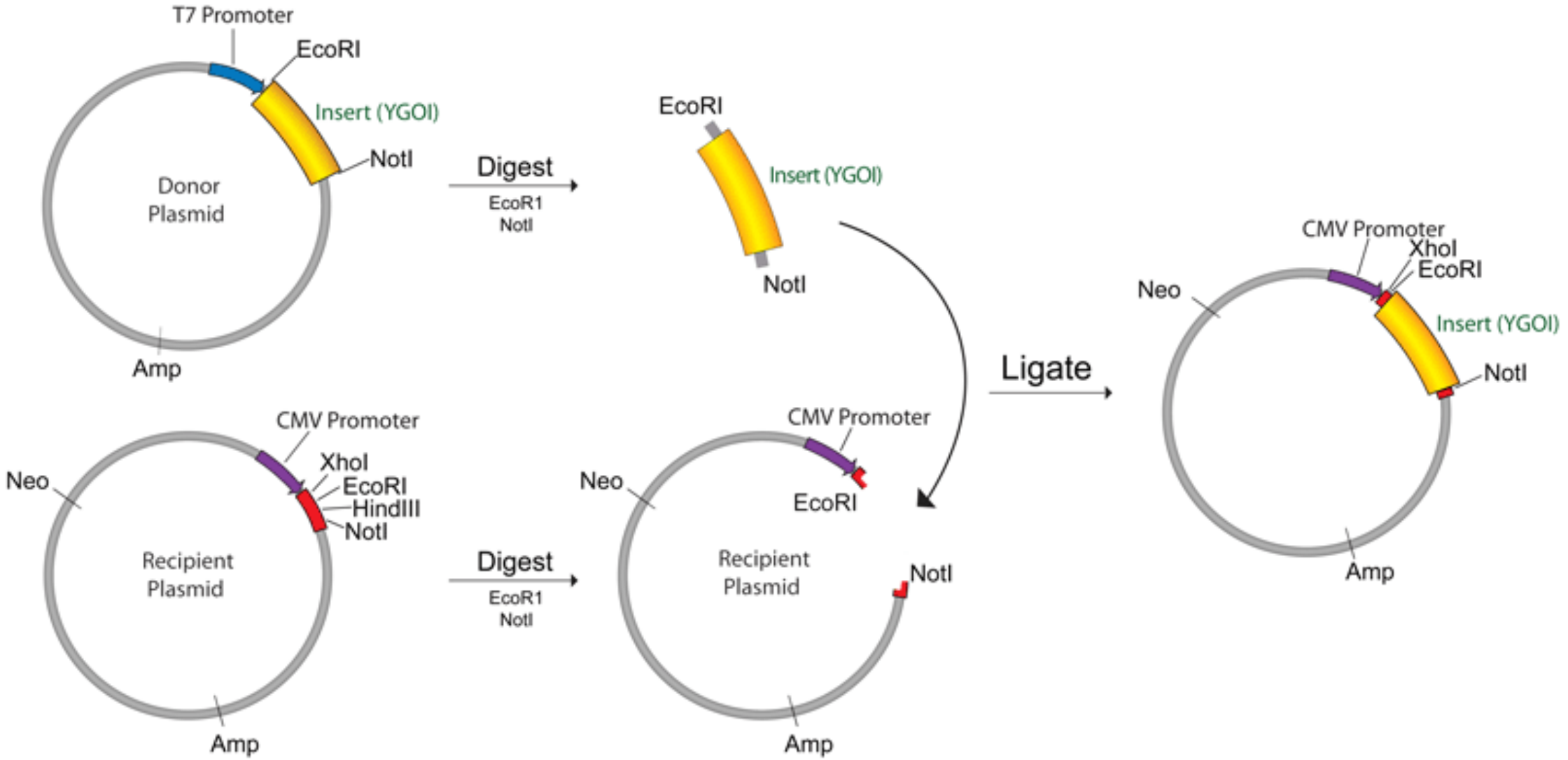


Рестриктаза Hpa I
(бактерия *Haemophilus parainfluenzae*)

https://www.snapgene.com/guides/restriction-enzymes

Microorganisms	*Restriction enzymes*	*Cleavage sites*	*Cleavage products*
Bacillus amyloliquefaciens H	*Bam* HI	5-G↓GATCC-3 3-CCTAGG↑-5	5-G GATCC-3 3-CCTAG G-5
B. globigii	*Bgl* II	5-A↓GATCT-3 3-TCTAGA↑-5	5-A GATCT-3 3-TCTAG A-5
Escherchia coli RY13	*Eco* RI	5-G↓AATTC-3 3-CTTAAG↑-5	5-G AATTC-3 3-CTTAA G-5
Haemophilus influenzae Rd	*Hin* dIII	5-A↓AGCTT-3 3-TTCGAA↑-5	5-A AGCTT-3 3 -TTCGA A-5
H. parainfluenzae	*Hpa* I	5-GTT↓AAC-3 3-CAATTG↑-5	5-GTT AAC-3 3-CAA TTG-5
Klebsiella pneumoniae OK 8	*Kpn* I	5-GGTAC↓C-5 3-C↑CATGG-3	5-GGTAC C-3 3-C CATGG-5
Streptomyces albus G	*Sal* I	5-G↓TCGAC-3 3-CAGCTG↑-5	5-G TCGAC-3 3-CAGCT G-5
Staphylococcus aureus 3AI	*Sau* 3AI	5-↓GATC-3 3-CTAG↑-5	5- GATC-3 3-CTAG 5

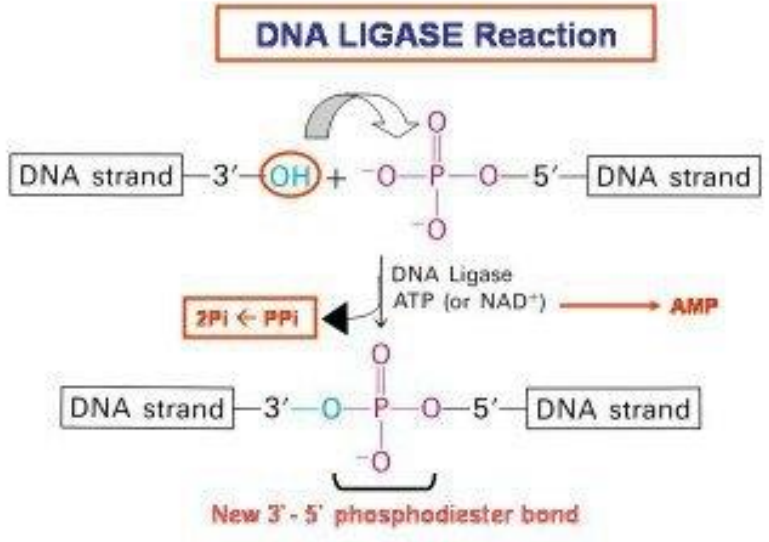
Source of restriction enzymes, cleavage sites and productions of cleavage



