

Laboratory Manual

Molecular Biology (BIO302)



Contents

S. No.	Practical	P. No.
1	Preparation of stock and working solutions	3
2	Isolation of DNA by organic method part A	6
3	Isolation of DNA by organic method part B	9
4	Isolation of DNA from Mammalian tissue by commercial kit	10
5	Quantification of DNA by Gel Electrophoresis	13
6	DNA Extraction from Plant Tissue: CTAB	14
7	Isolation of RNA by Trizol method	16
8	Quantification of RNA by Nanodrop (Spectrophotometer)	17
9	cDNA synthesis (RT-PCR)	18
10	Polymerase Chain Reaction (PCR) and types	22
11	Quantitative Real time PCR (qRT-PCR)	29
12	DNA fingerprinting	38
13	Restriction Fragment Length Polymorphism (RFLP)	43
14	Cloning and Preparation of Chemically Competent Cells	46
15	Isolation of bacterial Plasmid	49
16	Analysis of proteins by SDS-PAGE	52

Practical No: 1

Preparation of Reagents

The following general instructions are applicable in the preparation of all reagents. Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents. Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared, initials of scientist that prepared reagent, lot number, and expiration date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl (Tris Hydroxymethyl amino methane) pH 8

Tris base 121.1g

H₂O to 800ml

Adjust to desired pH with concentrated HCl. Mix and add H₂O to 1 Liter.

Store at room temperature

0.5 M EDTA (Ethylenediamine Tetra acetic Acid) pH 8.0

Na₂EDTA.2H₂O 186.1g

H₂O to 700ml

Adjust pH to 8.0 with 10M NaOH (almost 50ml) Mix and add H₂O to 1 Liter.

Store at room temperature

10M NaOH

NaOH 400 g

H₂O to 1 Liter Store at room temperature

10 mg/ml Ethidium Bromide

Ethidium Bromide 0.2 g H₂O to 20ml

Mix well and store at 4°C in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0 (Lysis Buffer)

1M Tris-HCl pH 8.0 10 ml

0.5 M EDTA pH 8.0 4 ml

H₂O to 1 Liter

Store at room temperature

Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0 for DNA storage

1M Tris-HCl pH 8.0 10 ml

0.5 M EDTA pH 8.0 0.4 ml

H₂O to 1 Lite

Store at room temperature

Proteinase K (10mg/ml)

Proteinase K 100 mg lyophilized powder in Ultra-pure H₂O to 10 ml.

Make the aliquot and store at approximately -20 °C.

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl pH 8.0 10 ml

5M NaCl 80 ml

0.5M EDTA 4 ml

H₂O to 1 Liter

Store at room temperature

SDS 10% w/v

Sodium dodecyl sulfate 100g H₂O to 700ml Heat to approximately 65°C to dissolve.

Bring to a final volume of 1.0 L with ultra-pure water.

Store at room temperature

CAUTION: SDS can be irritating to mucous membranes. Wear safety glasses, mask and gloves when handling

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base 242g

Glacial acetic acid 57.1 ml

0.5 M EDTA pH 8.0 100ml H₂O to 1 Liter

Store at room temperature

1x TAE (Tris 40mM, Acetate 20mM, EDTA 2mM) Electrophoresis working buffer

50x TAE 10 ml H₂O to 500 ml the pH of diluted buffer is 8.3

Store at room temperature

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base 108g

Boric Acid 55g

0.5M EDTA pH 8.0 40 ml H₂O to 1 Liter

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue 0.25 ml

2% Xylene cyanol 0.25 ml

Glycerol 7ml

H₂O 10ml

Store at room temperature

5M Sodium Chloride

Sodium Chloride 292.2 g

H₂O to 1 Liter

Store at room temperature

6M Sodium Chloride

Sodium Chloride 351g H₂O to 1 Liter

Store at room temperature

Practical no: 2

Isolation of DNA by organic method part A

Sources of DNA

Purified DNA is required for a variety of molecular biology applications. DNA can be purified from any living organism and its living parts

Origin of Samples:

1. Human tissues i.e. histological samples, prenatal samples, postmortem harvesting.
2. Blood, (EDTA).
3. Hair, (follicle part of the hair to be specific.
4. Rodent tissues, as rats are the most common lab mammals used in labs.
5. Leaf.
6. Bacteria, Bacterial cultures.
7. Yeast, yeast cultures.
8. Fungi.
9. Insect, i.e. *Drosophila melanogaster*
10. Stool.
11. Body fluids, i.e. semen.
12. Spores.
13. Soil.
14. Clinical samples (e.g. biopsy samples, fine needle aspirates).
15. Forensic samples (e.g. dried blood spots, buccal swabs finger prints).

DNA extraction is used to isolate

Types of DNA

Mitochondrial DNA

Genomic DNA

Plasmid DNA

DNA Extraction from Whole Blood

Materials

- Lysis buffer (TE)
- Proteinase K
- Phenol-chloroform isoamyl alcohol (PCI)
- SDS 10%

- TNE buffer
- Isopropanol
- Ice cold 95-100% ethanol
- Ultrapure (DNA- & DNase-free) water

Lab equipment needed

- Pippetes,
- 1.5mL sterile microcentrifuge tubes or 15, 50 mL Falcons,
- Racks,
- Tips
- Vortex
- Freezer
- Centrifuge

Blood Sample

Blood collection in anticoagulant i.e. Ethylenediamide tetra-acetic acid (0.5 M EDTA) containing tube 1.5 mL eppendorf or 15 mL Falcon tube

Storage of Blood Samples

Field blood samples should be place on ice immediately after their collection store in freezer at -20°C before

DNA extraction

Steps in Organic DNA Extraction

- 1- Lysis of Red Blood Cells, RBC
- 2- Digestion step (Lysis of White blood cells, WBC)
- 3- Phase Separation step (Extraction of Protein)
- 4- DNA Precipitation
- 5- Washing with ice cold Ethanol
- 6- Dilute the pellet

1. Lysis of Red Blood Cells, RBC

1. Lysis of red blood cells
2. Added 800 uL of Tris EDTA buffer (Tris HCl 10mM, EDTA 2mM) in 200 uL ml of the blood. Mixed by inverting several times.
3. Centrifuged at 5000 rpm for 10 min.

