**Course content**

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7. **Gels:** agarose gel, isolating DNA fragments from agarose gels, polyacrylamide gels, isolating DNA fragments from polyacrylamide gels, pulse-field electrophoresis, capillary electrophoresis
8. **Blotting:** southern blots, northern blots, dot blots and slot blots
9. **the polymerase chain reaction:** standard polymerase chain reaction, nested polymerase chain reaction, multiplex polymerase chain reaction, amplification of longer DNA fragments
10. **PCR application:** reverse transcription polymerase chain reaction, rapid amplification of cDNA ends, amplification of coincidental products, classic quantitative polymerase chain reaction, real-time polymerase chain reaction.
11. **methods of gene isolation:** various methods of gene isolation

**Molecular biology**

**The molecular world for beginners**

* Molecular biology is the study of biology at a molecular level. The field overlaps with other areas of biology and chemistry, particularly genetics and biochemistry.
* Molecular biology chiefly concerns itself with understanding the interactions between the various systems of a cell, including the interrelationship of DNA, RNA and protein synthesis and learning how these interactions are regulated.
* Researchers in molecular biology use specific techniques native to molecular biology, but increasingly combine these with techniques and ideas from genetics and biochemistry.
* Molecular biology is the study of molecular underpinnings of the process of replication, transcription and translation of the genetic material.
* The central dogma of molecular biology where genetic material is transcribed into RNA and then translated into protein, despite being an oversimplified picture of molecular biology, still provides a good starting point for understanding the field
* Much of the work in molecular biology is quantitative, and recently much work has been done at the interface of molecular biology and computer science in bioinformatics and computational biology

**Substrate in molecular biology**

Substrate channeling (Tunneling) is the process of non-covalent direct transfer of a reaction intermediate from the active site of one enzyme to the catalytic center of a second enzyme without prior dissociation into the solvent. Substrate channeling can occur within protein matrix tunnels or along electrostatic highways crossing the surface of multifunctional enzymes, of tightly associated multienzyme complexes, or of transient multienzyme complexes.

Substrate channeling has been proposed

1) To decease the transit time of reaction intermediates

2) To prevent the loss of reaction intermediate by diffusion

3) To protect labile reaction intermediates from solvent

4) To sequester reaction intermediate that is toxic to the cell

5) To circumvent unfavorable equilibrium

6) To forestall the entrance of reaction intermediate into competing metabolic pathways

**EXAMPLES**

Examples of substrate channeling in molecular biology have been reported for numerous biochemical pathways, including purine & pyrimidine biosynthesis, glycolysis, the tricarboxylic acid cycle , DNA replication , RNA synthesis & protein synthesis .

**Laboratory Safety Guidelines**

These are as follows: 1. Do not consume/store food or beverages in the laboratory

2. Do not allow visitors, including children, in laboratories where hazardous substances are stored or are in use or hazardous activities are in progress.

3. Do not wear open shoes or shorts in the lab.

4. Lab coats are mandatory in BSL1 laboratories. Wear gloves and safety goggles when handling hazardous material. It is strongly recommended not to wear contact lenses, also not in combination with safety goggles.

5 .When leaving the lab, remove gloves and lab coats. No lab coats and gloves in offices, seminar rooms, toilets and kitchen! Outside the lab area, gloves have to be removed especially in elevators and when touching doors!

6. Hands should be washed after handling biological material, after removing gloves and when leaving the laboratory.

7. Do not pipette by mouth; only mechanical pipetting devices are permitted.

8. Perform all procedures carefully to minimize the creation of splashes or aerosols.

9. The laboratory should be kept clean and free of material not pertinent to the work. Work surfaces should be decontaminated at least once a day with 70% alcohol.

10. Any spill has to be removed immediately. Put up warning signs if floor is wet.

11. The use of needles and syringes should be restricted. When used, care must be taken to avoid injuries. Dispose needles, syringes, broken glassware and other sharp things into appropriate containers.

12. Contaminated liquids or solid materials have to be decontaminated before disposal

**Fundamental methods**

**Differences in nucleic acid**:

Nucleic acid types differ in the structure of the sugar in their nucleotides–DNA contains 2'-deoxyribose while RNA contains ribose (where the only difference is the presence of a hydroxyl group). Transfer RNA (tRNA) molecules contain a particularly large number of modified nucleosides. Messenger RNA (mRNA) is a single-stranded RNA molecule. Ribosomal RNA (rRNA), molecule synthesizes protein molecule.

**Differences in DNA and RNA:**

|  |  |  |
| --- | --- | --- |
| **comparison** | **DNA** | **RNA** |
| Function | DNA replicates and stores genetic information. It contains all genetic information present within an organism. | RNA converts the genetic information contained within DNA for the synthesis of protein. |
| Structure | DNA consists of two strands, arranged in a double helix. | RNA only has one strand, and have chain shorter than DNA |
| Base pairs | Adenine and Thymine pair (A-T)  Cytosine and Guanine pair (C-G) | Adenine and Uracil pair (A-U)  Cytosine and Guanine pair (C-G) |
| Location | DNA is found in nucleus, and small amount of DNA also present in mitochondria. | RNA forms in the nucleolus, and then moves to specialized regions of the cytoplasm depending on the type of RNA formed. |
| Ultraviolet sensitivity | DNA is vulnerable to damage by ultraviolet light. | RNA is more resistant to damage from UV light than DNA. |

**Precipitation and concentration of alcohol:**

**Precipitation of alcohol:** Ethanol is alcohol; ethanol precipitation is a commonly used technique for concentrating and de-salting nucleic acids (DNA or RNA) preparations in aqueous solution. The basic procedure is that salt and ethanol are added to the aqueous solution, which forces the precipitation of nucleic acids out of solution.

**Concentration of alcohol:** The concentration of alcohol in a beverage is usually stated as the percentage of alcohol by volume (ABV the number of milliliters (ml) of pure ethanol in 100 ml of beverage) or as proof. In the United States, proof is twice the percentage of alcohol by volume at 60 degrees Fahrenheit (e.g. 80 proof = 40% ABV).

***Savant speedvac:***

The Savant speedvac is a high capacity DNA SpeedVac designed for efficient processing of large batches of DNA/RNA samples. The Savant DNA120 is a high performance DNA SpeedVac Concentrator offering rapid and efficient concentration and drying of small-volume DNA/RNA samples.

**Characteristics:**

* Ideal for drying nucleic acids, PCR preparations and synthetic trace elements
* Drying at room temperature or low temperature to ensure DNA/RNA protection
* Dual timer independent of heat.
* DNA 130 features:
* Small capacity
* Delivered with: complete system with RD36 rotor for 36 X 1.5 - 2 ml microtubes and integrated pump
* Display: LED (temperature, cycle duration and heating time)
* Solvent guide : Ethanol, methanol, water and ACN, PCR buffer
* Lid: transparent acrylic with safety lock

**Salting out:**

The process of "salting out" is a purification method that relies on the basis of protein solubility. It relies on the principle that most proteins are less soluble in solutions of high salt concentrations because the addition of salt ions shields proteins with multi-ion charges.

Salting out is an effect based on the electrolyte–non-electrolyte interaction, in which the non-electrolyte could be less soluble at high salt concentrations. It is used as a method of purification for proteins, as well as preventing protein denaturation due to excessively diluted samples during experiment. The salt concentration needed for the protein to precipitate out of the solution differs from protein to protein. This process is also used to concentrate dilute solutions of proteins. Dialysis can be used to remove the salt if needed.

**Purification of nucleic acids:**

We have developed a simple, rapid, and reliable protocol for the small-scale purification of DNA and RNA from, e.g., human serum and urine. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent. By using size-fractionated silica particles, nucleic acids (covalently closed circular, relaxed circular and linear double-stranded DNA; single-stranded DNA; and rRNA) could be purified from 12 different specimens in less than 1 h and were recovered in the initial reaction vessel. Purified DNA (although significantly sheared) was a good substrate for restriction endonucleases and DNA ligase and was recovered with high yields (usually over 50%) from the picogram to the microgram level. Co purified rRNA was recovered almost undegraded. Substituting size-fractionated silica particles for diatoms (the fossilized cell walls of unicellular algae) allowed for the purification of microgram amounts of genomic DNA, plasmid DNA, and rRNA from cell-rich sources, as exemplified for pathogenic gram-negative bacteria. In this paper, we show representative experiments illustrating some characteristics of the procedure which may have wide application in clinical microbiology.