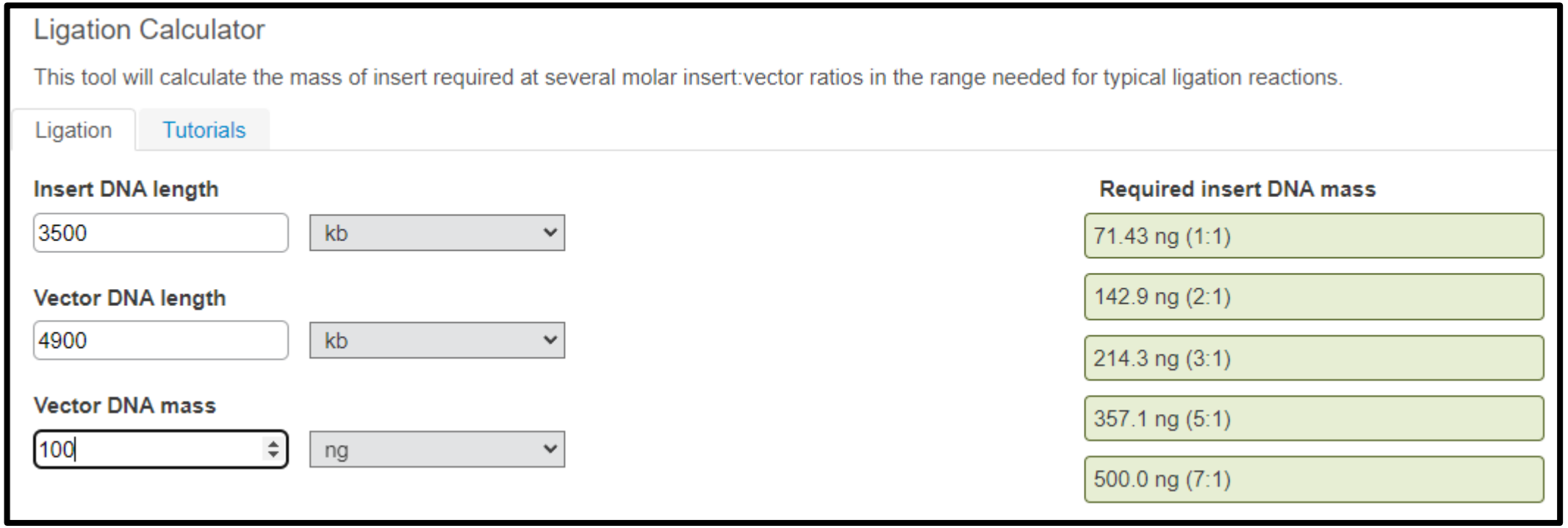
Cloning of the desired gene (YGOI) into the vector using the EcoRI and NotI sites as constraints.

➢ Ligation method

- In molecular biology, **ligation** is the joining of two nucleic acid fragments by the action of an enzyme "**Ligase**".
- In 1967, DNA ligase was discovered independently by the **Gellert**, **Lehman**, **Richardson**, and **Hurwitz** Institute.
- The ends of the DNA fragments join together, forming phosphodiester bonds between the 3'-hydroxyl of one end of the DNA and the 5'-phosphoryl of the other.
- Ligation in the laboratory is usually performed using **T4 DNA ligase**. This is an important laboratory procedure for molecular DNA cloning, in which DNA fragments are combined to create recombinant molecules.