4. Discarded the supernatant.
5. Break the pellet formed at the bottom of the eppendorf tube by tapping it gently. Add 1 mL TE buffer and mixed it gently.
6. Centrifuged at 5000 rpm for 10 min. this step may be repeated until pallet becomes light pink.

2. Digestion step (Lysis of White blood cells, WBC)

1. Pellet obtained after lysis of RBCs re-suspended in
2. 400 uL Buffer TNE (Tris HCL 10mM, EDTA 2mM, NaCl 400mM),
3. 200uL 10% SDS
4. 50 uL Proteinase K (50 μ l of 10 μ g/uL conc.).
5. Homogenize the tube with gentle rotation
6. Samples incubate at overnight in 58 °C in shaker water bath.
7. Next Day

<p style="text-align: center;">Practical no: 3 Isolation of DNA by organic method part B</p>
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3. Phase Separation step (Extraction of Protein)

1. In this step, we can remove the digested protein through
2. Phenol-chloroform isoamyl alcohol (PCI, in ratio 25:24:1 respectively) (Organic Method).
3. DNA released into solution is extracted with PCI to remove proteinaceous materials.
4. Add equal volume of phenol-chloroform-isoamyl (PCI) alcohol Mix gently for 2 min and centrifuge for 10 minutes at 10,000 rpm at 4°C.
5. Carefully remove the top (aqueous) phase containing the DNA using a 1000- μ L pipette transfer to a new tube.

4. DNA Precipitation

1. Precipitate the DNA with absolute isopropanol and inverted the tubes gently till DNA threads became visible and then left the tubes at room temperature for 10 minutes.
2. Centrifuged at 8000 rpm for 10 minutes and discarded the supernatant carefully and white pellet of DNA may visible at the bottom of the tube.

5. Washing with ice cold Ethanol

1. Washed DNA pellet with 1 mL of 70-100% ethanol, break and mix the pellet
2. Then centrifuged at 8000 rpm for 10 minutes and discarded the supernatant carefully
3. Air dried the DNA pellet at room temperature for at least 2 hours

6. Dilute the pellet

1. Add 50-100 μ L of low T.E. (Tris HCl 10 mM, EDTA 0.2mM) or DEPC water
2. Place the tubes in a shaking water bath at 70°C for one hour so that nucleases were inactivated. Finally DNA will store at -20°C.

Practical no: 4

Isolation of DNA from Mammalian tissue by Commercial kits

gDNA Extraction Kits from Thermo Fisher Scientific

Catalog number: K0721, K0722

Thermo Scientific GeneJET Genomic DNA Purification Kit is designed for rapid and efficient purification of high quality genomic DNA from various mammalian cell culture and tissue samples, whole blood, bacteria and yeast.

The kit utilizes silica-based membrane technology in the form of a convenient spin column, eliminating the need for expensive resins, toxic phenol-chloroform extractions, or time-consuming alcohol precipitation.

The standard procedure takes less than 20 minutes following cell lysis and yields purified DNA greater than 30 kb in size. Isolated DNA can be used directly in PCR, Southern blotting and enzymatic reactions.

Highlights

- Universal—can be used for both cell and tissue samples from a wide range of sources
- Efficient—high yields of high molecular weight genomic DNA
- Fast—20 minute procedure following cell lysis
- Convenient—spin columns are capped and assembled with collection tubes

Applications

- Isolated genomic DNA is ideal for use in common molecular biology procedures, including:
- PCR and qPCR
- FastDigest and conventional restriction digestion
- Southern blotting

Genomic DNA Purification Protocols

Protocols for genomic DNA purification from mammalian tissue and rodent tail, cultured mammalian cells, mammalian blood, gram-negative, gram-positive bacteria, yeast and buccal swabs

A. Mammalian Tissue and Rodent Tail Genomic DNA Purification Protocol

Step Procedure

1. Grind up to 20 mg of mammalian tissue (use up to 10 mg of spleen tissue), 0.6 cm (rat)

or 0.5 cm (mouse) tail clip in liquid nitrogen using a mortar and pestle. Alternatively, cut the tissue into small pieces or disrupt it using a homogenizer.

2. Collect the material into a 1.5 mL microcentrifuge tube (not provided) and resuspend in 180 μ L of Digestion Solution. Add 20 μ L of Proteinase K Solution and mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
3. Incubate the sample at 56 °C until the tissue is completely lysed and no particles remain. During incubation vortex the vial occasionally or use a shaking water bath, rocking platform or thermomixer.

Suggested incubation times:

Quantity Suggested incubation time

- 5 mg of tissue (except spleen) 1 hour
- 10 mg of tissue (except spleen) 2 hours
- 20 mg of tissue (except spleen) 3 hours
- 5 mg of spleen tissue 2 hours
- 10 mg of spleen tissue 3 hours
- Mouse tail (0.5 cm), rat tail (0.6 cm) 6 hours

Note. Lysis time varies on the type and amount of tissue processed. In some cases incubation time should be prolonged to 6-8 hours or overnight (for rodent tail) until complete lysis occurs.