**Phenol chloroform extraction:**

Phenol chloroform extraction is a liquid-liquid extraction technique in molecular biology used to separate nucleic acid from proteins and lipids.

**Process:**

Aqueous samples, lysed cells, or homogenized tissue are mixed with equal volumes of a phenol: chloroform mixture. After mixing, the mixture is centrifuged and two distinct phases are formed, because the phenol: chloroform mixture is immiscible with water. Phenol only has a slightly higher density than water therefore it is mixed with chloroform to form a mixture with a much higher density than water. The aqueous phase is on top because it is less dense than the organic phase (phenol: chloroform). The hydrophobic lipids will partition into the lower organic phase, the proteins will remain at the interphase, while the nucleic acids (as well as other contaminants such as salts, sugars, etc.) remain in the upper aqueous phase. The upper aqueous phase is pipetted off and care is taken to avoid pipetting any of the organic phase or material at the [interface](https://en.wikipedia.org/wiki/Interface_(chemistry)). This procedure is often performed multiple times to increase the purity of the DNA.

If the mixture is acidic, [DNA](https://en.wikipedia.org/wiki/DNA) will precipitate into the organic phase while [RNA](https://en.wikipedia.org/wiki/RNA) remains in the aqueous phase due to DNA being more readily neutralized than RNA.

This procedure yields large double stranded DNA that can be used in [PCR](https://en.wikipedia.org/wiki/Polymerase_chain_reaction) or [RFLP](https://en.wikipedia.org/wiki/Restriction_fragment_length_polymorphism). The disadvantages of this technique in forensic use, is that it's time-consuming, uses hazardous reagents and because it is a two-step process involving transfer of reagents between tubes it is at a greater risk from [contamination](https://en.wikipedia.org/wiki/Contamination)

**Precipitation of DNA by polyethylene glycol:**

We present here conditions for precipitation of DNA by PEG that gives improved recoveries of DNA so that precipitation by PEG is comparable to that by ethanol, in terms of recovery, over a wide range of DNA concentration. The problem of precipitation of low molecular weight DNA fragments by PEG was also investigated.

The recovery of DNA obtained on precipitation with PEG or ethanol was determined as follows: HindIII-digested Puc13 DNA was labeled with [α-³²P]dATP by end filling using klenow fragment of DNA polymerase 1 and the labeled DNA precipitated by adding 0.5 volumes of 40%(w/v)PEG 8000(sigma) or 2 volume of 95%(v/v) ethanol and keeping for 10 min at the volume of desired temperature . The precipitated DNA was then collected by centrifugation at the desired temperature for 30 min and the radioactivity in the pellet estimated by measuring Cerenkov radiation.

**Determination of Concentrations of Nucleic Acids in Solutions**

Before analysis, larger macroscopic organisms were removed from the sediment samples.

* All of the materials used for nucleic acid analysis were carefully cleaned by soaking in 1 N NaOH–10% HCl–MilliQ water to remove organic matter contamination.
* All of the solutions were prepared with MilliQ water and then autoclaved.

Amounts of DNA and RNA were determined by the spectrophotometric and fluorometric.

For each method, internal standards of calf thymus DNA and baker’s yeast RNA (5 to 10 μg) were added to replicate subsamples before extraction. The final yields of the internal standards of DNA and RNA were on average respectively, 60 and 85% for the spectrophotometric method and 55 and 80% for the fluorometric method.

1. **Spectrophotometric method.**

Determination of concentrations of nucleic acids by optical density with aid of absorption spectrometry is done by the use of spectrophotometer.

Nucleic acid extraction was done as following:

* 1 g of sediment (three replicates) was treated with 3.0 ml of 0.5 N perchloric acid, stirred for 3 min, and sonicated three times for 1 min (with intervals of 30 s).
* Nucleic acid extraction was carried out at 75°C for 30 min under continuous stirring.
* After centrifugation (3,000 × *g*, 10 min), the absorbance of the total nucleic acid content (TNA) in the supernatant was measured at 260 nm.
* DNA absorbance was determined with a diphenylamine (2% in acetic acid) light-activated reaction (40 W, 12 h) at 598 nm and converted to concentration, using standard solutions of calf thymus DNA.
* DNA concentration was then reported as equivalent of absorbance at 260 nm in order to calculate by difference the absorbance due to RNA as following:

ABSRNA = ABSTNA − ABSDNA

Where ABSRNA is the absorbance of RNA, ABSTNA is the absorbance of TNA, and ABSDNA is the absorbance of DNA.

* RNA absorbance (260 nm) was then converted to concentration, using standard solutions of baker’s yeast RNA.

1. **Fluorometric method**

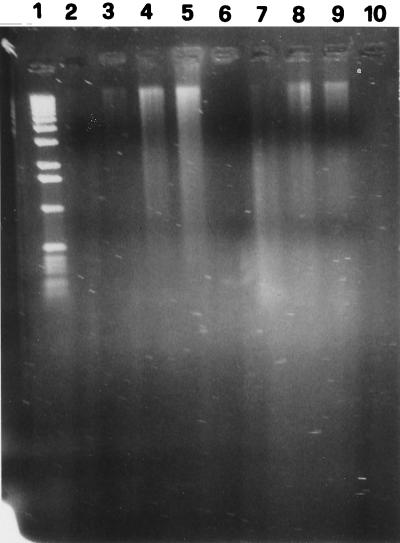
Marine sediments were selected for nucleic acids determination.

To extract nucleic acids from sediments, we used sodium dodecyl sulfate (SDS), which is efficient in cellular lysis and is commonly used in protocols for extracting nucleic acids from marine sediments. Nucleic acid extraction for fluorometric determination was carried out using organic method.

The sediments were centrifuged and the suspensions collected according to organic method and enough resuspention obtained for flourometric studies.

* Before fluorometric analysis, an aliquot of the resuspension was analyzed by gel electrophoresis to ensure the presence of nucleic acids in the extract. As seen below in figure.

**Procedure:**

Steps in flourometric method are following:

1. Subsamples were then analyzed with two fluorescent dyes.
2. Thiazole orange dye was used for TNA(total nucleic acids)
3. Hoechst 33258 (Hoechst) dye was used for DNA determination studies.
4. Fluorescence was measured with a Perkin-Elmer LS50B spectrofluorometer (thiazole orange, 511-nm excitation and 533-nm emission & Hoechst, 360-nm excitation and 460-nm emission).
5. DNA concentrations were calculated from calf thymus DNA standards stained with **Hoechst**. The same DNA standards were stained with thiazole orange to determine the DNA contribution to the **total** **thiazole** **orange** fluorescence.
6. The RNA contribution in the thiazole orange fluorescence (FTORNA) was estimated as:

FTORNA = FTOTNA − FTODNA

Where FTOTNA is the fluorescence of TNA after staining with thiazole orange and FTODNA is the calculated contribution of DNA in the thiazole orange fluorescence (as determined from Hoechst staining).

1. RNA concentrations were calculated from baker’s yeast RNA standards after thiazole orange staining.
2. **Agarose Gel Electrophoresis**

This method may also be used for determining concentrations of nucleic acids and visualizing DNA entities.

To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are required.

1. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field.
2. The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, the DNA is separated by size.
3. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity.
4. Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA will be distinct.
5. Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard.

For example, if a 2µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/µl (100ng divided by 2µl). Standards used for quantization should be labeled as such and be the same size as the sample DNA being analyzed.

* In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal.

**SMALL-SCALE PREPARATIONS OF PLASMID DNA**

Mini preparations of plasmid DNA can be obtained either by the alkaline lysis method or by the boiling method

**Boiling method**

**Introduction**:

The classic alternative to the alkaline lysis method for plasmid DNA preparation is that of Holmes and Quigely and is commonly known as rapid boiling method.

