

DNA, RNA, and Protein Extraction & PCR

1. Introduction

- Purpose: Extraction of DNA, RNA, and protein is the starting point in molecular biology, used for research, diagnostics, and downstream applications.
- Sources: Can be isolated from cells, tissues, viruses, or other biological materials.
- Goal: Obtain pure, high-quality biomolecules free of contaminants (proteins, lipids, carbohydrates, other nucleic acids).

DNA Extraction:

- 1869: Friedrich Miescher isolated DNA ("nuclein") from white blood cells. Early methods were crude and low-yield, later improved for larger-scale purification.

Key Steps for Nucleic Acid Purification:

- Break open cells/tissues (cell disruption).
- Separate nucleic acids from proteins (denaturation of nucleoproteins).
- Inactivate nucleases (RNase for RNA, DNase for DNA).
- Remove contaminants (DNA free of RNA, RNA free of DNA).

RNA special care:

- RNA is unstable, easily degraded by ubiquitous RNases. Requires RNase-free conditions, strong denaturants, and careful handling.

Step	Chemical/Enzyme	Function
Cell lysis	SDS, CTAB, Triton X-100	Break membranes
Protein removal	Proteinase K, phenol, chloroform	Remove/digest proteins
Nuclease inhibition	EDTA	Bind metal ions
RNA removal (for DNA)	RNase A	Degradate RNA
DNA removal (for RNA)	DNase I	Degradate DNA
Precipitation	Ethanol, isopropanol, salts	Recover nucleic acids
pH & storage	Tris-HCl, TE buffer	Stability

Column binding

Guanidinium salts

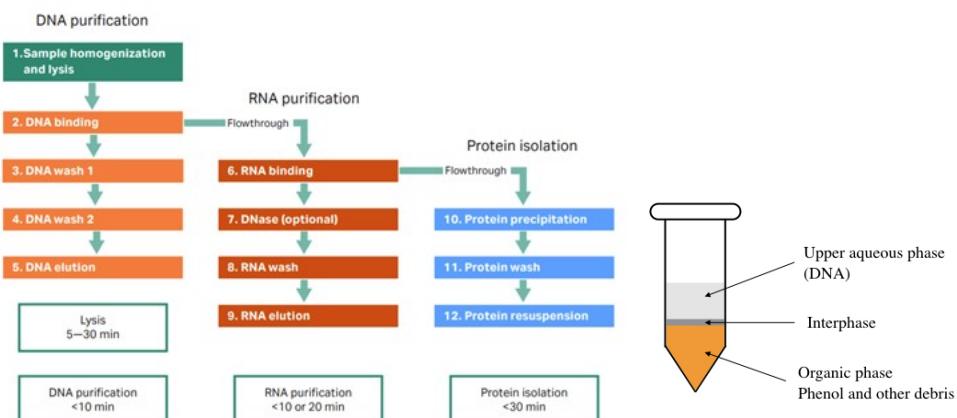
Bind nucleic acids to silica

Protein special care:

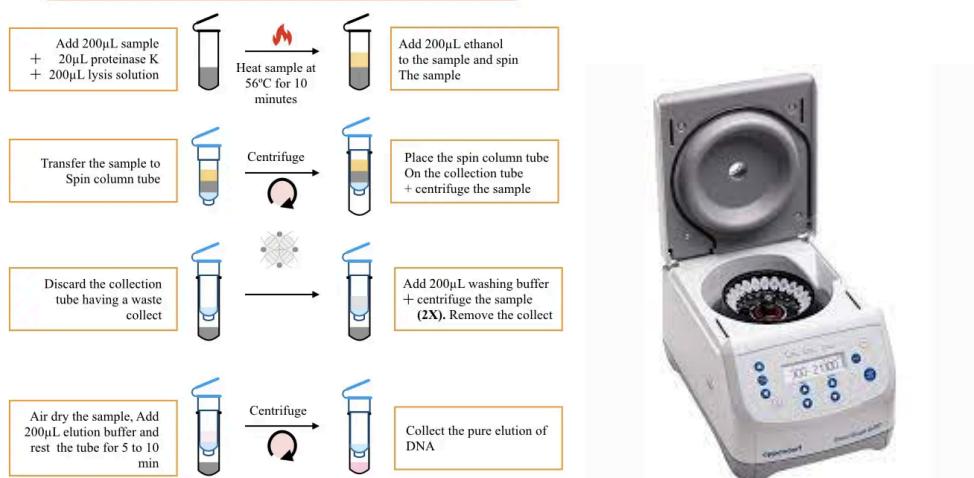
- Extracted at low temperature (4°C) to prevent denaturation.
Buffers, detergents, and reducing agents maintain protein stability and solubility.
Purification methods: ion exchange, gel filtration, affinity chromatography, electrophoresis.

Protein Extraction:

- 18th century: Antoine Fourcroy distinguished proteins as coagulating molecules.
1893: Mulder showed proteins are macromolecules (C, H, O, N \pm S, P).
WWII: Edwin Joseph Cohn developed protein purification for blood plasma.



Spin-Column DNA extraction



Centrifugation speeds up phase separation that would otherwise take a long time.

After adding PCI, the sample is spun at high speed to separate components quickly.

Centrifugal force separates the mixture into three layers — organic (bottom), interphase (middle), and aqueous (top).

The aqueous layer on top contains purified DNA.
DNA is collected by carefully pipetting the top aqueous layer into a new tube.