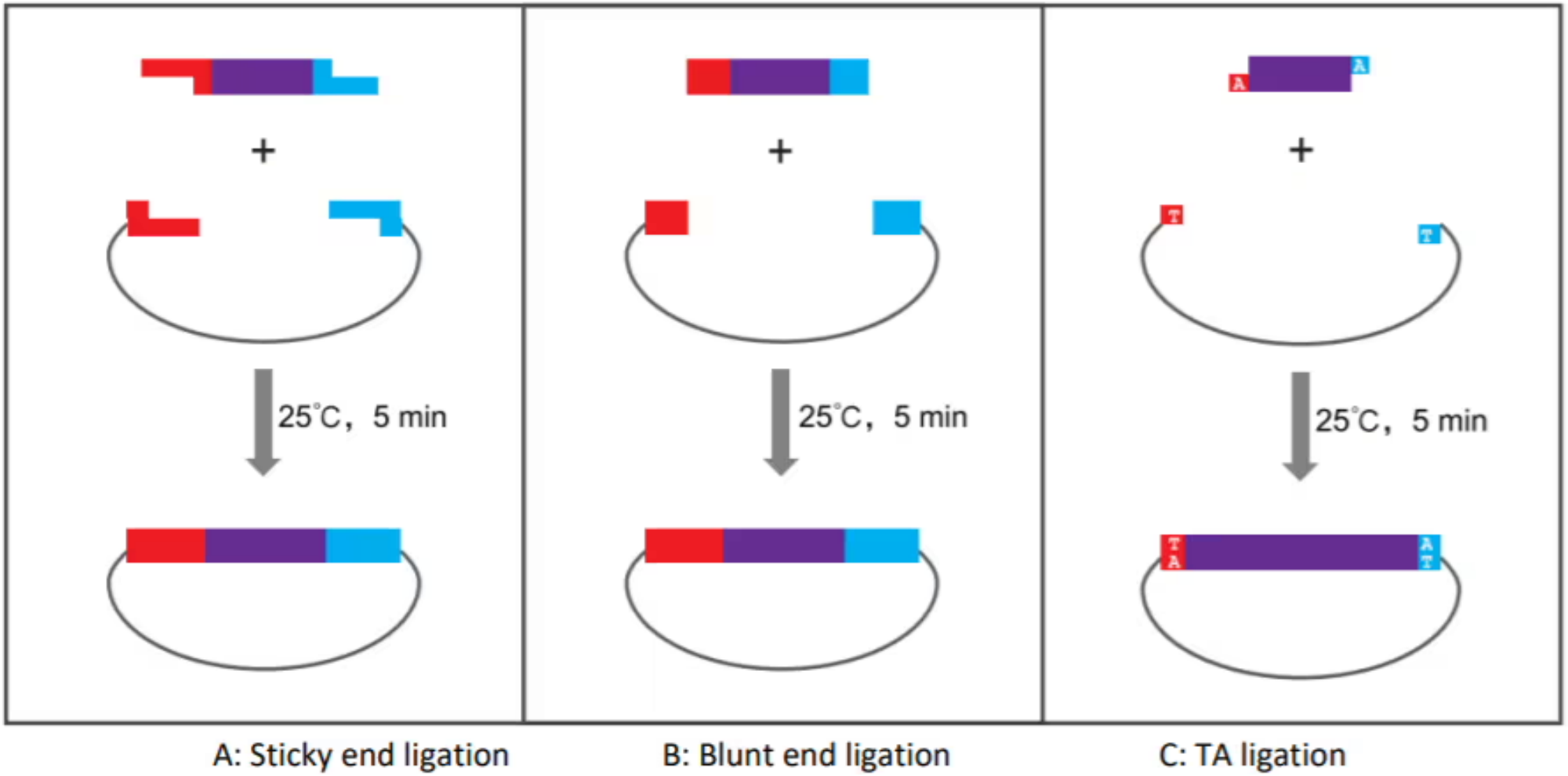
Ligation calculator



$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

https://nebiocalculator.neb.com/#!/ligation

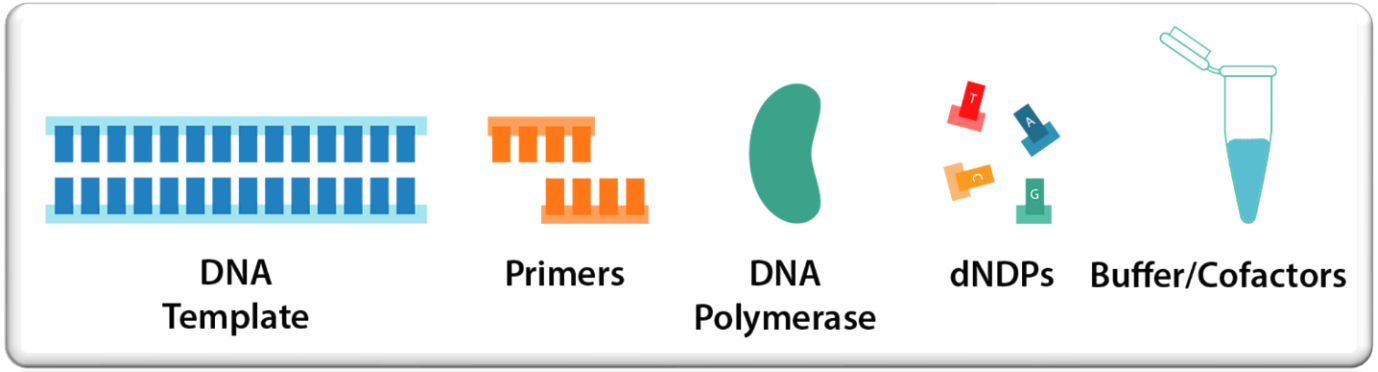
Ligation types



A: Sticky end ligation B: Blunt end ligation C: TA ligation

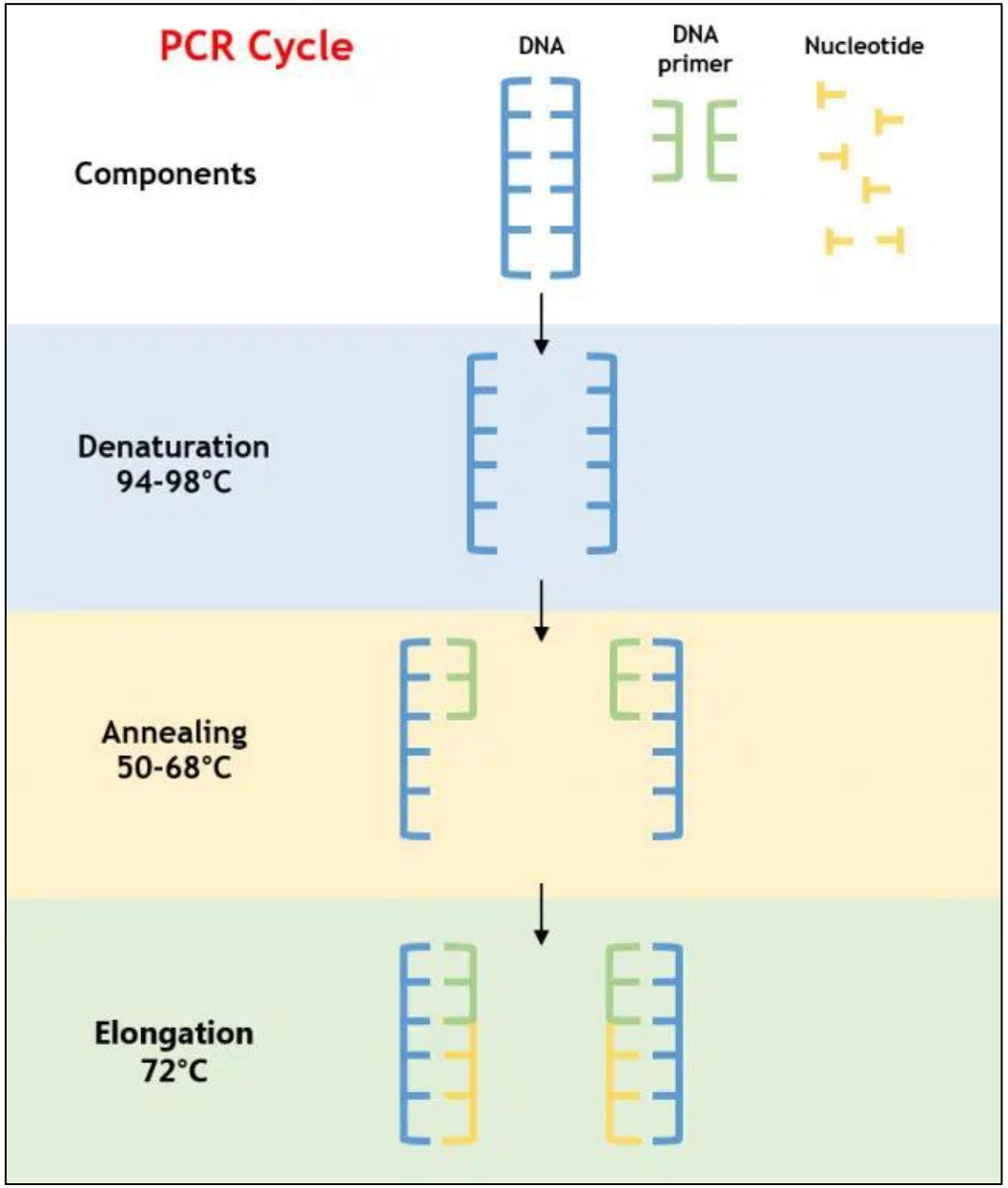
➢ Polymerase chain reaction (PCR)

- The **Polymerase Chain Reaction** (PCR) technique, invented in 1985 by **Kary B. Mullis**, allowed scientists to make millions of copies of a scarce sample of DNA.
- The history of modern PCR begins in 1976 with the isolation of ***Taq* DNA polymerase** from the thermophilic bacterium *Thermus aquaticus*.
- PCR is a simple yet elegant enzymatic assay that allows the amplification of a specific piece of DNA from a complex pool of DNA.
- Each PCR assay requires template DNA, primers, nucleotides, Reaction buffer and DNA polymerase.