This is rapid and convenient method for making large no of small scale plasmid preparations (minipreps). The DNA is of good quality and can be used directly for restriction enzyme digestion and sub coiling. With only small amount of further processing, it can be used as template for DNA sequencing. The rapid boiling method is however is less convenient for large-scale preparation, for which the alkaline lysis method is better suited.

**Material:**

1. STET: 5%tritonX-100, 50mM Tris –HCl (pH8.0), 50mM EDTA (pH8.0), and 8% sucrose. Can be stored at room temperature
2. Lysozyme; dry powder. Store at -20 0C
3. ISOPROPANOL: propan-2-ol
4. 70% ethanol
5. TE: 10mM tris-HCl ,pH 7.5, 1mM EDTA
6. A boiling water bath: a beaker of water heated by Bunsen burner will suffice. An opened bottom tube rack is required because the tubes must be directly placed in water to achieve rapid heating.

**Method:**

1. set up a culture for each miniprep by inoculating 2-3 mL of L-broth, containing an appropriate antibiotic (e.g.,100μg/Ml AMPICILLIN)WITH A BACTERIAL COLONY.GROW OVERNIGHT AT 37 0C with vigorous shaking.
2. Before starting the miniprep, start boiling the water and makeup a fresh 1mg/ml lysozyme STET mix
3. Fill a 1.5-mL labeled microfuge tube with an aliquot from each culture. Pellet the bacteria by centrifugation for 1min at 12000g. Carefully aspirate off the supernatant on a water pump using a drawn –out Pasteur pipette.
4. Vortex each pellets for a few seconds to break up the pellet. Add200μL of STET to each tube. The pellet should now easily resuspend by vortexing.
5. Immediately place the tubes in the open bottom rack and place in boiling water for eaxctly45s. Ensure each tube is least half submerged.
6. Centrifuge the tubes at 12000g for 10 mints. a large, sticky and loose pellet should form
7. Remove the pellet from each tube by fishing it out with a wooden toothpick. Since the pellet is quite slippery, it is useful to have a paper tissue at the top of the tube to catch the pellet and prevent it from slipping back down into the tube.
8. Add 200μL of isopropanol to each tube, and centrifuge at 12,000g for 5min.
9. Aspirate off the supernatant, and wash the pellet in 500 μL of 70%ethanol.

Air dries the pellet for 10min, and resuspends each in 100 μL of TE buffer. Vortex and shake for 10min before use to ensure complete dissolution.

1. Use2-10 μL (equivalent to 100mg of plasmid for most vectors) per restriction enzyme digest and analyze by gel electrophoresis.

**Notes:**

* If the plasmid has high copy no, growth time can be reduced to approx6h, enabling the whole process to be carried out in a day.
* The short centrifugation time leaves a loose pellet that is easier to resuspend.
* If the pellet does not readily resuspend, pipette the solution up and down to dislodge it. Don’t stick the pellet directly up into the pipette tip.

**Preparation of genomic DNA**

**Extraction of chromosomal bacterial DNA BY DIATOMS (Total or genomic DNA)**

1. **Preparation of diatoms suspension:**
2. Add50ml H2O and 500 μL of 35% HCl to 10g celite or diatoms.
3. Divide the suspension in aliquots into small bottles which are tightly closed and autoclave, keep in room temperature in the dark.
4. **Buffers:**
5. **Lysis buffer (L6)**

Dissolve 30g GuScN into 25ml of 0.1 M Tris HCl (pH 6.4) +5.5ml of 0.2M EDTA. Adjusted with NaOH to pH 8.0+ 0.65ml Triton X-100. Homogenize the solution in shaking water bath at 600C stable at room temp. in the dark for at least 3 weeks).

1. **Washing buffer(L2)**

Dissolve 30gm of GuscN IN 25 OF 0.1M Tris Hcl (pH 6.4) by heating in shaking water bath at 60 -650C (stable at room temperature in the dark for at least 3 weeks).

1. **Elution buffer(TE)**

* 10 mM tris –HCl
* 1 Mm EDTA pH8
* 100X concentrated stock solution must dilute to 1X working solution. These buffers prepared under fume hood.

**Reaction vessel:**

Add 900 μL of lysis buffer (L6) and 40 μL of diatoms suspension (shake well before use) to 1.5 ml ep-tube homogenize the solution by vortexing. Reaction vessel can be successfully used up to 1 week after assembly.

1. **Method:**
2. Vortex the reaction vessel.
3. Vortex the overnight bacterial broth culture and take 1ml in eppindrof tube or 50 μL sample( urine or serum)
4. Centrifuge at 13000 for 15 min, then bacterial pellet is suspended in 50 μL autoclaved distilled water or TE buffer vortex.
5. Add the suspended pellet to the reaction vessel.
6. Leave at room temperature for 10 min. vortex several times during the incubation time.
7. Centrifuge for 2min at 13000rpm.
8. Dispose the supernatant. Diatom NA pellet is washed twice with washing buffer L2 the twice. With ethanol 70% and once with acetone. Vortex them centrifuge for one minute each time.
9. Dry the vessel at 56 0C with open lid ep-tube ( use the thermal cycler or eppendrof heat block) for 10 min.
10. Add elution buffer TE with or without Rnase then vortex briefly and incubate for 10 min at 0C (100-75 μLTE ) vortex again and centrifuge at 13000 for 3min.
11. Use the supernatant containing DNA for further experiment.

**THE TOOLS**

**Restriction enzyme**

A **restriction enzyme** is a protein that recognizes a specific, short nucleotide sequence and cuts the DNA *only* at that specific site, which is known as **restriction site** or **target sequence**.

More than 400 restriction enzymes have been isolated from the bacteria that manufacture them. In live bacteria, restriction enzymes function to defend the cell against invading viral bacteriophages. Restrictions sites in the viral genome are cleaved by the bacterium's restriction enzymes, fragmenting and destroying the DNA of invading bacteriophages before it can incorporate into the host's genome and take over the cell.

A bacterium is immune to its own restriction enzymes, even if it has the target sequences ordinarily targeted by them. This is because the bacterial restriction sites are highly methylated, making them unrecognizable to the restriction enzyme.

As an example of how a restriction enzyme recognizes and cuts at a DNA sequence, let's consider *Eco*RI, a common restriction enzyme used in labs. *Eco*RI cuts at the following site:

5'-...GAATTC...-3'
3'-...CTTAAG...-5'

**EcoRI** site

5'-...GAATTC...-3' 3'-...CTTAAG...-5'

**EcoRI** site

When EcoRI recognizes and cuts this site, it produces ends with single-stranded DNA “overhangs”:

An *Eco*RI enzyme binds to an *Eco*RI site in a piece of DNA and makes a cut on both strands of the DNA. The pattern of the cut is:

5'-...G|AATTC...-3'
3'-...CTTAA|G...-5'

Thus, it produces an overhang  of 5'-AATT-3' on each end of the cut DNA.

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5'-...G|AATTC...-3' 3'-...CTTAA|G...-5'

Thus, it produces an overhang of 5'-AATT-3' on each end of the cut DNA.

If another piece of DNA has matching, the overhangs can stick together by complementary base pairing. For this reason, enzymes that leave single-stranded overhangs are said to produce **sticky ends**. Sticky ends are helpful in cloning because they hold two pieces of DNA together so they can be linked by DNA ligase.

Not all restriction enzymes produce sticky ends. Some are “blunt cutters,” which cut straight down the middle of a target sequence and leave no overhang. The restriction enzyme *Sma*I is an example of a blunt cutter:

A *Sma*I enzyme binds to the *Sma*I restriction site, which is:

5'-...CCCGGG...-3'
3'-...GGGCCC...5'

It makes a cut right in the middle of this sequence on both strands, producing blunt ends. The cut sites are:

5'-...CCC|GGG...-3'
3'-...GGG|CCC...5'

A *Sma*I enzyme binds to the *Sma*I restriction site, which is:

5'-...CCCGGG...-3' 3'-...GGGCCC...5'

It makes a cut right in the middle of this sequence on both strands, producing blunt ends. The cut sites are:

5'-...CCC|GGG...-3' 3'-...GGG|CCC...5'

Blunt-ended fragments can be joined to each other by DNA ligase. However, blunt-ended fragments are harder to ligate together (the ligation reaction is less efficient and more likely to fail) because there are no single-stranded overhangs to hold the DNA molecules in position.