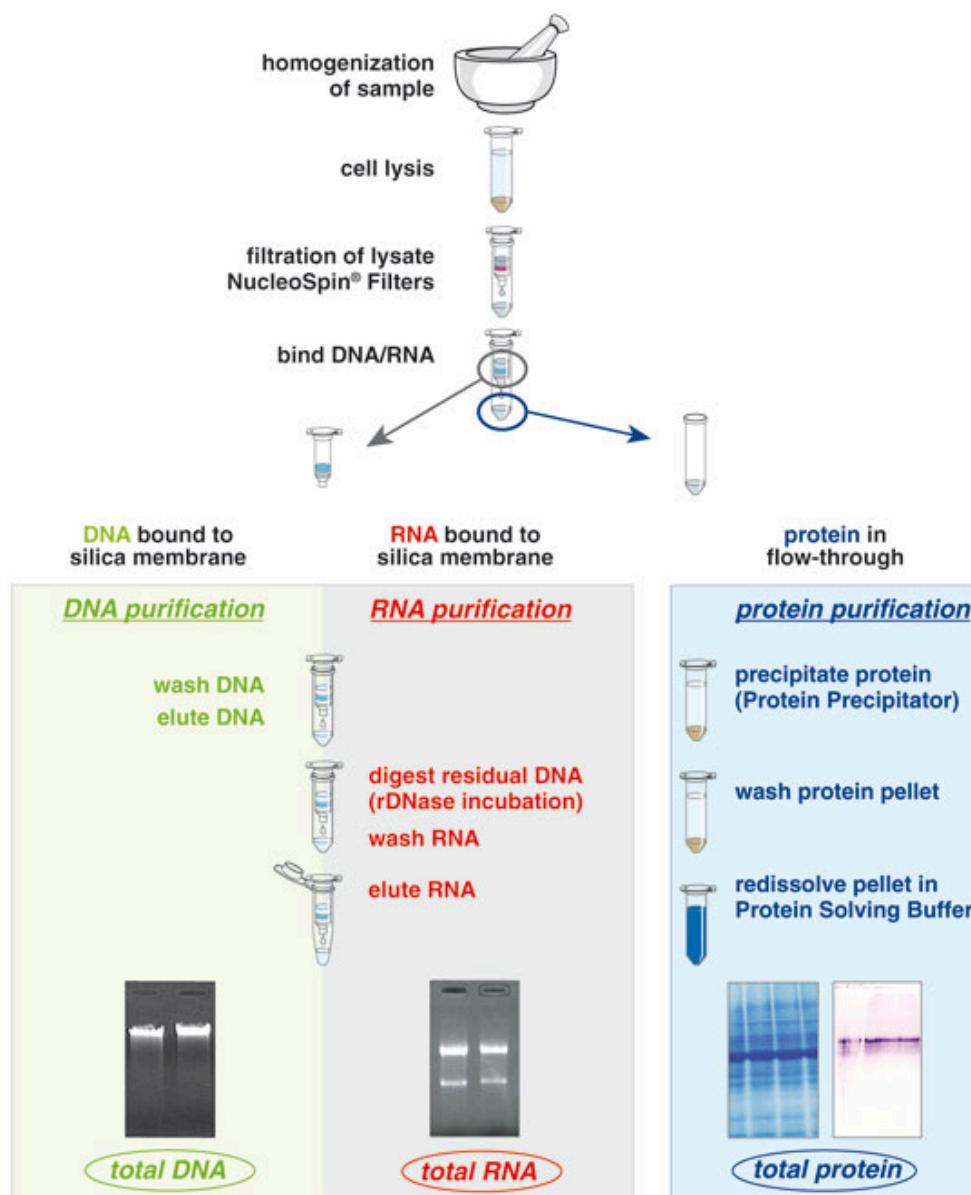


Purpose of PCI Chemicals

Phenol – Denatures and removes proteins by breaking hydrogen bonds; moves proteins into the organic phase.

Chloroform – Increases density for clearer layer separation and helps remove lipids and proteins.

Isoamyl alcohol – Prevents foam and emulsions, giving cleaner layer separation.

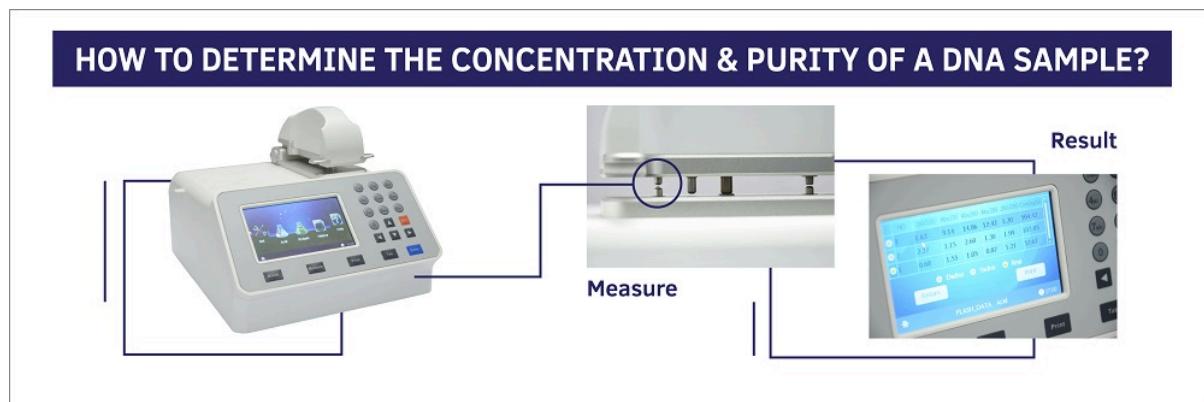


Spectrophotometric Analysis of DNA, RNA, and Protein

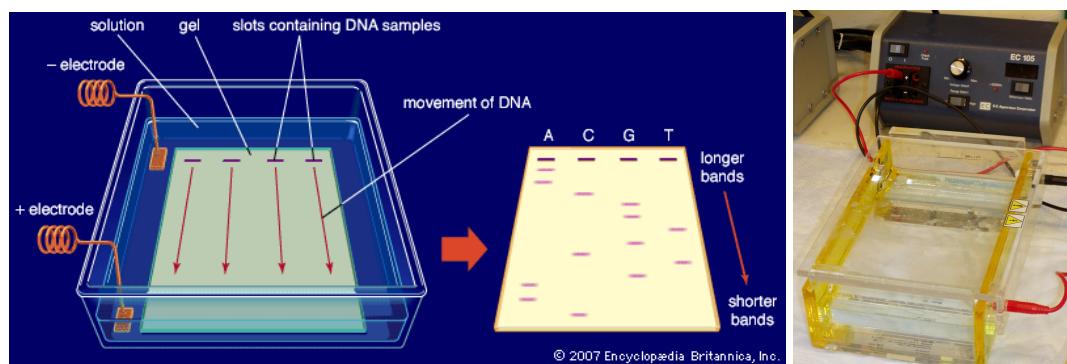
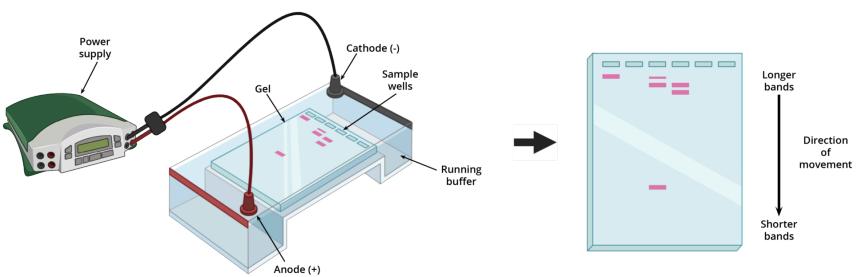
Principle