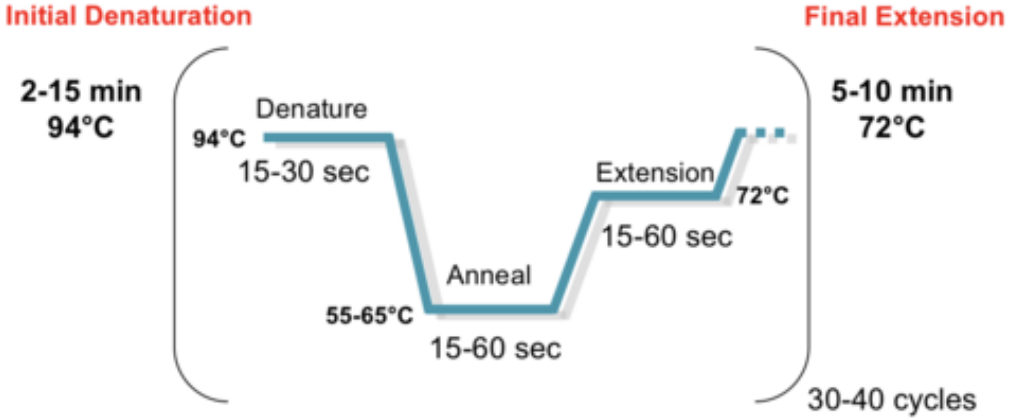


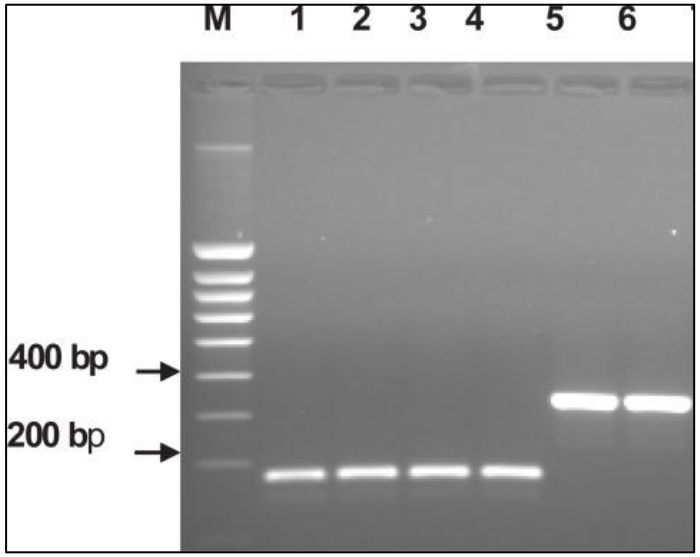
https://siarchives.si.edu/collections/siris_arc_217745#:~:text=The%20Polymerase%20Chain%20Reaction

Polymerase Chain Reaction principle



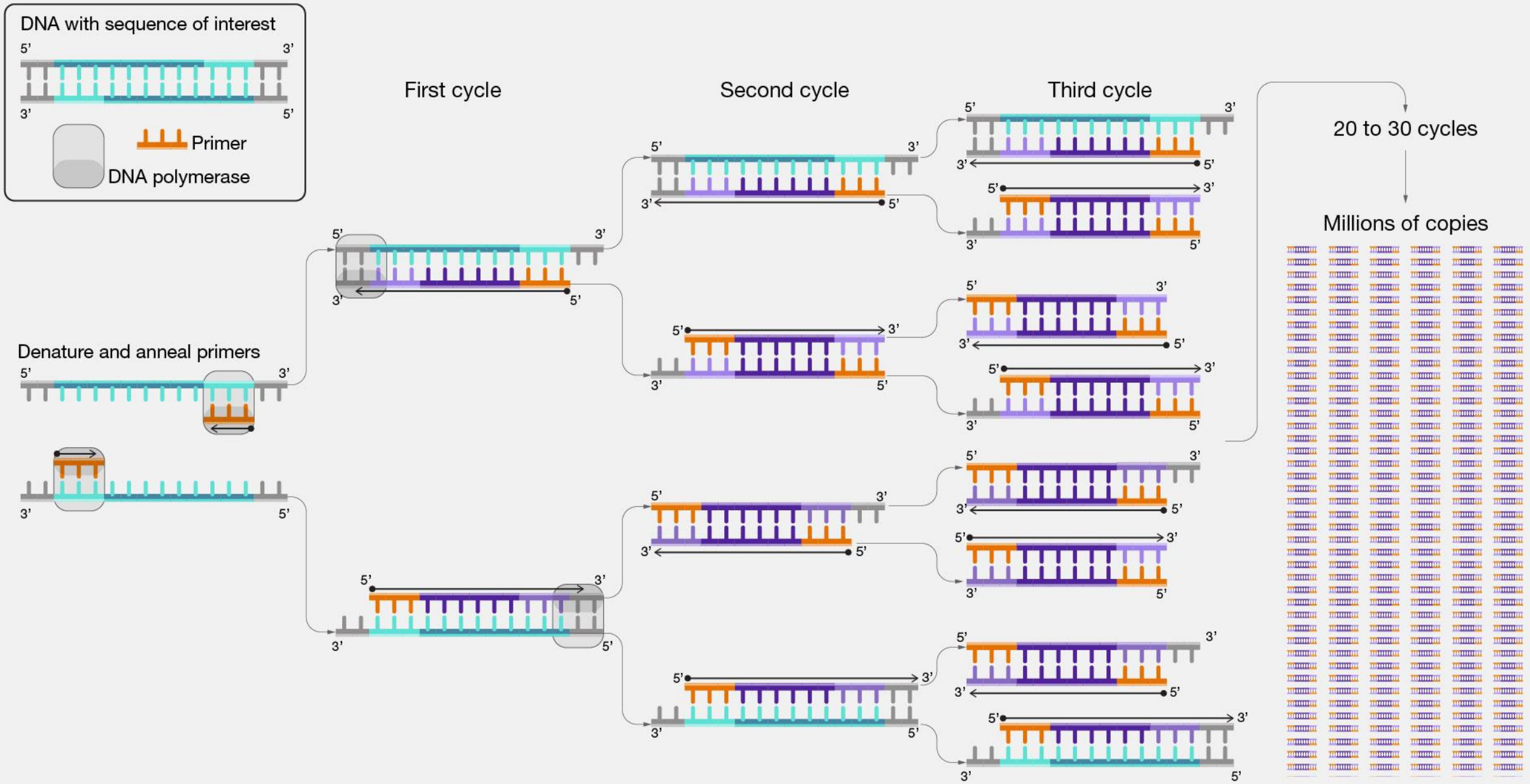
- **Consists of 3 basic steps:**
  1. Denaturation
  2. Annealing
  3. Extension