**Nomenclature**

Since their discovery in the 1970s, many restriction enzymes have been identified; for example, more than 3500 different Type II restriction enzymes have been characterized. Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial [genus](https://en.wikipedia.org/wiki/Genus), [species](https://en.wikipedia.org/wiki/Species) and [strain](https://en.wikipedia.org/wiki/Strain_(biology)).For example, the name of the [EcoRI](https://en.wikipedia.org/wiki/EcoRI" \o "EcoRI) restriction enzyme was derived as shown in the box.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  |  |  | | --- | --- | --- | | Abbreviation | Meaning | Description | | E | *Escherichia* | Genus | | Co | *coli* | Specific Species | | R | RY13 | Strain | | I | First identified | Order of identification in bacterium |   Derivation of EcoR1 name |

# Restriction digestion

Restriction enzyme digestion takes advantage of naturally occurring enzymes that cleave DNA at specific sequences. There are hundreds of different restriction enzymes, allowing scientists to target a wide variety of recognition sequences.

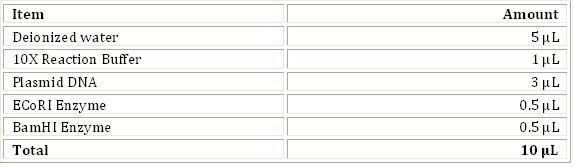
Restriction enzyme digestion is commonly used in molecular cloning techniques, such as [PCR](https://www.addgene.org/plasmid-protocols/pcr-cloning/) or [restriction cloning](https://www.addgene.org/plasmid-protocols/subcloning/). It is also used to quickly check the identity of a plasmid by [diagnostic digest](https://www.addgene.org/plasmid-protocols/diagnostic-digest/).

# Materials Required

* Microcentrifuge tubes
* Vial stand
* 10µl pipette
* Pipette tips
* Beaker
* Table top mini centrifuge
* Incubator
* Reagents
* Gel electrophoresis

# Procedure

* Select restriction enzymes to digest your plasmid
* Determine an appropriate reaction buffer for enzyme
* Transfer the following solutions in a micro centrifuge tube.



* Incubate the mixture at 37 o C for 1 h to overnight. Keep the tubes in -4o C freezer or in -20o C freezer, after the incubation
* To visualize the results of your digest, conduct [gel electrophoresis](https://www.addgene.org/plasmid-protocols/gel-electrophoresis/).

## Precaution

* Make sure that the restriction enzyme does not exceed more than 10% of the total reaction volume, Otherwise the glycerol and the EDTA in the enzyme storage buffer may inhibit digestion process.

**Difficulties associated with restriction digestion**

The restriction enzyme did not cleave efficiently. If cleaving near the end of a PCR **fragment** leave at least 6 bases past the restriction site. Test the restriction enzyme on a control substrate.  
\* The restriction enzyme was not completely inactivated. Phenol/ETOH purifies the DNA if the enzyme cannot be heat inactivated.  
\* Star activity from the restriction digest cleaved the vector or insert. Check the DNA on a gel. If there is an extra band, reduce the amount of enzyme or time for the restriction digest.  
\* The DNA or restriction enzyme contained exonuclease or phosphatase that damaged the ends. Phenol/ETOH purifies the DNA. Check the enzyme QC data and notes. If the ligation QC is poor or the exonuclease level is high reduce the amount of enzyme or incubation time.

**Gels**

1. **Agarose gels:**

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix.

**Isolating DNA fragments from agarose gel**

Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels used are between 0.7–2percent dissolved in a suitable electrophoresis buffer.

1. **Polyacrylamide gels**

Polyacrylamide gel electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Electrophoretic mobility is a function of the length, conformation and charge of the molecule.

Polyacrylamide gel electrophoresis is a powerful tool used to analyze RNA samples. When polyacrylamide gel is denatured after electrophoresis, it provides information on the sample composition of the RNA species. Hydration of acrylonitrile results in formation of acrylamide molecules(C 3H5 NO) by nitrile hydratase. Acrylamide monomer is in a powder state before addition of water. Acrylamide is toxic to the human nervous system, therefore all safety measures must be followed when working with it. Acrylamide is soluble in water and upon addition of water it polymerizes resulting in formation of polyacrylamide. It is useful to make polyacrylamide gel via acrylmide hydration because pore size can be regulated. Increased concentrations of acrylamide result in decreased pore size after polymerization. Polyacrylamide gel with small pores helps to examine smaller molecules better since the small molecules can enter the pores and travel through the gel while large molecules get trapped at the pore openings. As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure. This method is called native PAGE.

1. **Pulse Field Gel electrophoresis**

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction.

1. **Capillary electrophoresis**

Capillary electrophoresis (CE) is a family of electro kinetic separation methods performed in sub millimeter diameter capillaries and in micro- and nanofluidic channels.

**Blotting**

**Introduction**

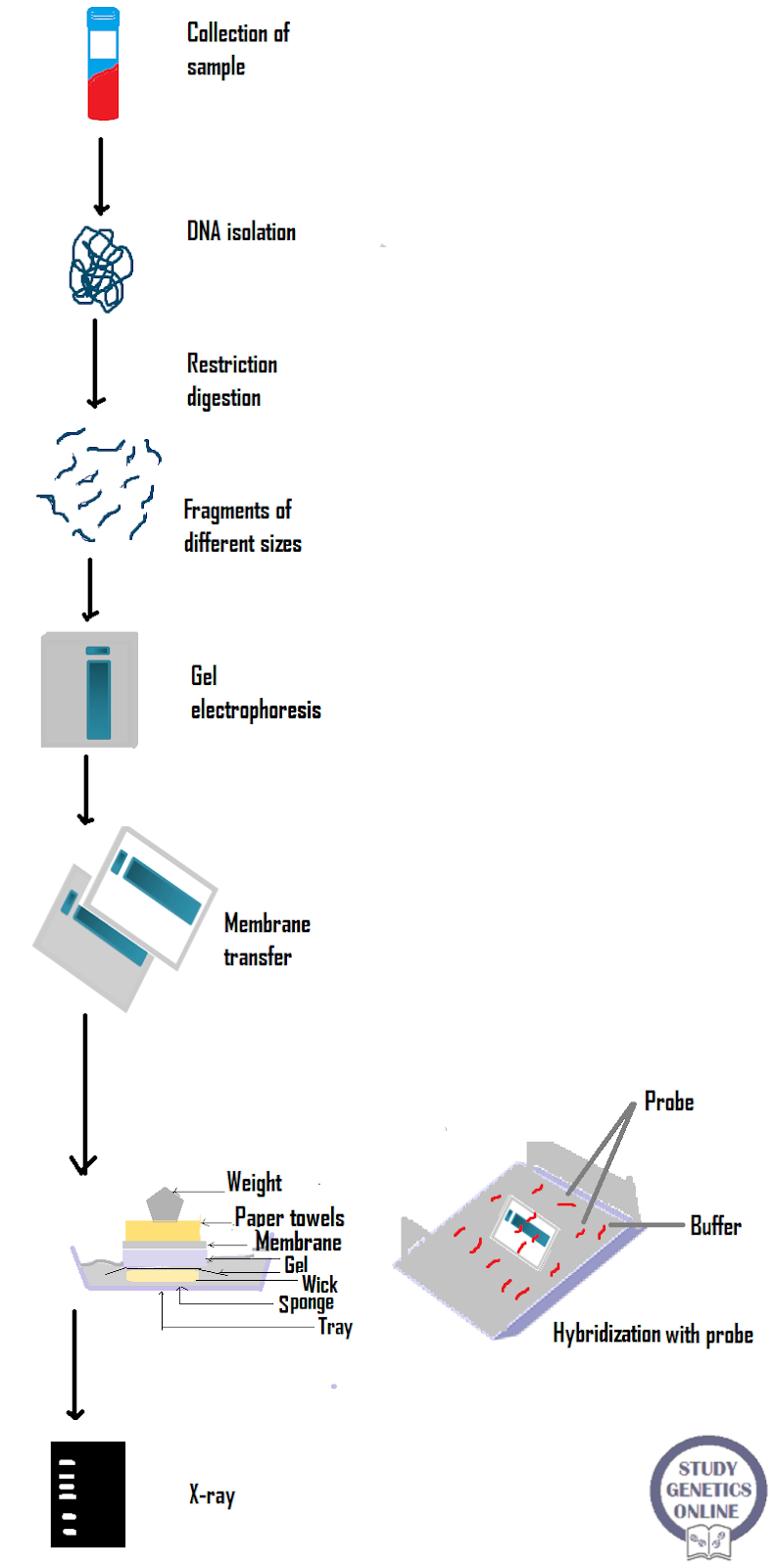
Blotting is technique in which nucleic acids i.e., RNA and DNA or proteins are transferred onto a specific membrane. This membrane may be nitrocellulose PVDF or nylon membrane. This process can be done just after the gel electrophoresis, by transferring the molecules from the gel onto the surface of blotting membrane. But sometimes it can be done by directly transferring the molecules onto the membrane. And then we can visualize these transferring molecules by using staining. Examples: Ethidium bromide, Crystal violet, Safranine and Ossmium tetroxide etc