- Nucleic acids and proteins absorb UV light at specific wavelengths:
DNA & RNA → 260 nm (A₂₆₀)
Proteins → 280 nm (A₂₈₀)
- A₂₆₀/A₂₈₀ ratio indicates sample purity:
DNA → ~1.8
RNA → ~2.0
Lower ratio → protein contamination
A₂₆₀/A₂₃₀ ratio (~2.0–2.2) indicates other contaminants like phenol or guanidine salts.

Nanodrop spectrophotometer → uses tiny samples (~1 µL) directly, no cuvettes needed.

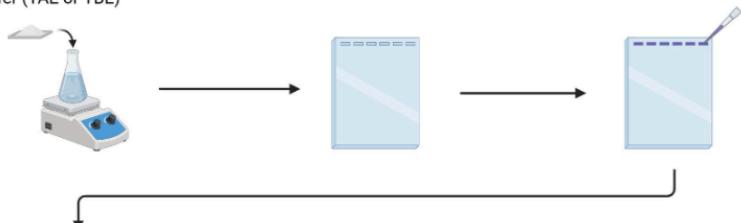


Sample Type	Expected A ₂₆₀ /A ₂₈₀	Interpretation
DNA	~1.8	Pure DNA
RNA	~2.0	Pure RNA
Any sample	<1.8	Protein contamination
Any sample	Low A ₂₆₀ /A ₂₃₀	Contamination by phenol, guanidine, or salts

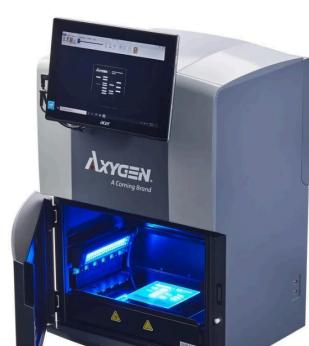
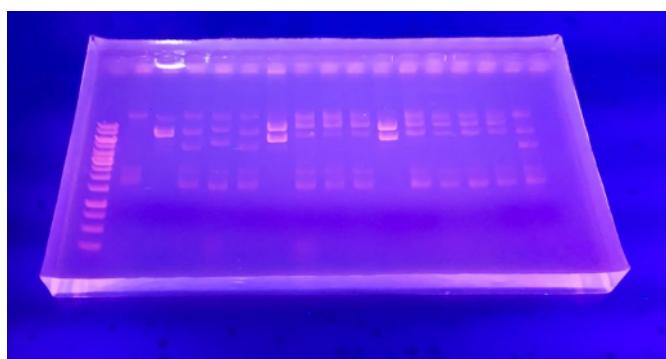


Agarose Gel Electrophoresis

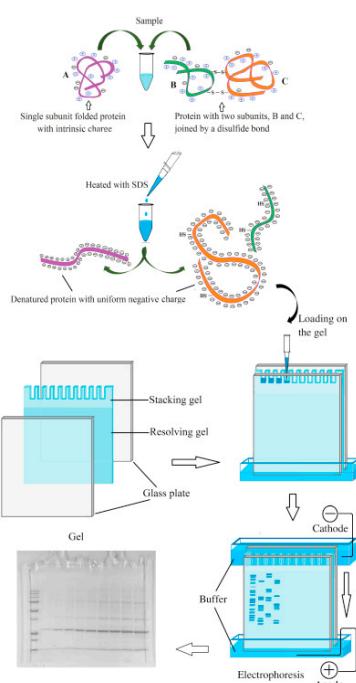
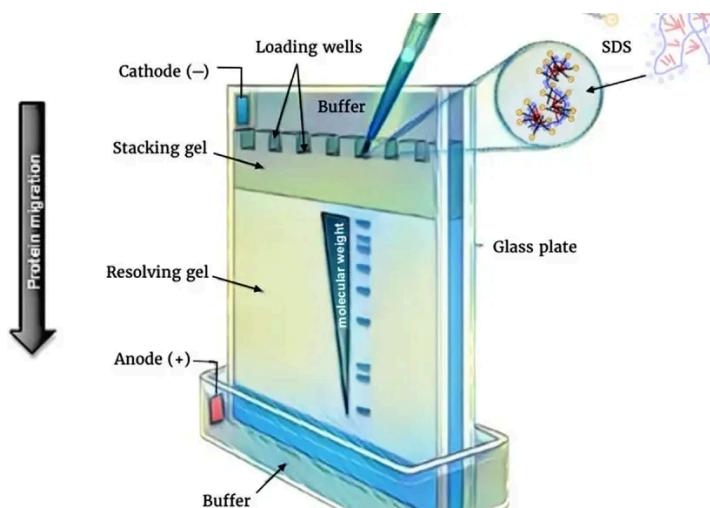
- ① Dissolve agarose powder in buffer (TAE or TBE)
- ② Agarose gel polymerization
- ③ Load DNA samples