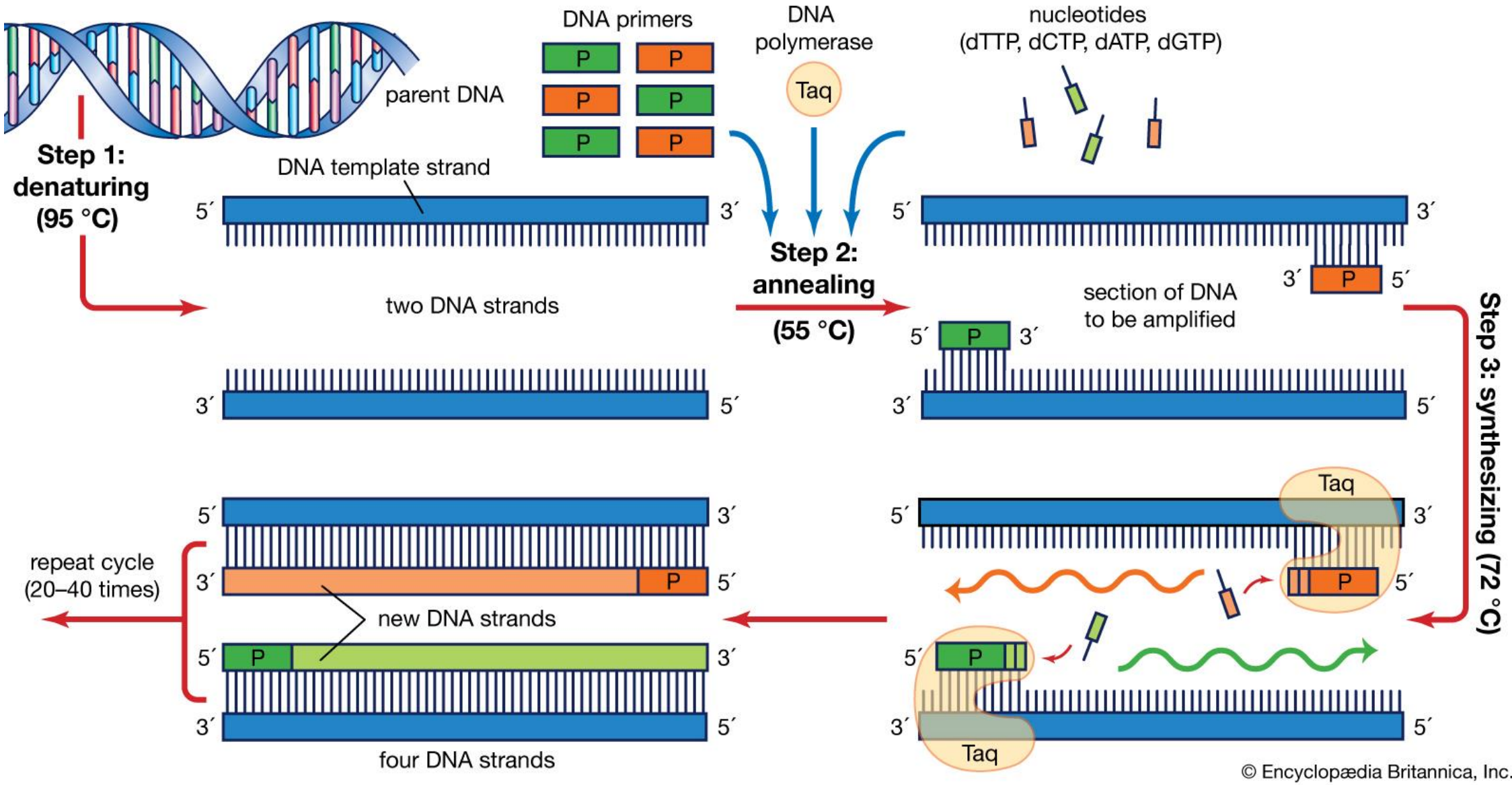




Agarose electrophoresis of PCR products

Thermocycler


DNA with sequence of interest
5'
3'
3'
5'
Primer
DNA polymerase
First cycle
Second cycle
Third cycle
20 to 30 cycles
Millions of copies
Denature and anneal primers



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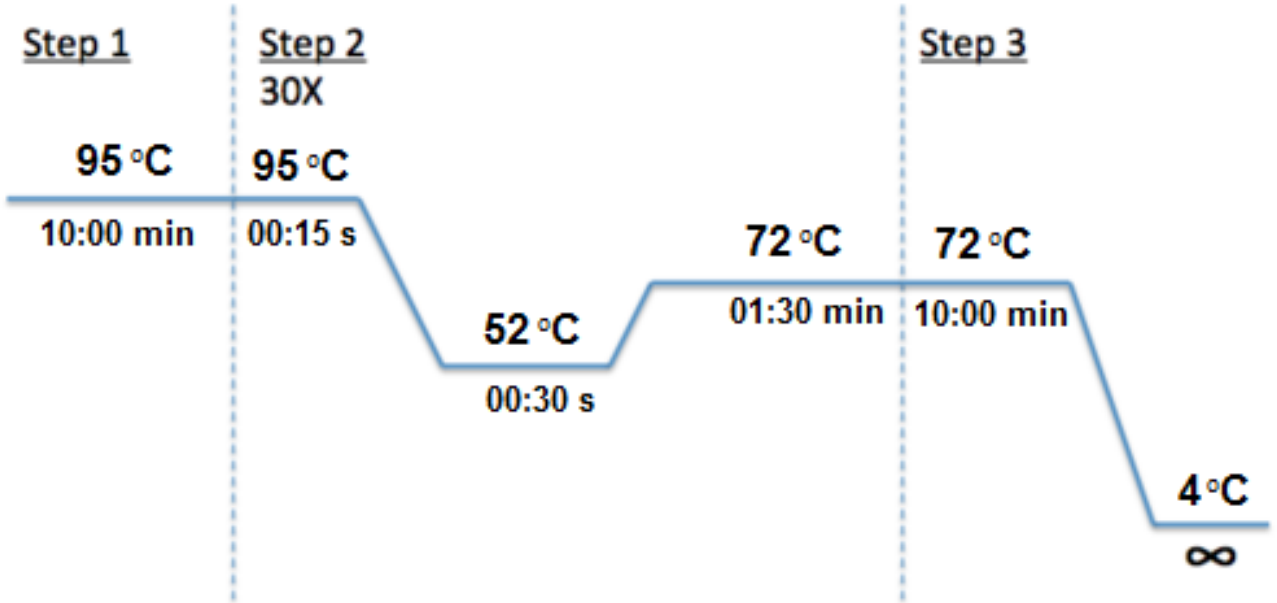
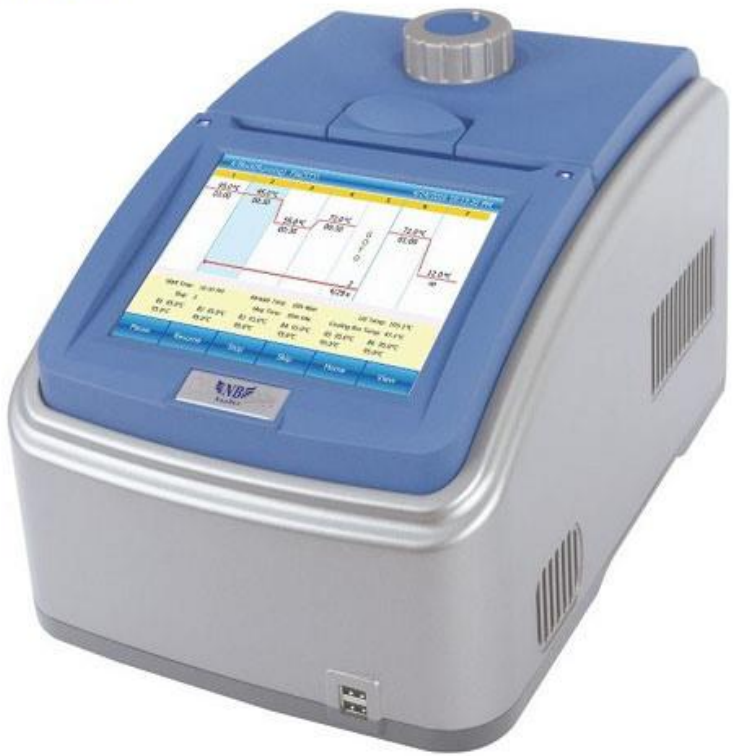


Figure 1: PCR conditions for three hypervariable regions of mtDNA

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	
Annealing	50°C - 65°C*	30 sec	30 - 35
Elongation	72°C	1 min – 3 min#	
Final Elongation	72°C	5 min	1
Soak	10°C -25°C	1 min	1

* Annealing temperature was dependent on oligonucleotide melting temperatures.