**Types of Blotting**

These are:

1. Southern blotting
2. Northern blotting
3. Dot /slot blotting
4. **Southern blotting**

Southern blotting is named after Edward M. Southern. This method is used for analysis of DNA sequences It involves the following steps:

• Firstly, large weighted DNA is cut into small fragments by using Restriction endonucleases

• Then, these fragments are electrophoresed on separating gel so that they can separate according to their size.

• If DNA fragments are much larger in size so firstly the gel should be treated with HCl, causes depurination of DNA fragments

• After separating these fragments, placed a nitrocellulose sheet over the separating gel. Apply pressure over the membrane so that proper interaction can occur between these two.

• After that the membrane is exposed to ultraviolet radiation so that the fragments are permanently attached to the membrane

• Then the membrane is exposed to hybridization probe. But the DNA probe is labeled so that it can easily detect, when the molecule is tagged with a chromogenic dye.

• After hybridization process, excess probe is washed away by using SSC buffer and it can be visualized on X-ray film with the help of autoradiography.

**Applications**

1. It is used in the technique called RFLP (Restriction fragment length polymorphism) mapping
2. Also used in phylogenetic analysis
3. To identify the gene rearrangements.

**2. Northern blotting**

Northern blotting is given by Alwine. This method is used to analyze and detection of RNA in a sample.

• Firstly, extract and purify mRNA from the cells.

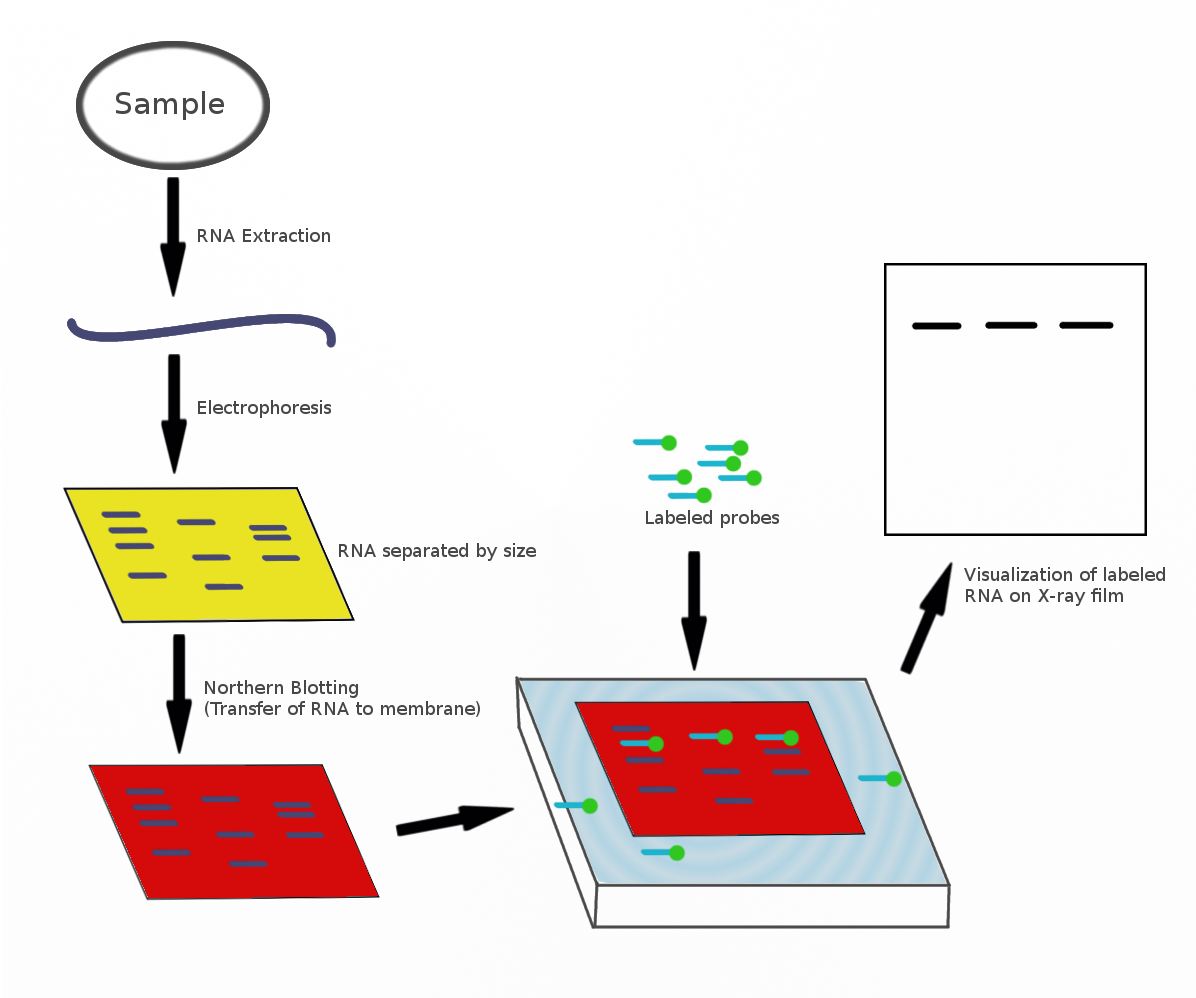
• Separate these RNA on agarose gels containing formaldehyde as a denaturing agent for the RNA.

• This gel is immersed in depurination buffer for 5-10 minutes and washed with water

• Then transfer these RNA fragments onto the carrier membrane i.e. aminobenzyloxymethyl filter paper.

• After transferring the RNA, it is fixed to the membrane by using UV or heat.

• Add DNA labeled probe for hybridization



Wash off the unbound probe and at the end mRNA-DNA hybrid are then detected by X-ray film

**Applications:**

1. Used in screening

**3. Dot/ slot blotting**

A **dot blot** (or **slot blot**) is a technique in [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology) used to detect proteins. It represents a simplification of the [western blot](https://en.wikipedia.org/wiki/Western_blot) method, with the exception that the proteins to be detected are not first separated by [electrophoresis](https://en.wikipedia.org/wiki/Electrophoresis). Instead, the sample is applied directly on a membrane in a single spot, and the blotting procedure is performed.