4. Add 20 μ L of RNase A Solution, mix by vortexing then incubate for 10 min at room temperature.
5. Add 200 μ L of Lysis Solution. Mix thoroughly by vortexing for 15 s until a homogeneous mixture is obtained.
6. Add 400 μ L of 50% ethanol and mix by pipetting or vortexing.
7. Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 g. Discard the collection tube containing the flow-through solution. Place the GeneJET Genomic

DNA Purification Column into a new 2 mL collection tube (included).

Note. Close the bag with GeneJET Genomic DNA Purification Columns tightly after each use!

8. Add 500 μ L of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 g. Discard the flow-through and place the purification column back into the collection tube.

9. Add 500 μ L of Wash Buffer II (with ethanol added) to the GeneJET Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥ 12000 g). Optional. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJET Genomic DNA Purification Column to a sterile 1.5 mL microcentrifuge tube (not included).
10. Add 200 μ L of Elution Buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 g.
11. Note

For maximum DNA yield, repeat the elution step with additional 200 μ L of Elution Buffer.

If more concentrated DNA is required or DNA is isolated from a small amount of starting material (e.g., <5 mg of tissue) the volume of the Elution Buffer added to the column can be reduced to 50-100 μ L. Please be aware that smaller volumes of Elution Buffer will result in smaller final quantity of eluted DNA.
12. Discard the purification column. Use the purified DNA immediately in downstream applications or store at -20 $^{\circ}$ C.

Practical no: 5

Quantification of DNA by Gel Electrophoresis

DNA Quantification

DNA yield can be assessed using various methods including absorbance (optical density), agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose gel electrophoresis is another way to quickly estimate DNA concentration. 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. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field.

For the quantification and quality of DNA,

0.8 % Agarose gel was prepared by boiling 0.8 g of Agarose in 100 mL of 1X TAE buffer.

As the temperature of gel solution reduced to approximately 55°C, Ethidium bromide (Conc.10mg/mL) add at the rate of 5 μ L/100 ml of gel solution.

Melted agarose was poured into gel casting tray with comb (10-20 wells) position appropriately.

When the gel get solidify, transfer into electrophoresis tank filled with 1X TAE buffer.

The level of buffer keep at least 1 cm above the gel

The wells carefully load with 5 μ L DNA mixed with 3 μ L 3X Bromophenol blue (gel loading dye).

Standard DNA ladder load along with the DNA samples in a separate well

Electrophoresis carries out at 100V for 30-60 min.

On completion of electrophoresis, the gel visualizes under UV light in Gel documentation system and estimate the quality and quantity.

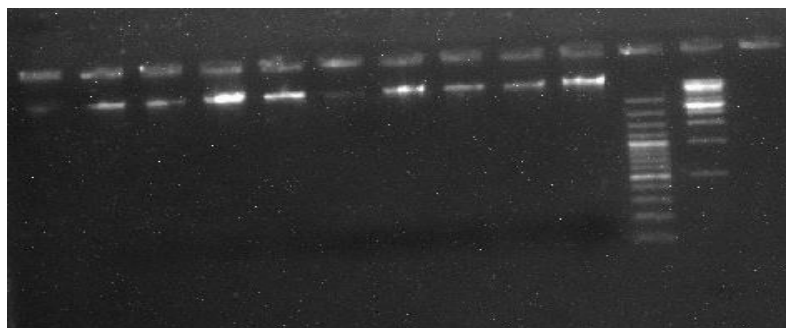


Fig 5.1: DNA bands in gel electrophoresis

Practical no: 6

DNA Extraction from Plant Tissue: CTAB

Materials Required

- CTAB buffer: 2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA
- Centrifuge (up to 14,000 x g)
- Isopropanol
- 70% Ethanol
- 2 ml centrifuge tubes
- SpeedVac
- TE Buffer (10 mM Tris, pH 8, 1 mM EDTA)

Method

Plant samples can be prepared by cryogenically grinding tissue in a mortar and pestle after chilling in liquid nitrogen. Freeze dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA.

1. For each 100 mg homogenized tissue use 500 μ l of CTAB Extraction Buffer. Mix and thoroughly vortex. Transfer the homogenate to a 60°C bath for 30 minutes.
2. Following the incubation period, centrifuge the homogenate for 5 minutes at 14,000 x g.
3. Transfer supernatant to a new tube. Add 5 μ l of RNase solution A and incubate at 32°C for 20 minutes
4. Add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex for 5 seconds then centrifuge the sample for 1 min. at 14,000 x g to separate the phases. Transfer the aqueous upper phase to a new tube. Repeat this extraction until the upper phase is clear.
5. Transfer the upper aqueous phase to a new tube. Precipitate the DNA by adding 0.7 volume cold isopropanol and incubate at -20°C for 15 minutes.
6. Centrifuge the sample at 14,000 x g for 10 minutes. Decant the supernatant without disturbing the pellet and subsequently wash with 500 μ l ice cold 70% ethanol. Decant the ethanol. Remove residual ethanol by drying in a SpeedVac.

7. Dry the pellet long enough to remove alcohol, but without completely drying the DNA. Dissolve DNA in 20 μ l TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The pellet may need warming in order to dissolve.