NanBei



3. Add restriction enzymes sites to the 5' ends of your primers (see primer design). Often the same enzymes that were used to clone the gene are used.

4. Add GGAGAA to the 5' end after the restriction site to give the enzyme sequence to "grab on" to. Do not include sequences that do not hybridize when calculating tm.

Figure 2: PCR truncation primers showing restriction sites (NotI and BamHI) and additional sequence for restriction enzymes to "grab on" to.

5. Do a PCR reaction using your RE designed primers

Polymerase Chain Reaction (PCR) has numerous applications in various fields of biology, medicine, and biotechnology:

1. Infectious Disease Detection: PCR is used to detect the presence of pathogens (viruses, bacteria, fungi) in patient samples. For example, it's widely used to test for COVID-19, HIV, hepatitis, and tuberculosis by amplifying specific genetic sequences of the pathogens.

2. Genetic Disorder Screening: PCR can identify mutations associated with genetic disorders like cystic fibrosis, sickle cell anemia, and Huntington's disease by amplifying and analyzing the mutated genes.

3. DNA Profiling: PCR is used to amplify specific regions of DNA in forensic samples (such as blood, hair, or saliva) to create genetic fingerprints. This helps in criminal investigations, identifying suspects, or verifying familial relationships.

4. Paternity Testing: PCR can amplify DNA from a potential father and child to compare genetic markers and determine biological relationships.

5. Gene Cloning: PCR is employed to amplify specific genes of interest, which can then be inserted into vectors for further research, study, or modification.

6. Gene Expression Analysis: PCR, especially quantitative PCR (qPCR), is used to measure the expression levels of genes in different tissues or conditions, providing insights into gene regulation and function.

7. Oncogene Detection: PCR can detect specific mutations in cancer-related genes (oncogenes) like BRCA1/BRCA2, which are linked to breast cancer, or mutations in genes like EGFR, which are associated with lung cancer.

8. Minimal Residual Disease: PCR is used in monitoring cancer recurrence by detecting minimal amounts of cancer cells left in the body after treatment.

9. Evolutionary Studies: PCR is used to amplify DNA from ancient or preserved samples, such as fossils, to study evolutionary relationships between species.

10. Species Identification: In biodiversity studies, PCR can amplify DNA from different organisms to help identify species, even in cases where the organism is poorly preserved or partially degraded.

11. Detection of Genetically Modified Organisms (GMOs): PCR is used to detect the presence of GMOs in food by amplifying specific transgenic sequences.

12. Pathogen Detection in Food: PCR helps in identifying harmful bacteria, such as *Salmonella*, *E. coli*, or *Listeria*, in food samples to ensure safety.

13. Fetal Genetic Testing: PCR can analyze fetal DNA from amniotic fluid or maternal blood to detect genetic abnormalities like Down syndrome or inherited diseases.

14. Newborn Screening: PCR is used to test newborns for certain genetic conditions early in life, allowing for prompt medical intervention if necessary.

15. HIV and Hepatitis Monitoring: PCR is used to measure the viral load in patients infected with viruses like HIV and hepatitis B or C. This helps monitor disease progression and the effectiveness of treatment.

16. Detection of Environmental Contaminants: PCR is applied in testing water, soil, or air samples for microbial contamination, including bacteria, fungi, and viruses.

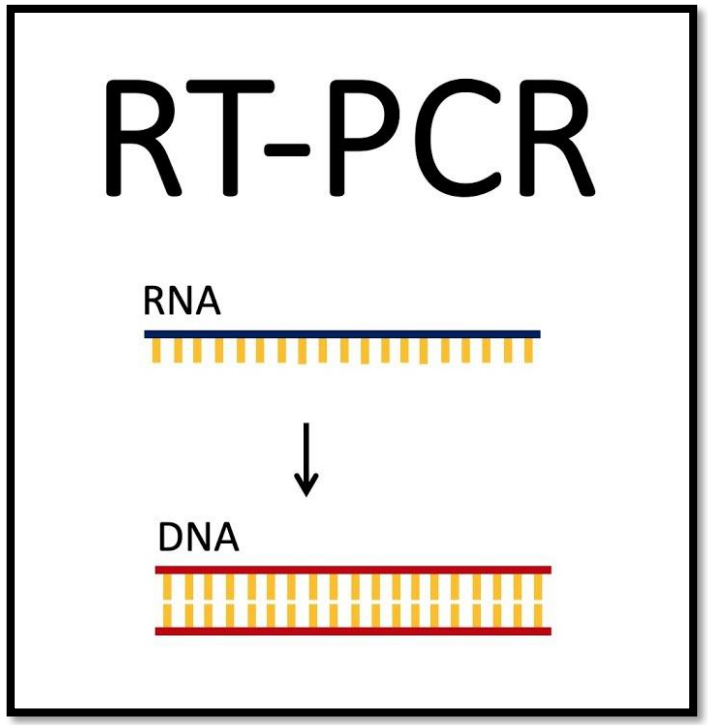
17. Biodiversity Assessment: PCR-based methods are used to assess the diversity of microorganisms in environmental samples, helping to monitor ecosystem health and track pollution.

18. Next-Generation Sequencing (NGS): PCR is a critical step in amplifying target DNA sequences for high-throughput sequencing, enabling the study of entire genomes or specific genes.

19. Sanger Sequencing: PCR helps amplify specific DNA fragments for traditional sequencing, providing detailed information on genetic sequences.

Reverse transcription PCR

- In molecular biology, reverse transcription polymerase chain reaction is abbreviated as RT-PCR;
- PCR using reverse transcription, respectively, as RT-PCR. This is a method for amplifying a specific fragment of ribonucleic acid (RNA).