The technique offers significant savings in time, as [chromatography](https://en.wikipedia.org/wiki/Chromatography) or [gel electrophoresis](https://en.wikipedia.org/wiki/Gel_electrophoresis), and the complex blotting procedures for the gel are not required. However, it offers no information on the size of the target protein

**Method:**

1. A general dot blot protocol involves spotting 1–2 microliters of a samples onto a [nitrocellulose](https://en.wikipedia.org/wiki/Nitrocellulose) or [PVDF](https://en.wikipedia.org/wiki/PVDF) membrane
2. letting it air dry
3. [](https://en.wikipedia.org/wiki/File:Dot_blot_de_ADN.jpg)Samples can be in the form of tissue culture supernatants, blood serum, cell extracts, or other preparations.
4. The membrane is incubated in blocking buffer to prevent non-specific binding.
5. It is then incubated with a primary antibody followed by detection antibody or a primary antibody conjugated to a detection molecule (commonly [HRP](https://en.wikipedia.org/wiki/Horseradish_peroxidase) or [alkaline phosphatase](https://en.wikipedia.org/wiki/Alkaline_phosphatase)).
6. After antibody binding, the membrane is incubated with a [chemiluminescent](https://en.wikipedia.org/wiki/Chemiluminescent" \o "Chemiluminescent) substrate and imaged.

**Uses:**

* Performing a dot blot is similar in idea to performing a western blot, with the advantage of faster speed and lower cost.
* Dot blots are also performed to screen the binding capabilities of an antibody

**Polymerase chain reaction (PCR)**

Polymerase chain reaction (PCR) is a common laboratory technique used to make many copies (millions or billions!) of a particular region of DNA. The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro

The goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for [sequencing](https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/dna-sequencing), visualized by [gel electrophoresis](https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/gel-electrophoresis), or [cloned](https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-cloning-tutorial/a/overview-dna-cloning) into a plasmid for further experiments.

**Taq polymerase**

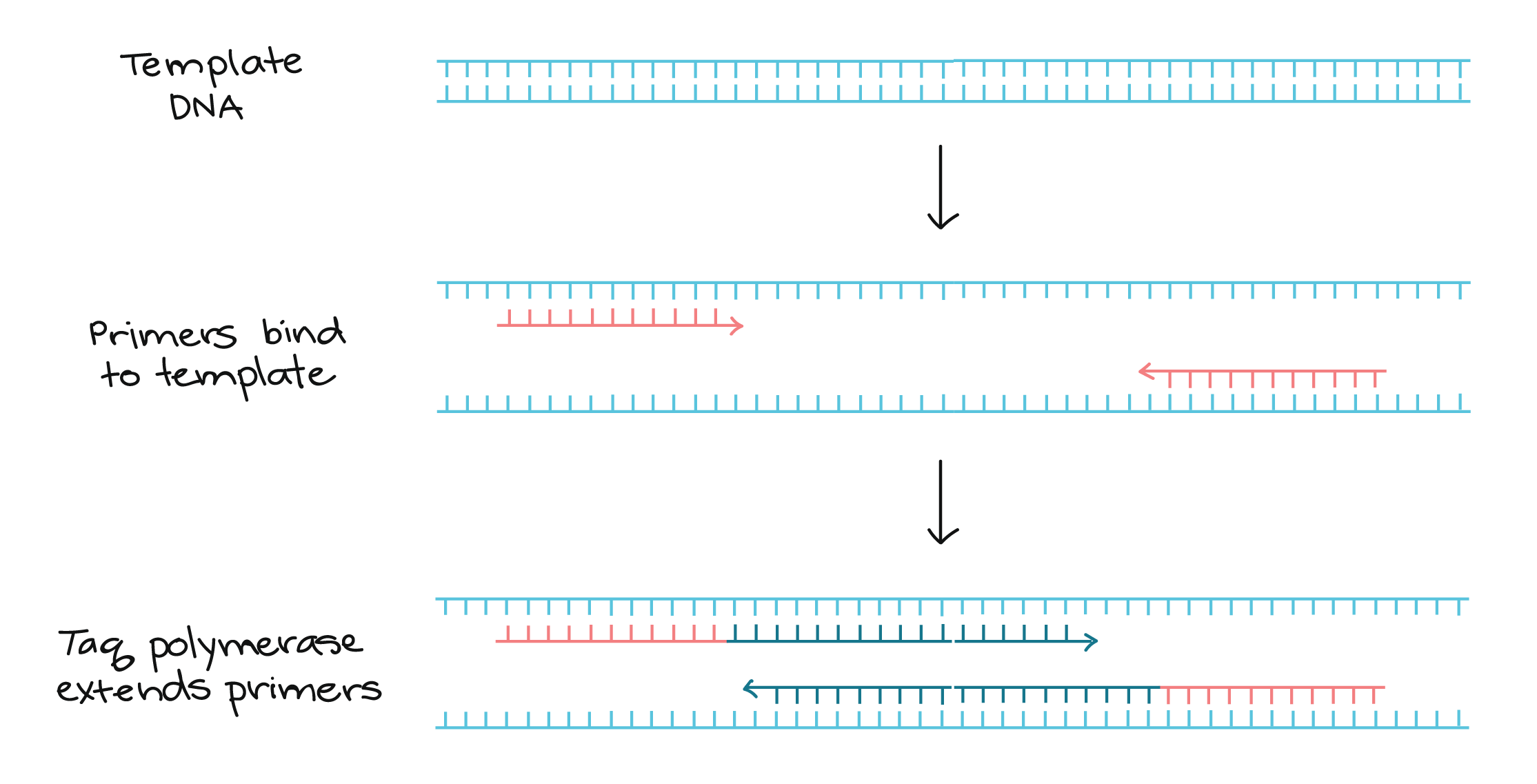
Like [DNA replication](https://www.khanacademy.org/science/biology/dna-as-the-genetic-material/dna-replication/a/molecular-mechanism-of-dna-replication) in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (Thermus aquaticus).

**PCR primers**

Like other DNA polymerases, Taq polymerase can only make DNA if it's given a primer, a short sequence of nucleotides that provides a starting point for DNA synthesis. In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the primers.

PCR primers are short pieces of single-stranded DNA, usually around 202020 nucleotides in length. Two primers are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied). That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied. The primers bind to the template by complementary base pairing.

When the primers are bound to the template, they can be extended by the polymerase, and the region that lies between them will get copied.