Table 6.1: CTAB extraction buffer

Reagent	Amount to add (for 10 mL)	Final concentration
Cetyltrimethyl ammonium bromide (CTAB) (10% in H ₂ O) ^a	3 mL	3%
5 M NaCl	2.8 mL	28%
0.5 M EDTA (pH 8.0)	0.4 mL	4%
1 M Tris-Cl (pH 8.0)	1 mL	10%
Polyvinylpyrrolidone (PVP) (MW 40 kDa)	0.3 g	3%
β -Mercaptoethanol	0.02 mL	0.2%
H ₂ O	2.48 mL	24.8%
Prepare CTAB extraction buffer immediately before use; buffer is only good when freshly prepared.		
^a Store 10% CTAB stock solution at 37°C to avoid precipitation. It may be stored at 37°C for several years.		

<p style="text-align: center;">Practical no: 7 Isolation of RNA by Trizol method</p>
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RNA extraction by Trizol reagents

1. Collect 250 uL samples to a 1.5 mL eppendorff and add 750uL Trizol and samples is overtex for 15 sec. incubate at room temperature for 7 minutes.
2. Pulse spin to remove liquid from the tube lid.
3. Add 200uL 100% chloroform to the sample, vortex for 15 sec and incubate at room temperature for 7 minutes.
4. Centrifuge at 12,000g for minutes at room temperature.
5. Transfer 450uL of the upper aqueous layer to a separate microcentrifuge tube.
6. Add 500uL 100% isopropanol, invert tube several time to mix and hold at room temperature for 10 minutes.
7. Discard supernatant. Care should be taken to assure that the RNA pellet is not disturbed.
8. Add 1 mL of 80% ethanol. Mix gently.
9. Centrifuge at 10,000g for 5 minute at 4C.
10. Discard ethanol. Invert tube on a clean tissue wipe and allow to air dry for 10 minutes. It is important not to let the RNA pellet over dry as this will decrease its solubility.
11. Hydrate pellet in 50 uL of RNase free water and Hydrate pellet in 50 uL of RNase free water and store at -40 to -80 °C.

Practical no: 8

Quantification of RNA by Nanodrop (Spectrophotometer)

Nanodrop (Spectrophotometer)

The most common technique to determine DNARNA yield and purity is measurement of absorbance using Nanodrop. A spectrophotometer is able to determine the average concentrations of the nucleic acids DNA or RNA present in a mixture, as well as their purity. The absorbance method is a spectrophotometer equipped with a UV lamp, and a solution of purified DNA/RNA sample. DNA concentration is estimated by measuring the absorbance at 260nm. To evaluate DNA purity, measure absorbance from 230 nm to detect other possible contaminants. The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm (Protein detection). Good-quality DNA will have an $A_{260/280}$ is widely considered ~1.8 and for RNA is ~2.0. A reading of 1.7, 1.6 etc does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present.



Fig 8.1: Nanodrop

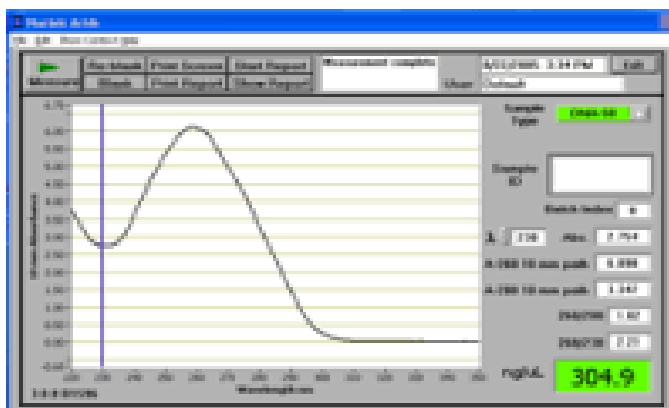


Fig 8.2: Concentration of samples view on Nanodrop

Practical no: 9

cDNA synthesis (RT-PCR)

The synthesis of DNA from an RNA template, via reverse transcription, results in complementary DNA (cDNA). cDNA can then serve as template in a variety of downstream applications for RNA studies such as gene expression; therefore, cDNA synthesis is the first step for many protocols in molecular biology.

Step 1. Prepare sample

RNA serves as the template in cDNA synthesis. Total RNA is routinely used in cDNA synthesis for downstream applications such as RT-(q)PCR, whereas specific types of RNAs (e.g., messenger RNA (mRNA) and small RNAs such as miRNA) may be enriched for certain applications like cDNA library construction and miRNA profiling.

Maintaining RNA integrity is critical and requires special precautions during extraction, processing, storage, and experimental use. Best practices to prevent degradation of RNA include wearing gloves, pipetting with aerosol-barrier tips, using nuclease-free labware and reagents, and decontamination of work areas.

To isolate and purify RNA, a variety of strategies are available depending on the type of source materials (e.g., blood, tissues, cells, plants) and goals of the experiments. The main goals of isolation workflows are to stabilize RNA molecules, to inhibit RNases, and to maximize yield with proper storage and extraction methods. Optimal purification methods remove endogenous compounds, like complex polysaccharides and humic acid from plant tissues that interfere with enzyme activity; and common inhibitors of reverse transcriptases, such as salts, metal ions, ethanol, and phenol. Once purified, RNA should be stored at -80°C with minimal freeze-thaw cycles.

Step 2. Remove genomic DNA

Trace amounts of genomic DNA (gDNA) may be co-purified with RNA. Contaminating gDNA can interfere with reverse transcription and may lead to false positives, higher background, or lower detection in sensitive applications such as RT-qPCR.

The traditional method of gDNA removal is the addition of DNase I to preparations of isolated RNA. DNase I must be removed prior to cDNA synthesis since any residual enzyme would degrade single-stranded DNA. Unfortunately, RNA loss or damage can occur during DNase I inactivation treatment.