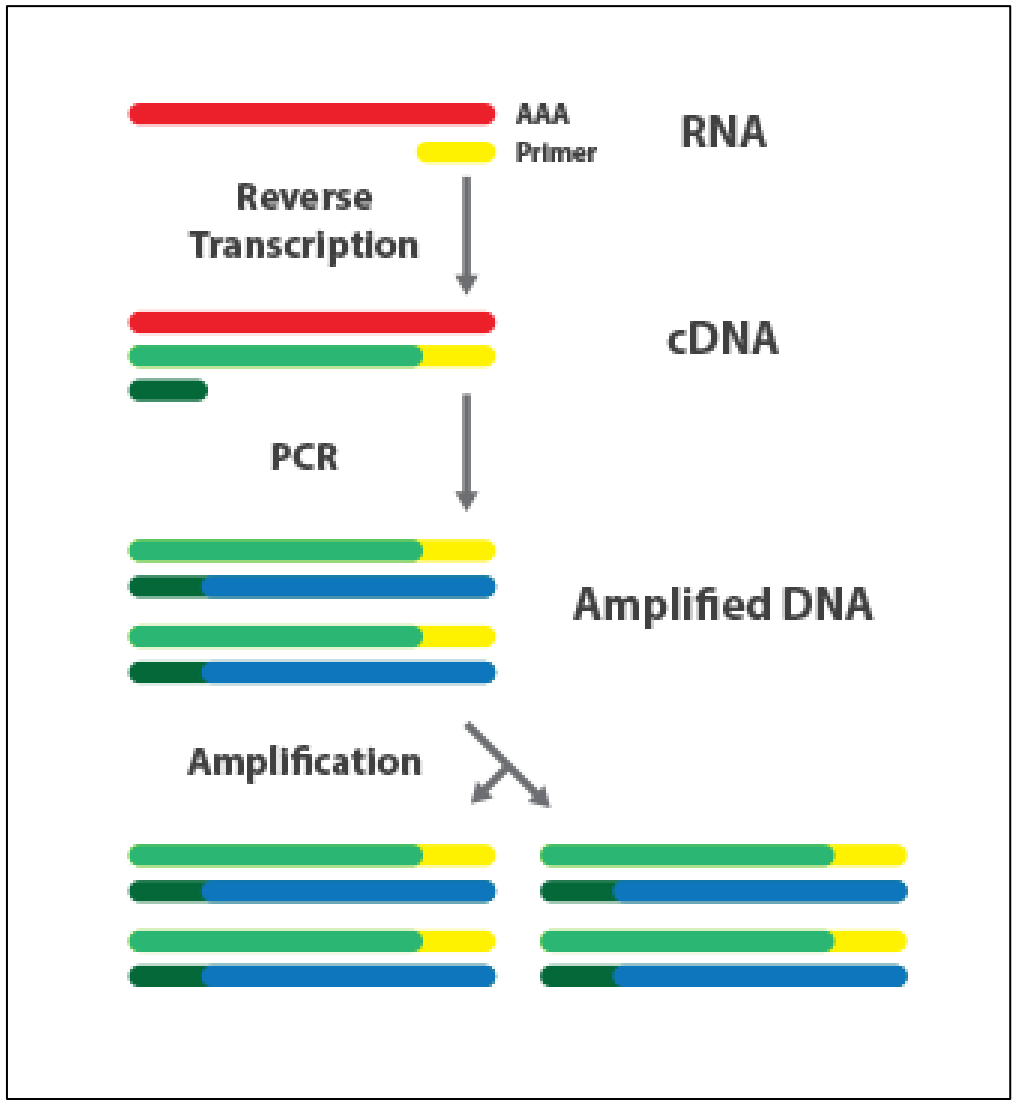


➢ RT-PCR or qPCR

RT-qPCR, or quantitative reverse transcription PCR, combines the effects of reverse transcription and quantitative or real-time PCR to amplify and detect specific targets.

RT-qPCR has many uses. The RT-qPCR technique is used to:

- quantify gene expression levels
- RNA validation and to study the loss of function of selective genes
- detect pathogens such as viruses to diagnose infectious diseases
- detect genetically modified organisms (GMOs)



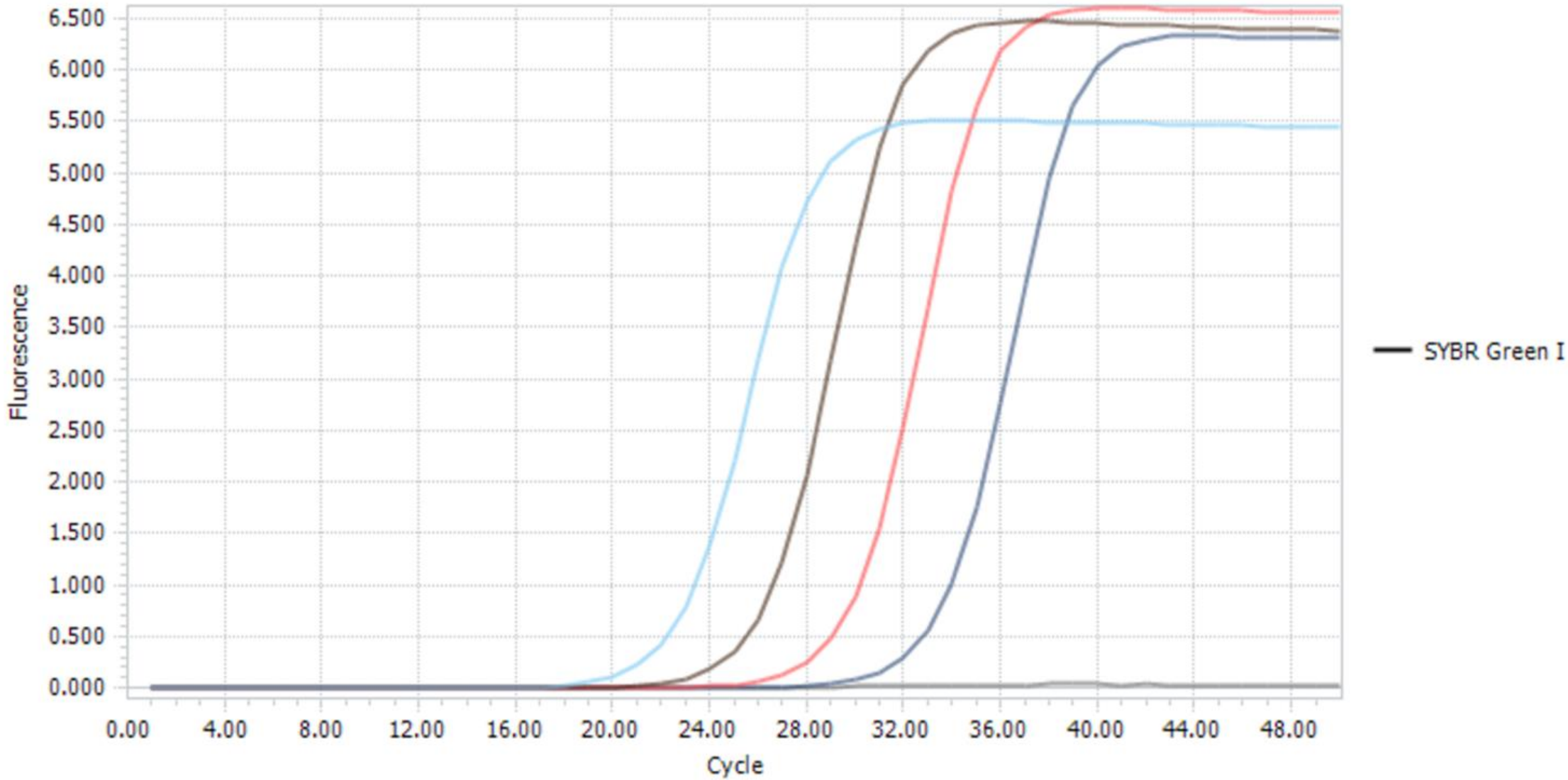
- A single-stranded RNA molecule is converted in reverse transcription reactions (RT, RT, reverse transcription) into complementary DNA (cDNA) and then the already single-stranded DNA molecule is amplified using traditional PCR.
- Reverse transcription PCR (RT-PCR) should not be confused with Real-time PCR (Q-PCR), which is also sometimes incorrectly abbreviated as RT-PCR.