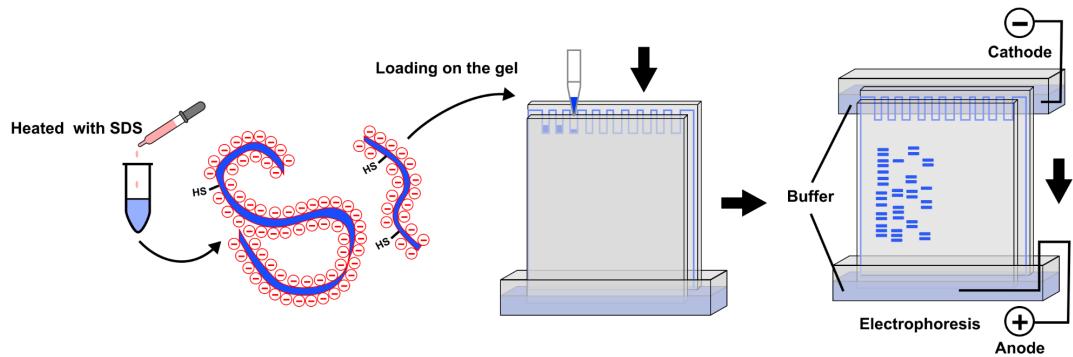


- ④ Apply current to pull DNA toward the positive electrode
- ⑤ DNA fragments separate based on size
- ⑥ Stain the gel and visualize under UV light



Feature	Agarose Gel Electrophoresis	SDS-PAGE
Used for	DNA or RNA separation	Proteins separation
Gel type	Agarose (polysaccharide)	Polyacrylamide (synthetic polymer)
Pore size	Large, suitable for big molecules (100 bp – 20 kb)	Small, suitable for proteins (5–250 kDa)
Molecule charge	DNA/RNA naturally negative; migrate toward anode	Proteins coated with SDS (negative charge); migrate toward anode
Denaturing	Usually native (can do denaturing with formaldehyde for RNA)	Denaturing with SDS (unfolds proteins)
Separation principle	Size/length of nucleic acids	Size (molecular weight) of proteins
Visualization	Ethidium bromide or other DNA dyes under UV	Coomassie Blue, silver stain, or western blot
Applications	DNA/RNA analysis, restriction digestion, PCR products	Protein purity, molecular weight determination, western blot





SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

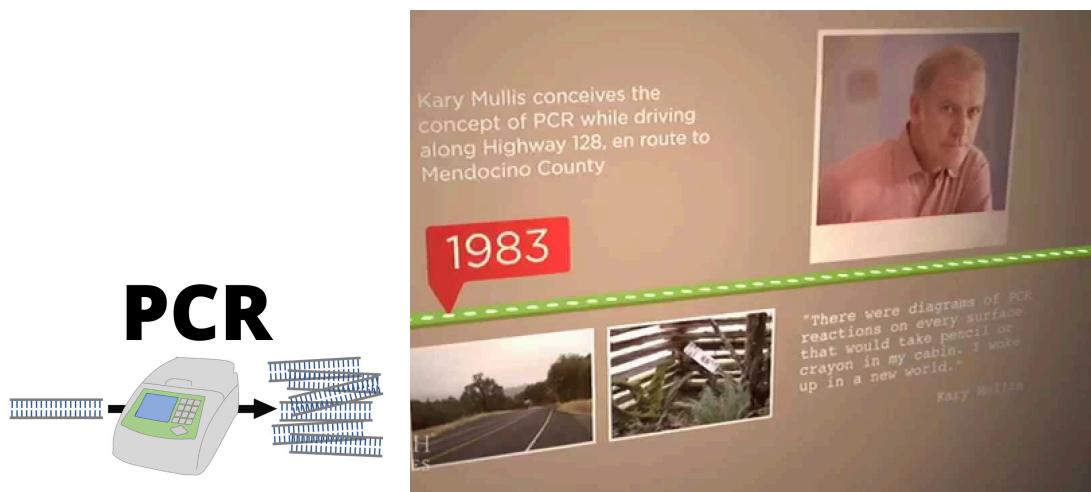
Purpose: Separates proteins based on their size (molecular weight).

How it works:

1. Denaturation: Proteins are treated with SDS, which unfolds them into linear chains.
- Uniform Negative Charge: SDS coats proteins with a negative charge, masking their natural charges.
- Reducing Agents: Chemicals like beta-mercaptoethanol or DTT break disulfide bonds, fully separating protein subunits.
- Polyacrylamide Gel: Acts as a sieve; smaller proteins move faster, larger ones move slower.
- Electrophoresis: An electric current pulls SDS-coated proteins toward the positive electrode.
- Size Separation: Proteins are separated solely based on size because all have similar negative charge.

Applications:

- Check protein purity
Estimate molecular weight
Assess protein expression levels
Analyze complex protein mixtures (e.g., in research or food science)
- Quantification cycle (Cq): Number of cycles for fluorescence to reach threshold.
PCR efficiency: Fold increase per cycle; 2 = 100% efficiency.
qPCR analysis: Can use ΔCq or $\Delta\Delta Cq$ for gene expression comparisons.



- Serial testing helps track disease progression and evaluate pathogen load.

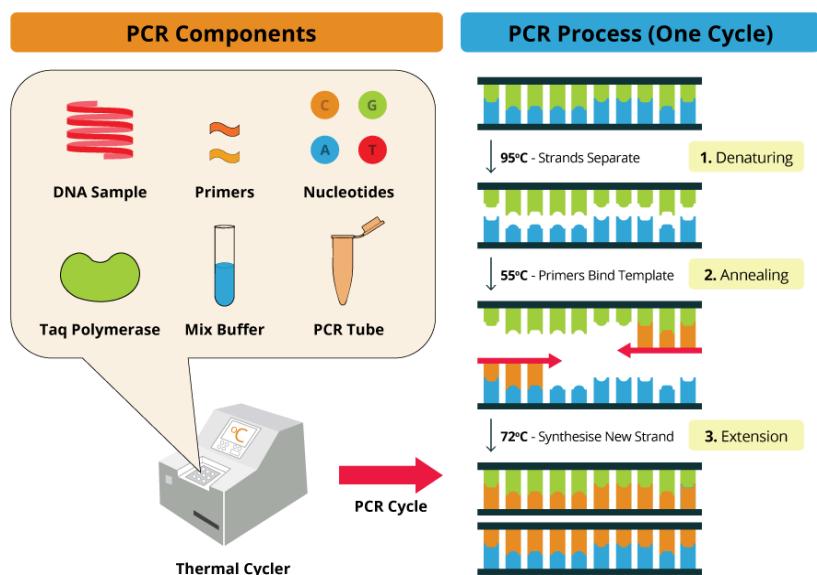
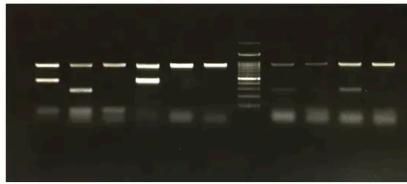
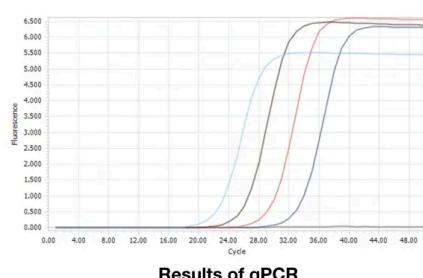


Figure 3. Interchangeable blocks of a thermal cycler for various throughputs. An example of a thermal cycler with interchangeable blocks is the ProFlex PCR System. Available blocks for this PCR machine include 3 x 32-well, 96-well, dual 96-well, dual 384-well, and dual flat blocks.