Step 3. Select reverse transcriptase

Most reverse transcriptases used in molecular biology are derived from the pol gene of avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV). The AMV reverse transcriptase was one of the first enzymes isolated for cDNA synthesis in the lab. The enzyme possesses strong RNase H activity that degrades RNA in RNA:cDNA hybrids, resulting in shorter cDNA fragments (<5 kb).

The MMLV reverse transcriptase became a popular alternative due to its monomeric structure, which allowed for simpler cloning and modifications to the recombinant enzyme. Although MMLV is less thermostable than AMV reverse transcriptase, MMLV reverse transcriptase is capable of synthesizing longer cDNA (<7 kb) at a higher efficiency, due to its lower RNase H activity.

To further improve cDNA synthesis, MMLV reverse transcriptase has been engineered for even lower RNase H activity (i.e., mutated RNase H domain, or RNaseH⁻), higher thermostability (up to 55°C), and enhanced processivity (65 times higher). These attributes result in increased cDNA length and yield, higher sensitivity, improved resistance to inhibitors, and faster reaction times

Step 4. Prepare reaction mix

In addition to enzyme and primers, the main reaction components for reverse transcription include RNA template (pre-treated to remove genomic DNA), buffer, dNTPs, DTT, RNase inhibitor, and RNase-free water.

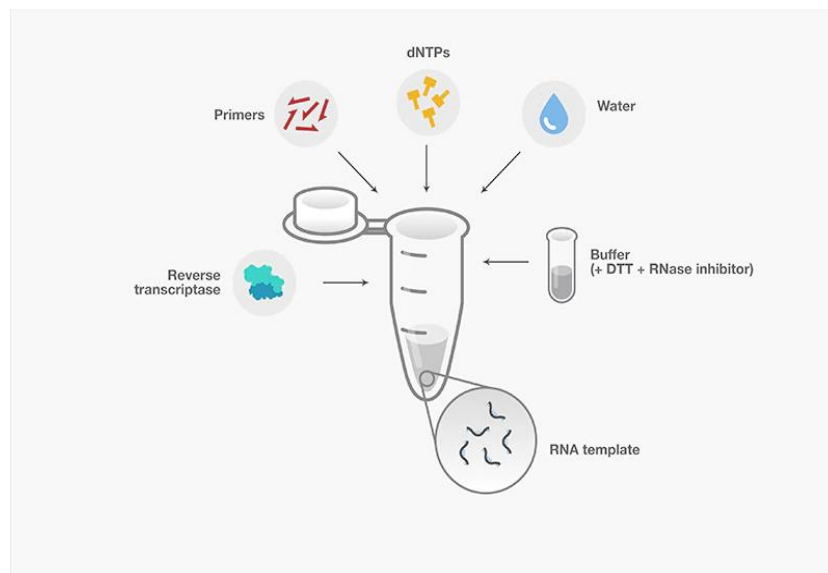


Fig 9.1: The diagram shows reverse transcription reaction with its main components

Table 9.1: Reverse transcription reaction with its main components

Component	Key features
RNA template	Maintaining RNA integrity is critical and requires special precautions during extraction, processing, storage, and experimental use (see step 1): <ul style="list-style-type: none">• Total RNA - routinely used in cDNA synthesis for downstream applications such as RT-(q)PCR• Messenger RNA (mRNA) and small RNAs such as miRNA) may be enriched for certain applications like cDNA library construction and miRNA profiling
Reaction buffer	<ul style="list-style-type: none">• Maintains a favorable pH and ionic strength for the reaction.• The supplied buffer may also contain additives to enhance the efficiency of reverse transcription.
dNTPs	<ul style="list-style-type: none">• Generally, should be at 0.5–1 mM each, preferably at equimolar concentrations• High-quality dNTPs, freshly diluted, are recommended to ensure proficient reverse transcription
DTT	<ul style="list-style-type: none">• Reducing agent, often included for optimal enzyme activity.• Reaction efficiencies may be compromised if DTT or other additives precipitate; hence, reaction components should be dissolved and well mixed.
RNase inhibitor	Often included in the reaction buffer or added to the reverse transcription reaction to prevent RNA degradation. They may be: <ul style="list-style-type: none">• Co-purified during isolation• Introduced during reaction setup A number of known RNases exist, and appropriate RNase inhibitors should be chosen based on their mode of actions and reaction requirements.
Water	Eliminate any RNases by using: <ul style="list-style-type: none">• Nuclease-free from a commercial source• DEPC (diethylpyrocarbonate)-treated water Contaminating RNases cannot be removed by simple filtration, and autoclaved water is not adequate because RNases are heat stable.

Step 5. Perform cDNA synthesis

Reverse transcription reactions involve three main steps: primer annealing, DNA polymerization, and enzyme deactivation. The temperature and duration of these steps vary by primer choice, target RNA, and reverse transcriptase used. The critical step is during DNA polymerization. In this step, reaction temperature and duration may vary according to the primer choice and reverse transcriptase used. If using random hexamers, then we recommend incubating the reverse transcription reaction at room temperature (~25 °C) for 10 min after enzyme addition to extend the primers. Among reverse transcriptases there are differences in thermostability, which in turn determines the highest optimal polymerization temperature for each. Using a thermostable reverse transcriptase allows, a higher reaction temperature (e.g.,

50°C), to help denature RNA with high GC content or secondary structures without impacting enzyme activity. With such enzymes, high-temperature incubation can result in an increase in cDNA yield, length, and representation.