**The steps of PCR**

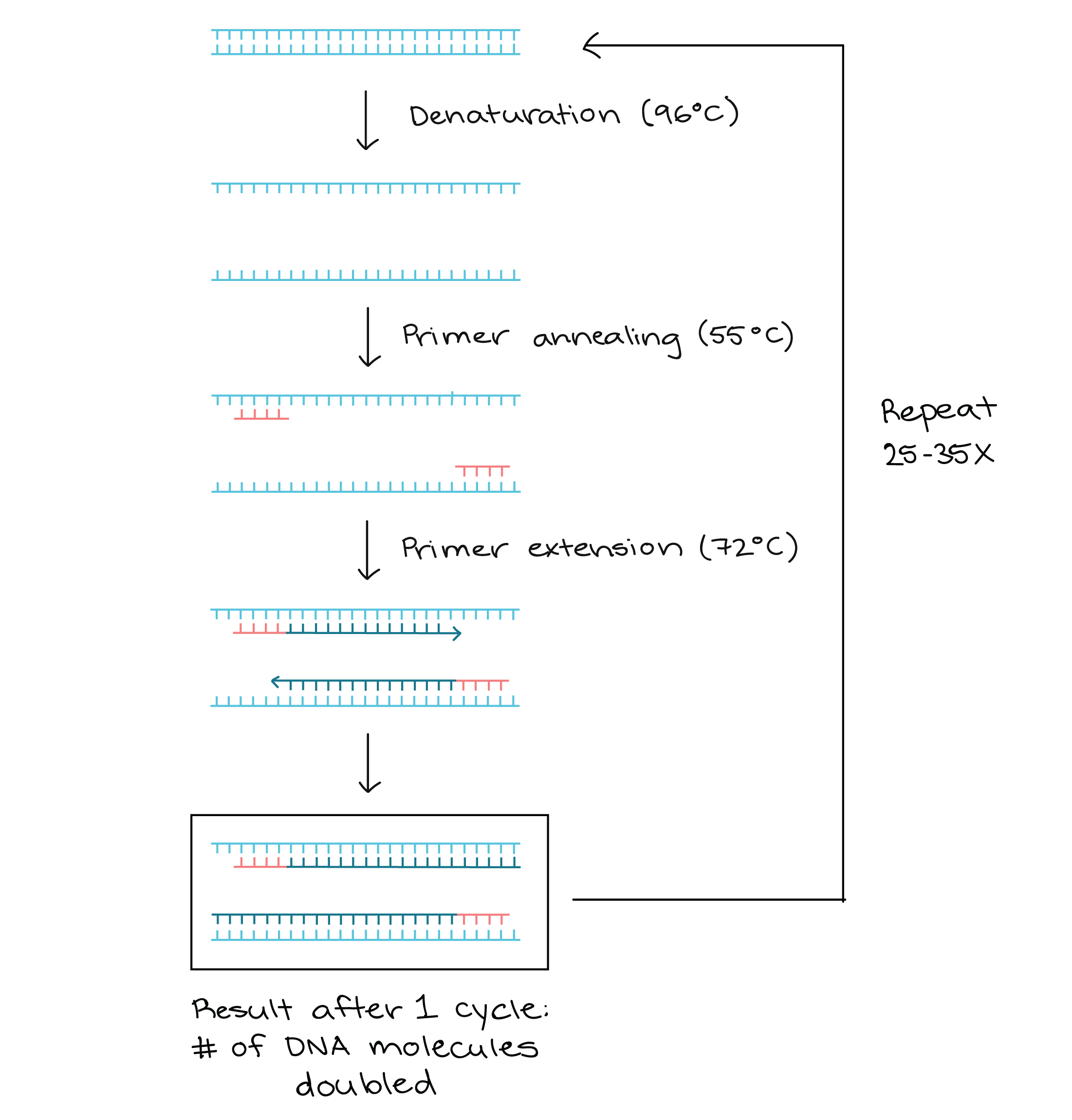
The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

**The basic steps are:**

**Denaturation**(at 96°C): Heat the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

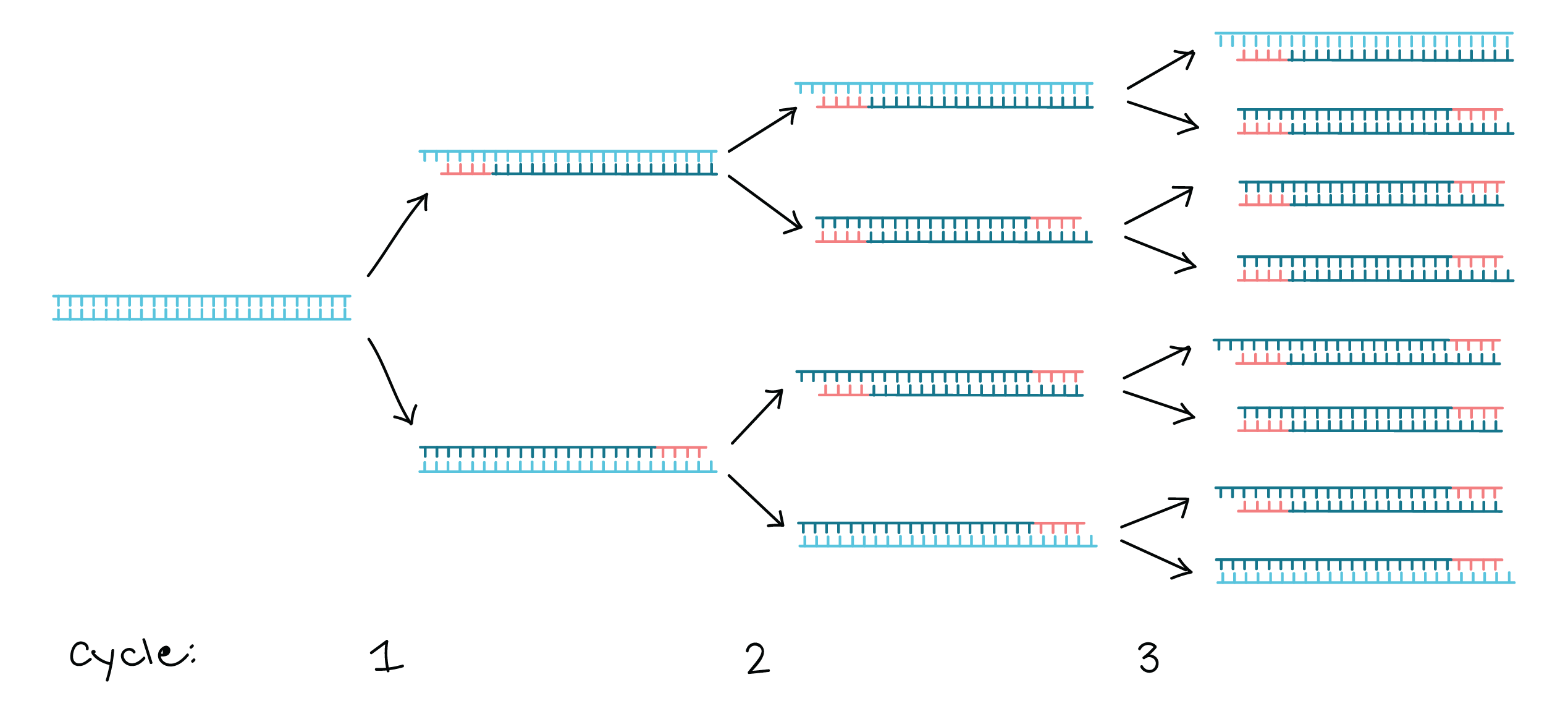
**Annealing** (at 55°C): Cool the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.

**Extension** (at 72°C.): Raise the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.



This cycle repeats 252525 - 353535 times in a typical PCR reaction, which generally takes 222 - 444 hours, depending on the length of the DNA region being copied. If the reaction is efficient (works well), the target region can go from just one or a few copies to billions.