Results of PCR

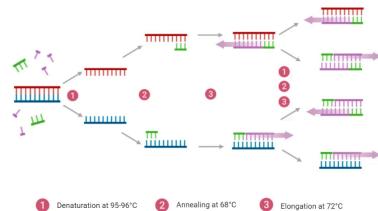
Real Time PCR / quantitative PCR (qPCR)



Results of qPCR



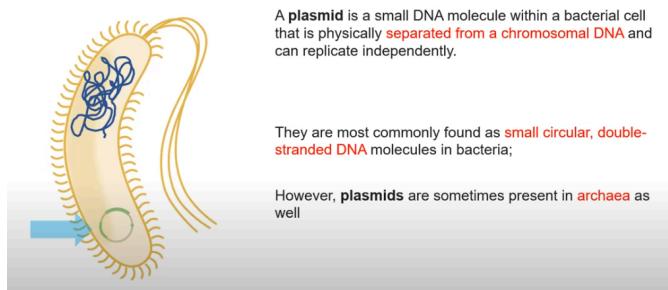
Polymerase chain reaction - PCR



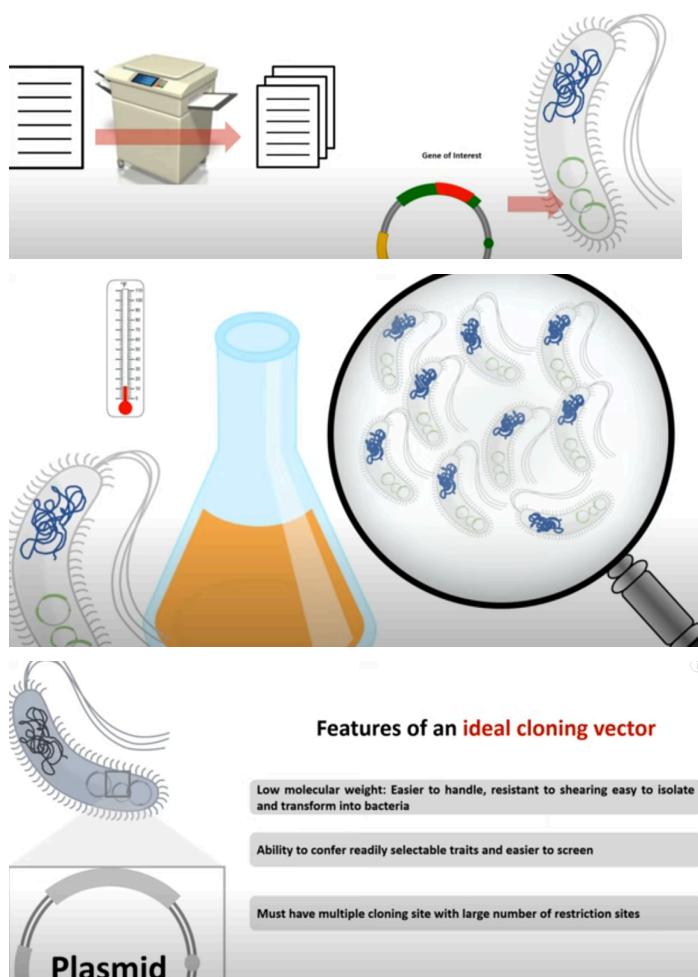
<https://www.youtube.com/watch?v=iQsu3Kz9NYo>

Colony PCR: An Overview

Colony PCR is a rapid and efficient technique used to validate the presence of a DNA insert (such as a gene of interest, GOI) in a bacterial plasmid directly from bacterial colonies grown on selective media. This method is widely used in gene cloning, genetic engineering, and gene transfer experiments. Unlike traditional methods, such as **restriction digestion**, **colony PCR** eliminates the need for time-consuming DNA extraction, and instead, bacterial colonies can be directly used as a template for PCR amplification. This makes the process faster and cost-effective.



Bacteria have two types of DNA because their chromosomal DNA contains the essential genes for survival, growth, and reproduction, while plasmids are extra, non-essential DNA that provide beneficial traits, such as antibiotic resistance, and can be shared among bacteria. This dual system allows bacteria to maintain core functions while quickly adapting to new challenges, like a new antibiotic in the environment.