Reagents

- RNA template
- 5X First Strand Buffer
- 10mM dNTP Set
- 0.1M DTT
- Random Primers
- RNaseOUT Ribonuclease Inhibitor
- SuperScript II RNase H- Reverse Transcriptase
- Water

Procedure

1. Combine 10 µl total RNA
2. Add 1µl Random Primers
3. Molecular grade water 1 µl
4. Vortex and then spin down tube.
5. Incubate at 65°C for 5 min.
6. Place tube on ice.
7. Add 4 µl 5X Buffer
8. Add 2µl dNTP mix
9. Add 1µl SuperScript II RNase H- Reverse Transcriptase
10. RNase inhibitor

PCR protocol (RT-PCR)

11. Incubate at 25°C for 5 min
12. Incubate at 42°C for 60 min.
13. Incubate at 70°C for 5 min.
14. Add x µl molecular grade water.

Practical no: 10

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is the cardinal laboratory technology of molecular biology. Arguably one of the most powerful laboratory techniques ever discovered, PCR combines the unique attributes of being very sensitive and specific with a great degree of flexibility. With the PCR it is possible to specifically address a particular DNA sequence and to amplify this sequence to extremely high copy numbers. Since its initial development in the early 1980's, dozens of variations in the basic theme of PCR have successfully been carried out. In fact, the very flexibility and application-specific variation of PCR make it seem like there are as many ways to do a PCR reaction as there are researchers doing them. Here, a basic, straight-forward PCR protocol is presented. Where appropriate, some of the choices for modifying this standard reaction that are routinely available to researchers are discussed.

Materials and Reagents

1. Tris-HCl
2. MgCl₂
3. Taq DNA polymerase
4. dNTPs
5. Template DNA (genomic, plasmid, cosmid, bacterial/yeast colony, etc.)
6. Primers
7. Water

Equipment

Thermal cycler (MJ Research)

Primers

Designing appropriate primers is essential to the successful outcome of a PCR experiment. When designing a set of primers to a specific region of DNA desired for amplification, one primer should anneal to the plus strand, which by convention is oriented in the 5' → 3' direction (also known as the sense or nontemplate strand) and the other primer should complement the minus strand, which is oriented in the 3' → 5' direction (antisense or template strand).

There are a few common problems that arise when designing primers:

- 1) self-annealing of primers resulting in formation of secondary structures such as hairpin loops
- 2) Primer annealing to each other, rather than the DNA template, creating primer dimers
- 3) drastically different melting temperatures (T_m) for each primer, making it difficult to select an annealing temperature that will allow both primers to efficiently bind to their target sequence during thermal cycling

Below is a list of characteristics that should be considered when designing primers.

1. Primer length should be 15-30 nucleotide residues (bases).
2. Optimal G-C content should range between 40-60%.
3. The 3' end of primers should contain a G or C in order to clamp the primer and prevent "breathing" of ends, increasing priming efficiency. DNA "breathing" occurs when ends do not stay annealed but fray or split apart. The three hydrogen bonds in GC pairs help prevent breathing but also increase the melting temperature of the primers.
4. The 3' ends of a primer set, which includes a plus strand primer and a minus strand primer, should not be complementary to each other, nor can the 3' end of a single primer be complementary to other sequences in the primer. These two scenarios result in formation of primer dimers and hairpin loop structures, respectively.
5. Optimal melting temperatures (T_m) for primers range between 52-58 °C, although the range can be expanded to 45-65 °C. The final T_m for both primers should differ by no more than 5 °C.
6. Di-nucleotide repeats (e.g., GCGCGCGCGC or ATATATATAT) or single base runs (e.g., AAAAA or CCCCC) should be avoided as they can cause slipping along the primed segment of DNA and or hairpin loop structures to form. If unavoidable due to nature of the DNA template, then only include repeats or single base runs with a maximum of 4 bases.

Setting up a Reaction Mixture

1. Start by making a table of reagents that will be added to the reaction mixture.
2. label PCR tube(s) with the ethanol-resistant marker.
3. Reaction volumes will vary depending on the concentrations of the stock reagents. The final concentrations (CF) for a typical 50 µl reaction are as follows.
 1. X buffer (usually supplied by the manufacturer of the DNA polymerase; may contain 15 mM $MgCl_2$). Add 5 µl of 10X buffer per reaction.

- 200 μM dNTPs (50 μM of each of the four nucleotides). Add 1 μl of 10 mM dNTPs per reaction (dATP, dCTP, dTTP and dGTP are at 2.5 mM each).
- 1.5 mM Mg^{2+} . Add only if it is not present in the 10X buffer or as needed for PCR optimization. For example, to obtain the 4.0 mM Mg^{2+} required for optimal amplicon production of a conserved 566 bp DNA segment found in an uncharacterized Mycobacteriophage add 8 μl of 25 mM MgCl_2 to the reaction.
- 20 to 50 pmol of each primer. Add 1 μl of each 20 μM primer.
- Add 10^4 to 10^7 molecules (or about 1 to 1000 ng) DNA template. Add 0.5 μl of 2ng/ μl genomic Mycobacteriophage DNA.
- Add 0.5 to 2.5 units of DNA polymerase per 50 μl reaction, For example, add 0.5 μl of Sigma 0.5 Units/ μl *Taq* DNA polymerase.
- Add Q.S. sterile distilled water to obtain a 50 μl final volume per reaction as pre-determined in the table of reagents. Thus, 33 μl per reaction is required to bring the volume up to 50 μl . However, it should be noted that water is added first but requires initially making a table of reagents and determining the volumes of all other reagents added to the reaction.