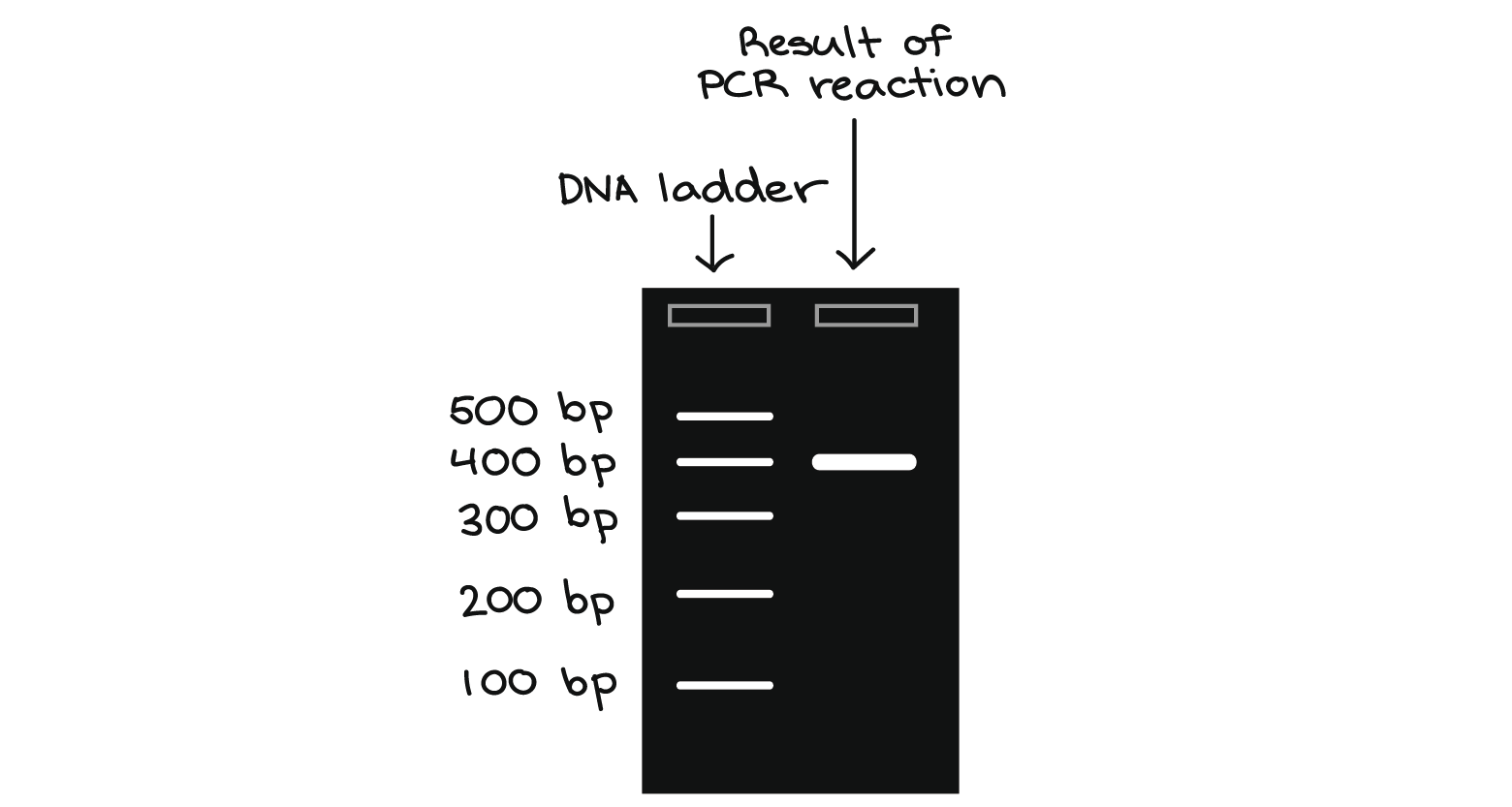
That’s because it’s not just the original DNA that’s used as a template each time. Instead, the new DNA that’s made in one round can serve as a template in the next round of DNA synthesis. There are many copies of the primers and many molecules of Taq polymerase floating around in the reaction, so the number of DNA molecules can roughly double in each round of cycling. This pattern of exponential growth is shown in the image below.



**Using gel electrophoresis to visualize the results of PCR**

The results of a PCR reaction are usually visualized (made visible) using [gel electrophoresis](https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/gel-electrophoresis). Gel electrophoresis is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. A standard, or DNA ladder, is typically included so that the size of the fragments in the PCR sample can be determined.

DNA fragments of the same length form a "band" on the gel, which can be seen by eye if the gel is stained with a DNA-binding dye. For example, a PCR reaction producing a 400400400 base pair (bp) fragment would look like this on a gel:



Left lane: DNA ladder with 100, 200, 300, 400, 500 bp bands.

Right lane: result of PCR reaction, a band at 400 bp.

A DNA band contains many, many copies of the target DNA region, not just one or a few copies. Because DNA is microscopic, lots of copies of it must be present before we can see it by eye. This is a big part of why PCR is an important tool: it produces enough copies of a DNA sequence that we can see or manipulate that region of DNA.

**Nested PCR**

[Nested PCR](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/nested-polymerase-chain-reaction) is a modification of PCR that was designed to improve sensitivity and specificity. Nested PCR involves the use of two primer sets. It is performed by two successive PCRs. The first reaction is performed with primers that cover the target sequence and some additional sequence flanking both ends of the target sequence. After the first reaction, a second reaction is performed on the products of the first PCR with primers that bind to the target sequence and are within the amplified sequence of the first PCR. This reduces the amount of nonspecific binding because in the second reaction, most of the [amplicons](https://www.sciencedirect.com/topics/medicine-and-dentistry/amplicon" \o "Learn more about Amplicon from ScienceDirect's AI-generated Topic Pages) of the first reaction only contain the target sequence and its surrounding sequences.

**Multiplex PCR**

Multiplex PCR is the simultaneous detection of multiple targets in a single reaction well, with a different pair of primers for each target. This technique requires two or more probes that can be distinguished from each other and detected simultaneously. There is a range of different probe technologies available, all using fluorophores.

**Applications of PCR**

**Reverse Transcription PCR**

Reverse transcription PCR (RT-PCR) uses mRNA rather than DNA as the starting template. First, the enzyme [reverse transcriptase](https://www.sciencedirect.com/topics/neuroscience/reverse-transcriptase) uses the mRNA template to produce a complementary [single-stranded DNA](https://www.sciencedirect.com/topics/neuroscience/single-stranded-dna) strand called cDNA in a process known as [reverse transcription](https://www.sciencedirect.com/topics/neuroscience/reverse-transcription). Next, [DNA polymerase](https://www.sciencedirect.com/topics/neuroscience/dna-polymerase) is used to convert the single-stranded cDNA into [double-stranded DNA](https://www.sciencedirect.com/topics/neuroscience/double-stranded-dna). These DNA molecules can now be used as templates for a PCR reaction as described above.

**Rapid Amplification of cDNA**

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA. This methodology of amplification with single-sided specificity has been described by others as “one-sided” PCR or “anchored” PCR.

**A real-time polymerase chain reaction**

A real-time polymerase chain reaction (real-time PCR), also known as quantitative polymerase chain reaction (qPCR), is a [laboratory technique](https://en.wikipedia.org/wiki/Laboratory_technique) of [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology) based on the [polymerase chain reaction](https://en.wikipedia.org/wiki/Polymerase_chain_reaction) (PCR). It monitors the amplification of a targeted [DNA](https://en.wikipedia.org/wiki/DNA) molecule during the PCR (i.e., in real time), not at its end

**Methods of Gene isolation**

There are three methods for obtaining genes

1. Synthesis of gene using an automated gene machine
2. Gene cloning
3. Using the PCR
4. **Gene Machine**

Recently, fully automated commercial instrument called automated polynucleotide synthesizer or gene machine is available in market which synthesizes predetermined polynucleotide sequence.

Therefore, the genes can be synthesized rapidly & in high amount. For examples, a gene for t RNA can be synthesized within a few days through gene machine. Gene machine automatically synthesizes the short segments of single stranded DNA under the control of microscope

The working principle of a gene machine includes

1) Development of insoluble silica based support in the form of beads which provides support for solid phase synthesis of DNA chain

2) Development of stable deoxyribonucleoside phosphoramidites as synthons which are stable to oxidation & hydrolysis & ideal for DNA synthesis.

1. **Gene Cloning**

Gene cloning is a common practice in molecular biology labs that is used by researcher to create of a particular gene for downstream applications, such as sequencing, mutagenesis, genotyping or heterologous expression of a protein.

The traditional technique for gene cloning involves the transfer of a DNA segment of interest from one organism to a self - replicating genetic element, such as a bacterial plasmid

This technique is commonly used today for isolating long or unstudied genes & protein expression.

**Steps in gene cloning experiments**

1. A fragment of DNA , containing the gene to be cloned , is inserted into a circular DNA molecule called a vector , to produce a recombinant DNA molecule
2. The vector transport the gene into a host cell , which is usually a bacterium , When the host cell divides , copies of recombinant DNA molecule are passed to the progeny & further vector replication takes place .
3. Within the host cell the vector multiplies producing numerous identical copies, not only of itself but also of the gene that it carries.
4. Although other types of living cells can be used
5. After a large number of cell divisions, a colony or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule, the gene carried by the recombinant molecule is now said to be cloned.
6. **PCR**

The polymerase chain reaction can also be obtaining a pure sample of gene. This is because the region of the starting DNA molecule that is copied during PCR is the segment whose boundaries are marked by the annealing position of the two oligonucleotides primers.

If the primer anneals either side of the gene of interest, many copies of that gene will be synthesized. The outcome is the same as with a gene cloning experiment, although the problem of selection does not arise because the desired gene is automatically ‘’ selected ‘’ as a result of the position at which primers anneal.

An alternative to cloning, polymerase chain reaction (PCR) can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known.