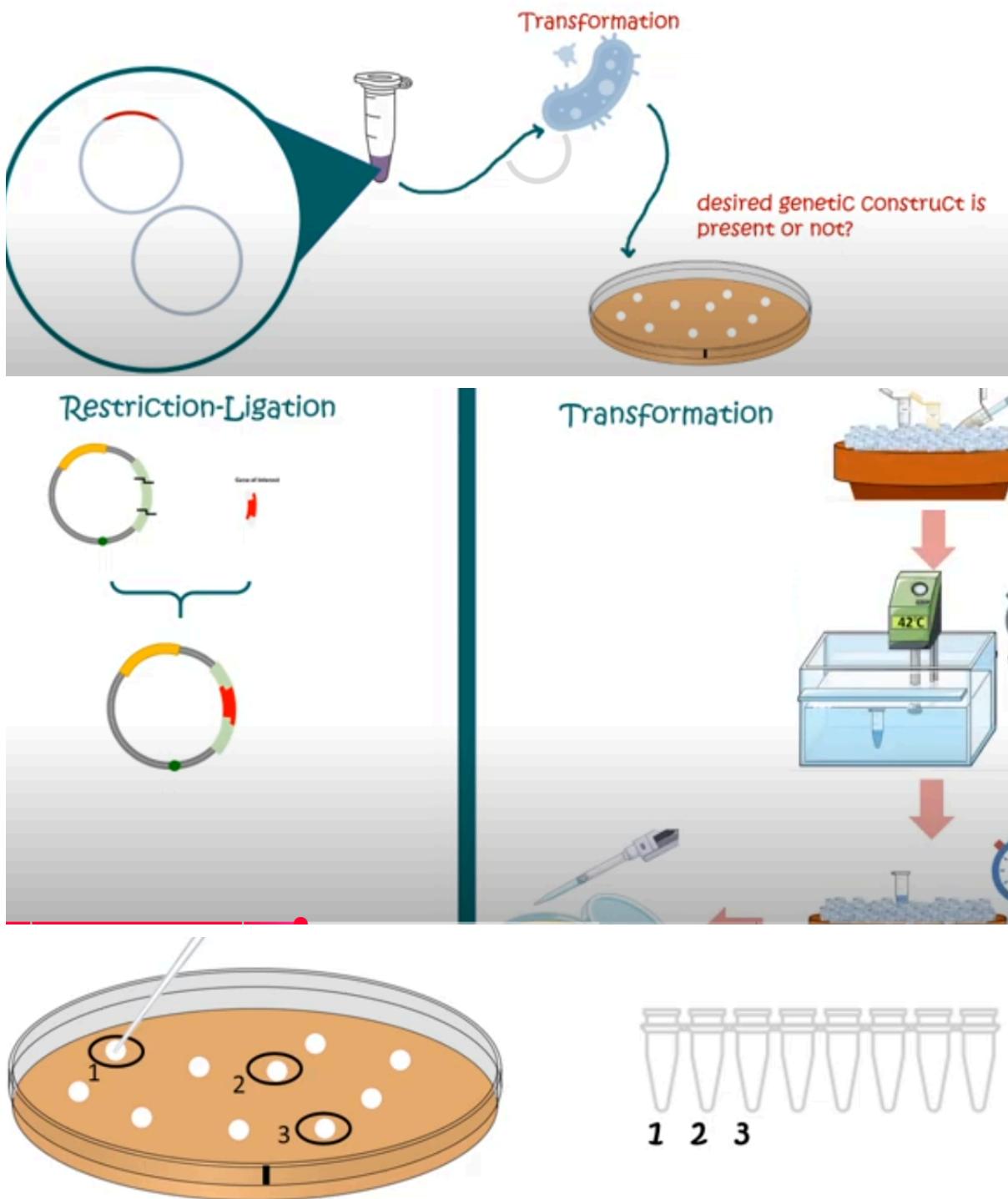


Vector is an artificially synthesized/ manipulated DNA whereas a plasmid naturally occurs in bacterial cells.

Principle of Colony PCR

The principle behind colony PCR relies on the amplification of specific DNA sequences from bacterial colonies.

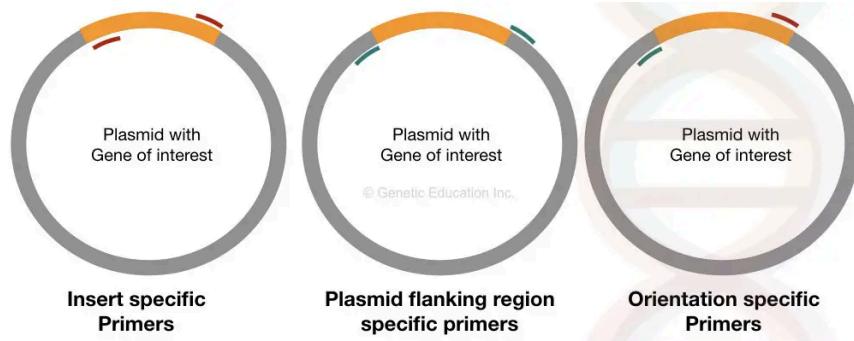
Colony PCR is a method for rapidly screening colonies of bacteria that have grown up on selective media following a transformation step, **to verify that the desired genetic construct is present**, or to amplify a portion of the construct.



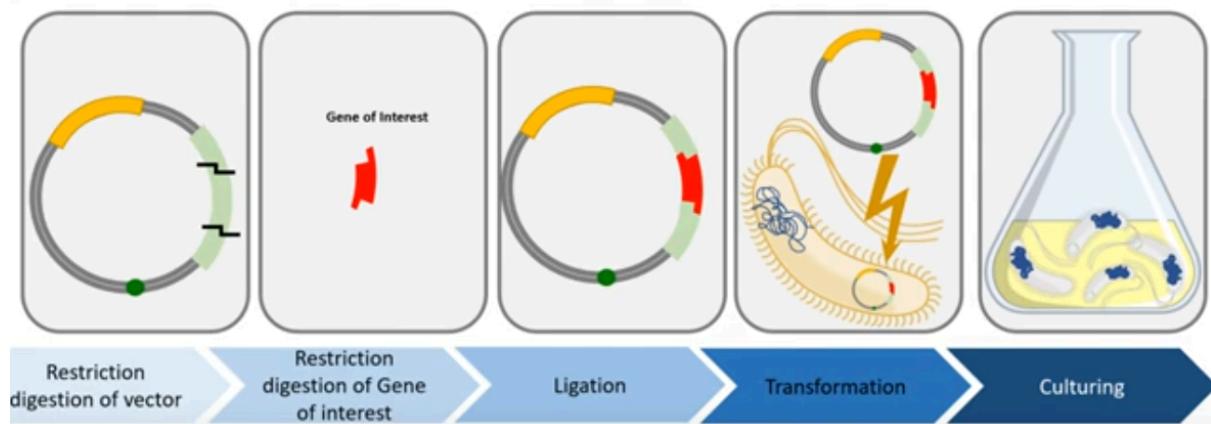
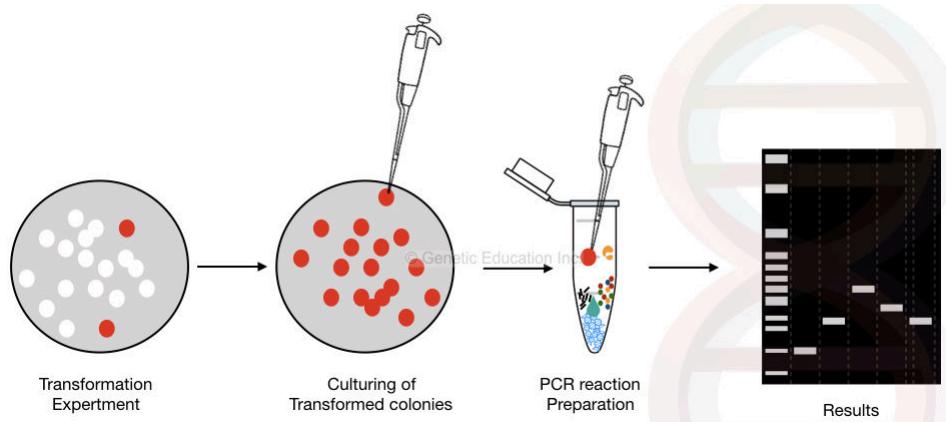
After bacterial transformation, individual colonies are picked and subjected to PCR using specific primers. These primers can be designed to:

- **Amplify the insert DNA** (Insert-specific primers)
- **Amplify the flanking regions around the insert** (Plasmid-specific primers)

Check the orientation of the inserted gene (Orientation-specific primers)



The PCR reaction selectively amplifies the plasmid DNA if it contains the insert, allowing researchers to directly determine the presence and orientation of the inserted DNA. The results are typically analyzed by **gel electrophoresis**.



Process of Colony PCR

1. Transformation:

After transformation (insertion of the gene of interest into a plasmid), the transformed bacteria are plated on selective media to allow for the growth of colonies containing plasmids.

2. Colony Selection:

Individual colonies are picked from the agar plate. These colonies contain bacteria that may or may not carry the plasmid with the insert.

3. Template Preparation:

Instead of extracting DNA, a colony is directly added to the PCR reaction. The bacterial cell wall is broken by a brief heat treatment to release the plasmid DNA. This step eliminates the need for DNA extraction.

4. PCR Amplification:

A PCR reaction mix is prepared with a mastermix containing Taq polymerase, primers, dNTPs, and reaction buffer. The primers used in the reaction are critical for amplifying specific regions of interest.

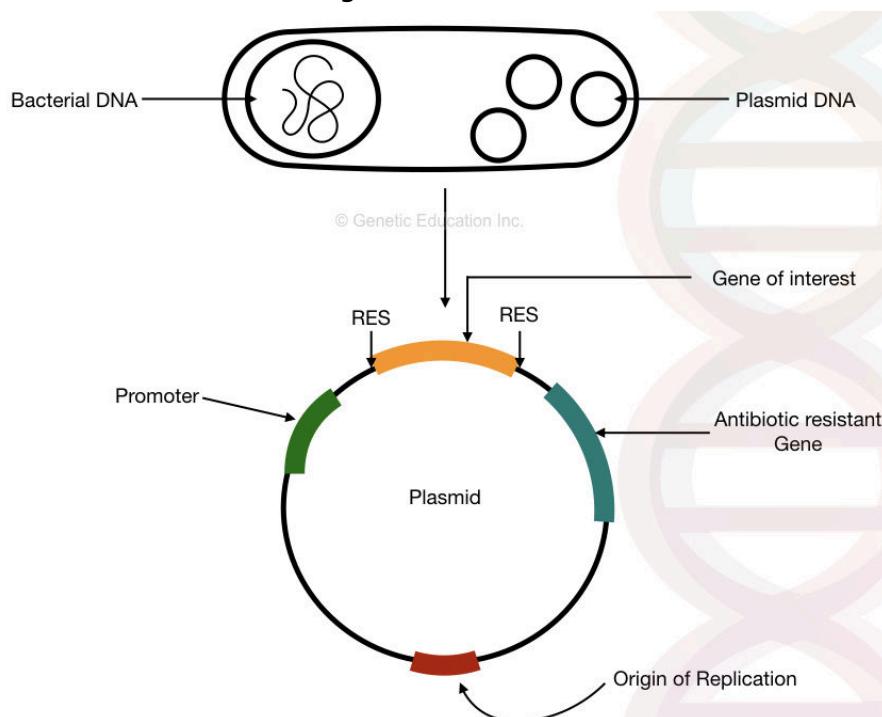
- **Insert-Specific Primers:** To check if the insert is present.
- Plasmid-Flanking Primers:** To amplify the region surrounding the insert and determine its size.
- Orientation-Specific Primers:** To verify if the insert is in the correct orientation.

5. PCR Cycling:

The PCR is carried out for 30–40 cycles, typically involving denaturation, annealing, and extension at appropriate temperatures.

6. Gel Electrophoresis:

The PCR products are loaded onto an agarose gel to assess the presence, size, and orientation of the insert. Bands corresponding to the correct amplicons indicate successful cloning.



Colony PCR Protocol

Here's a standard protocol for performing colony PCR:

1. Pick Colonies:

Use a sterile tool to pick several bacterial colonies and transfer them to a PCR tube.

Prepare Template:

Add 20 µL of **TE buffer or nuclease-free water** to the tube and heat the sample at **65°C for 20 minutes**. This step breaks open the bacterial cells and releases the plasmid DNA.

Centrifuge:

After heating, vortex the sample gently and centrifuge it at high speed for 2 minutes. This will pellet cellular debris, leaving the plasmid DNA in the supernatant.

Prepare PCR Reaction:

To the PCR tube, add 12 µL of **master mix**, 5 µL of **PCR reaction buffer**, 1 µL each of **forward** and **reverse primers**, and 3 µL of the supernatant.

PCR Setup:

Run the PCR reaction for 25 cycles with the following conditions:

- **Initial Denaturation:** 95°C for 3 minutes
- Denaturation:** 95°C for 10 seconds
- Annealing:** 55–65°C for 45 seconds
- Extension:** 72°C for 50 seconds
- Final Extension:** 72°C for 5 minutes

2. Gel Electrophoresis:

Analyze the PCR products on a **2% agarose gel** to check for the presence and size of the amplified DNA fragments.