Table 10.1: PCR reactions components

Component	Final Concentration/amount
water	to 50 μL
buffer	1 x
Taq polymerase	0.05 units/ μL
dNTP mix	200 μM
MgCl_2	0.1-0.5 mM
Forward primer	0.1-0.5 μM
Reverse primer	0.1-0.5 μM
template	200 pg/ μL
DMSO (optional)	1 to 10% w/v

Setting Up Thermal Cycling Conditions

- Denaturation (strand separation): The separation of the two hydrogen-bonded complementary chains of DNA into a pair of single-stranded polynucleotide molecules by a process of heating (94°C to 96°C)

2. Annealing (primer binding): The temperature is lowered (45-60 °C) so the primers can attach themselves to the single-stranded DNA strands.
3. Extension (synthesis of new DNA): It starts at the annealed primer and works its way along the DNA strand (72°C).

In Details

1. PCR thermal cyclers rapidly heat and cool the reaction mixture, allowing for heat-induced denaturation of duplex DNA (strand separation), annealing of primers to the plus and minus strands of the DNA template, and elongation of the PCR product. Cycling times are calculated based on the size of the template and the GC content of the DNA. The general formula starts with an initial denaturation step at 94 °C to 98 °C depending on the optimal temperature for DNA polymerase activity and G-C content of the template DNA. A typical reaction will start with a one minute denaturation at 94 °C. Any longer than 3 minutes may inactivate the DNA polymerase, destroying its enzymatic activity. One method, known as hot-start PCR, drastically extends the initial denaturation time from 3 minutes up to 9 minutes. With hot-start PCR, the DNA polymerase is added after the initial exaggerated denaturation step is finished. This protocol modification avoids likely inactivation of the DNA polymerase enzyme. Refer to the Troubleshooting section of this protocol for more information about hot start PCR and other alternative methods.
2. The next step is to set the thermal cycler to initiate the first of 25 to 35 rounds of a three-step temperature cycle (Table 2). While increasing the number of cycles above 35 will result in a greater quantity of PCR products, too many rounds often results in the enrichment of undesirable secondary products. The three temperature steps in a single cycle accomplish three tasks: the first step denatures the template (and in later cycles, the amplicons as well), the second step allows optimal annealing of primers, and the third step permits the DNA polymerase to bind to the DNA template and synthesize the PCR product. The duration and temperature of each step within a cycle may be altered to optimize production of the desired amplicon. The time for the denaturation step is kept as short as possible. Usually 10 to 60 seconds is sufficient for most DNA templates. The denaturation time and temperature may vary depending on the G-C content of the template DNA, as well as the ramp rate, which is the time it takes the thermal cycler to change from one temperature to the next. The temperature for this step is usually the same as that used for the initial denaturation phase (step #1 above; e.g., 94 °C). A 30 second annealing step follows within the cycle at a temperature set about 5 °C below the apparent T_m of the primers (ideally between 52 °C to 58 °C). The cycle

concludes with an elongation step. The temperature depends on the DNA polymerase selected for the experiment. For example, Taq DNA polymerase has an optimal elongation temperature of 70 °C to 80 °C and requires 1 minute to elongate the first 2 kb, then requires an extra minute for each additional 1 kb amplified. Pfu DNA Polymerase is another thermostable enzyme that has an optimal elongation temperature of 75 °C. Pfu DNA Polymerase is recommended for use in PCR and primer extension reactions that require high fidelity and requires 2 minutes for every 1 kb to be amplified. See manufacturer recommendations for exact elongation temperatures and elongation time indicated for each specific DNA polymerase.

3. The final phase of thermal cycling incorporates an extended elongation period of 5 minutes or longer. This last step allows synthesis of many uncompleted amplicons to finish and, in the case of Taq DNA polymerase, permits the addition of an adenine residue to the 3' ends of all PCR products. This modification is mediated by the terminal transferase activity of Taq DNA polymerase and is useful for subsequent molecular cloning procedures that require a 3'-overhang.
4. Termination of the reaction is achieved by chilling the mixture to 4 °C and/or by the addition of EDTA to a final concentration of 10 mM.

Table 10.2: Thermocycler condition

Step	Temp	Time	# of cycles
Initial Denaturation	94°C	5 min	30-35
Denaturation	94°C	30 sec	
Primer Annealing	$T_m - 5^\circ\text{C}$	45 sec	
Extension	72°C	1 min per kb	
Final Extension	72°C	5 min	