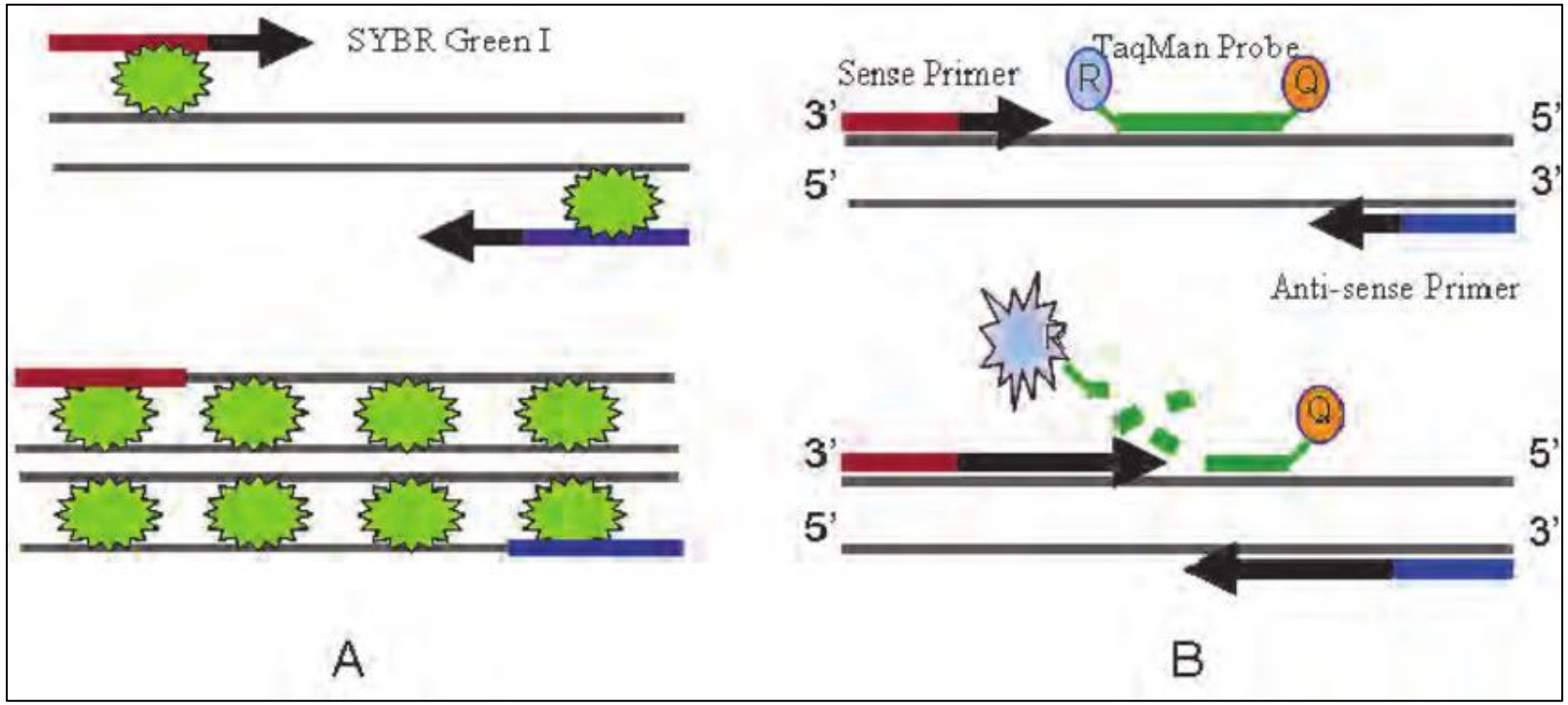
DESCRIPTION OF THE METHOD

Reverse transcriptase is used to convert an RNA sequence into complementary DNA:

1. Reaction of the first chain: Complementary DNA (cDNA) is formed on the mRNA template from dNTP by the enzyme reverse transcriptase. The reaction components are mixed with DNA primer and reverse transcriptase buffer for one hour at 37°C.
2. Reaction of the second chain: After reverse transcription is completed and cDNA is formed on the mRNA template, the next cycles are performed according to the standard PCR method.
3. After about 30 cycles of amplification, millions of copies of the desired sequence are produced.

Amplification Curves




SYBR Green I
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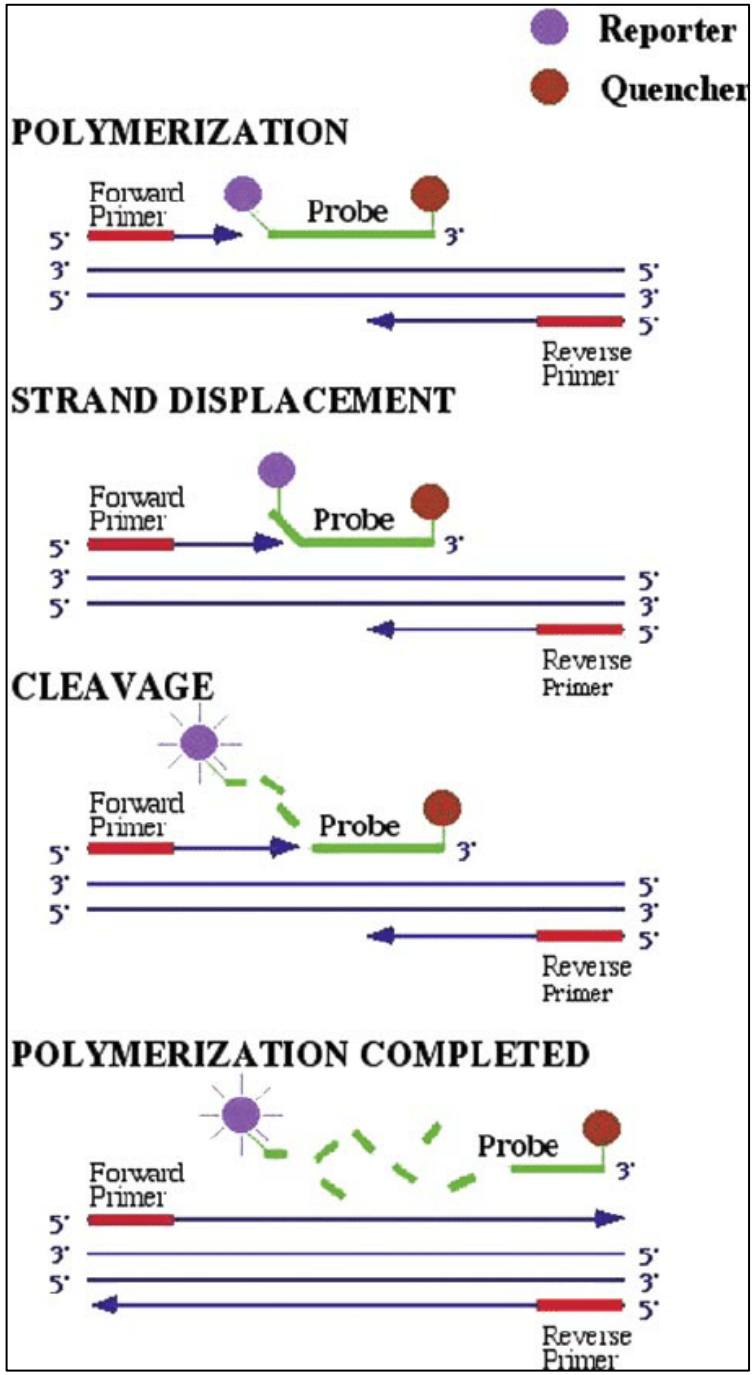
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