Advantages of Colony PCR

- **Rapid and Cost-Effective:** Colony PCR is much faster than traditional DNA extraction and restriction digestion methods. Results can be obtained within a few hours.
- No DNA Extraction Required:** The technique uses bacterial colonies directly as templates, eliminating the need for time-consuming DNA purification steps.
- High Sensitivity:** The PCR amplification can detect even low amounts of DNA.
- Simple Setup:** The PCR setup is similar to conventional PCR, making it easy to implement in most labs.
- Avoids False Negative Results:** By using colony PCR, false negatives that may arise from incomplete DNA extractions are minimized.

Disadvantages of Colony PCR

- **Mutation Detection:** Colony PCR cannot detect mutations or SNPs (single nucleotide polymorphisms) within the insert. Sequencing is required for this purpose.
- **False Positives:** There is a risk of false-positive results, especially if the primers are not well-designed or if there is contamination.
- **Size Limitation:** The method may be less effective for very large inserts or for constructs with complex rearrangements.

Applications of Colony PCR

Colony PCR is primarily used in gene cloning and genetic engineering applications, including:

- **Gene Transfer:** Verifying the presence of the insert in transformed bacteria.
- **Gene Cloning:** Confirming the successful ligation of the gene of interest into a plasmid.
- **CRISPR-Cas9 Experiments:** Checking for successful edits or insertions in gene editing experiments.
- **Plasmid Screening:** Confirming that a plasmid contains the correct insert for downstream applications like protein expression.

Optimization of Colony PCR

For optimal results, several factors need to be carefully controlled:

- Primer Design:** Ensure primers are designed for specificity and efficiency. Insert-specific primers should amplify only the target insert, while plasmid-flanking primers should amplify the regions around the insert.
- Use of Controls:** Positive and negative controls help ensure the reaction is working as expected. Positive controls could involve using plasmids known to contain the insert, while negative controls could use untransformed bacteria.
- Colony Selection:** Using too many colonies can lead to non-specific amplification. Limit the number of colonies used for each reaction.
- PCR Cycles:** Adjust the cycle number to prevent over-amplification or premature termination of the reaction.
- Gel Electrophoresis:** Use an appropriate gel concentration (typically 2%) to resolve PCR products effectively.

Result Analysis in Colony PCR

After running the PCR products on an agarose gel, the results can be interpreted as follows:

Insert-Specific Primer: If the band corresponds to the expected size of the insert, it indicates that the gene of interest is present.

Flanking Region Primer: A larger band indicates the presence of the plasmid, along with the insert.

Orientation-Specific Primer: The band size can reveal whether the insert is in the correct orientation.

Example Gel Analysis:

Lane 1: DNA ladder (reference marker).

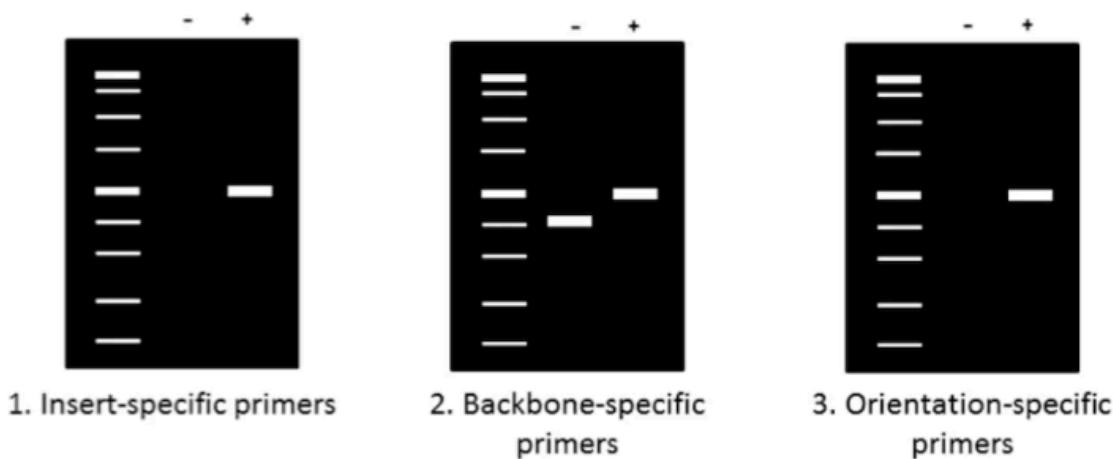
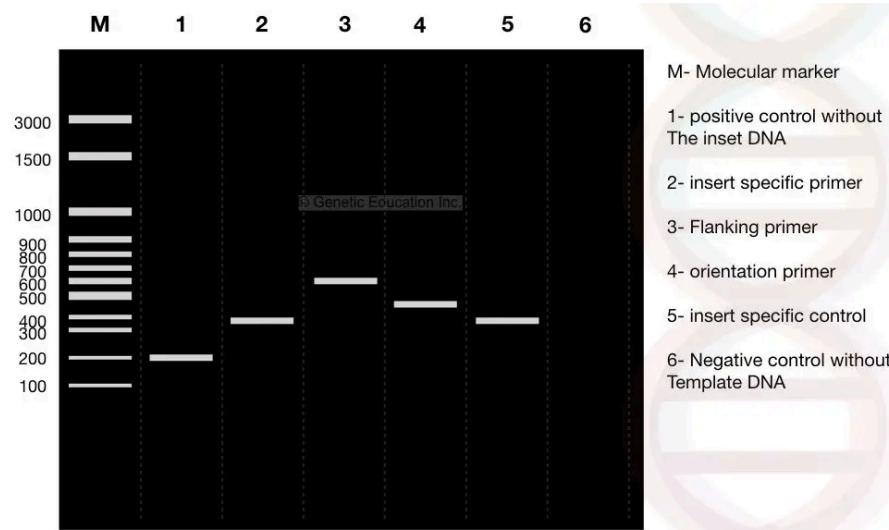
Lane 2: Insert-specific PCR product (400 bp).

Lane 3: Flanking region PCR product (600 bp).

Lane 4: Orientation-specific PCR product (between 400–600 bp).

Lane 5: Positive control for insert-specific primers.

Lane 6: Negative control for orientation-specific primers.



DNA is negatively charged because of its phosphate backbone, which contains negatively charged oxygen atoms in the phosphate groups (PO_3^{4-}). This negative charge is crucial for processes like DNA replication and transcription and allows DNA to be manipulated using electric fields, such as in gel electrophoresis where it moves toward the positive electrode.