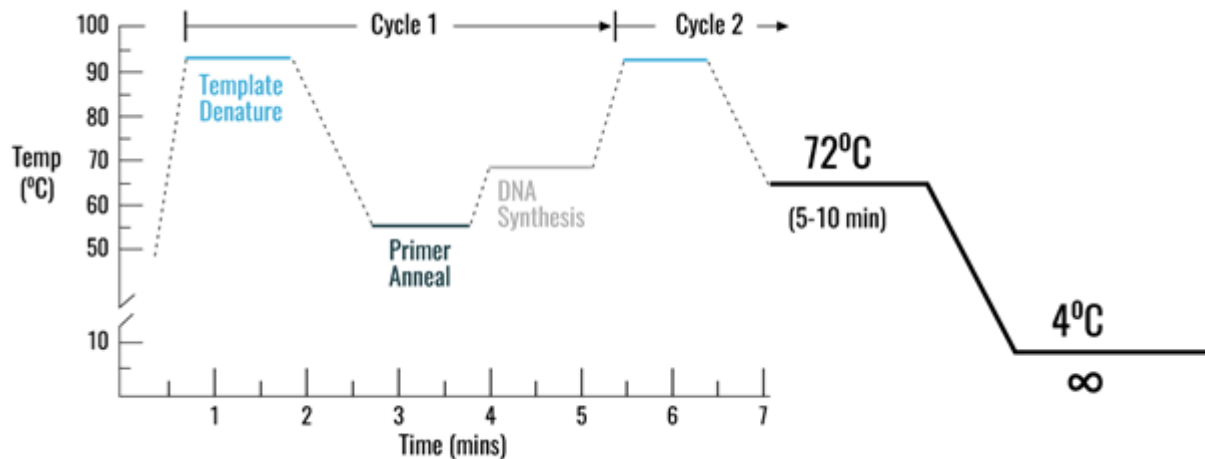


Fig 10.2: PCR conditions

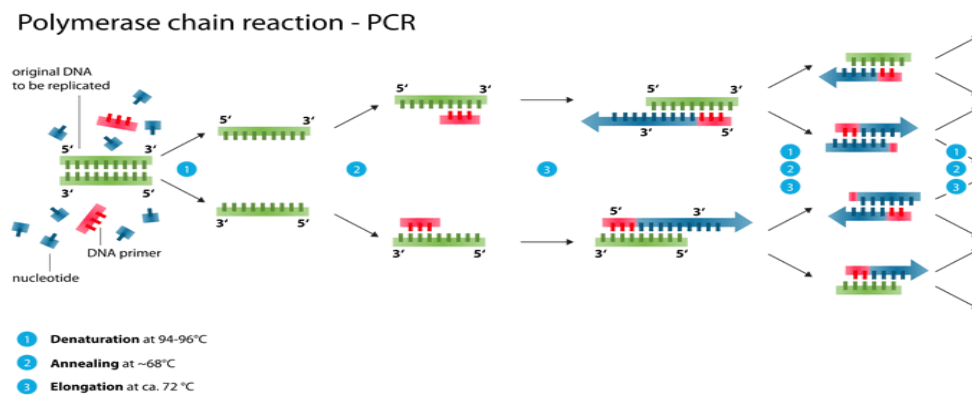


Fig 10.3: PCR process

Types of polymerase chain reaction-PCR

Several modifications of PCR methods have been developed to enhance the utility of this method in diagnostic settings based on their applications. Some of the common types of PCR are;

1. Real-Time PCR (quantitative PCR or qPCR)
2. Reverse-Transcriptase (RT-PCR)
3. Gradient PCR
4. Touch down PCR
5. Multiplex PCR
6. Nested PCR
7. High Fidelity PCR

8. Fast PCR

9. Hot Start PCR

Applications of PCR

- Identification and characterization of infectious agents
- Direct detection of microorganisms in patient specimens
- Identification of microorganisms grown in culture
- Detection of antimicrobial resistance
- Investigation of strain relatedness of a pathogen of interest
- Genetic fingerprinting (forensic application/paternity testing)
- Detection of mutation (investigation of genetic diseases)
- Cloning genes
- PCR sequencing

Practical no: 11

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

A real-time polymerase chain reaction (real-time PCR), also known as quantitative polymerase chain reaction (qPCR), is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR) and semi-quantitatively (i.e., above/below a certain amount of DNA molecules) (semi-quantitative real-time PCR).

Two common methods for the detection of PCR products in real-time PCR are (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence.

Real-time polymerase chain reaction (real-time PCR) is commonly used to measure gene expression. It is more sensitive than microarrays in detecting small changes in expression but requires more input RNA and is less adaptable to high-throughput studies. It is best suited for studies of small subsets of genes. Its one major shortcoming is that the sequence of the specific target gene of interest must be known (so you can design the PCR primers), hence real-time PCR can only be used for studying known genes.

In the traditional PCR method after the amplification, the PCR products or the amplicon are run on the agarose gel or PAGE to detect the presence or absence of DNA amplification. But in the real-time PCR, the amplification during each PCR cycle is monitored in a real-time manner, instead.

A camera or detector detects each amplicon produced during the amplification of template DNA.

Usually, the chemistry behind real-time monitoring relies on the use of fluorescent dye. As the amplification progressed, the detector detects the amount of fluorescence emitted. This is the basic and global principle behind all the types of real-time PCR.

However, some platform uses labeled probes instead of dNTPs.

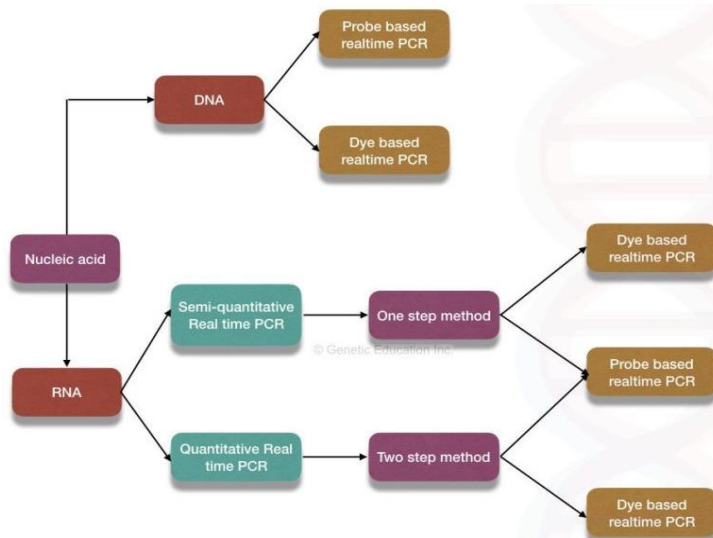


Fig 11.1: qRT-PCR steps

The principle of real-time PCR:

The principle of real-time PCR relies on the use of fluorescent dye. In general, the principle of the present method is stated below,

The amount of the nucleic acid present into the sample is quantified using the fluorescent dye or using the fluorescent labeled oligos.”

Two types of chemistry are available for the real-time quantitative PCR:

DNA binding dye (Intercalating dye-based method)

Sequence-specific probe (Hydrolysis Probe-based detection method)

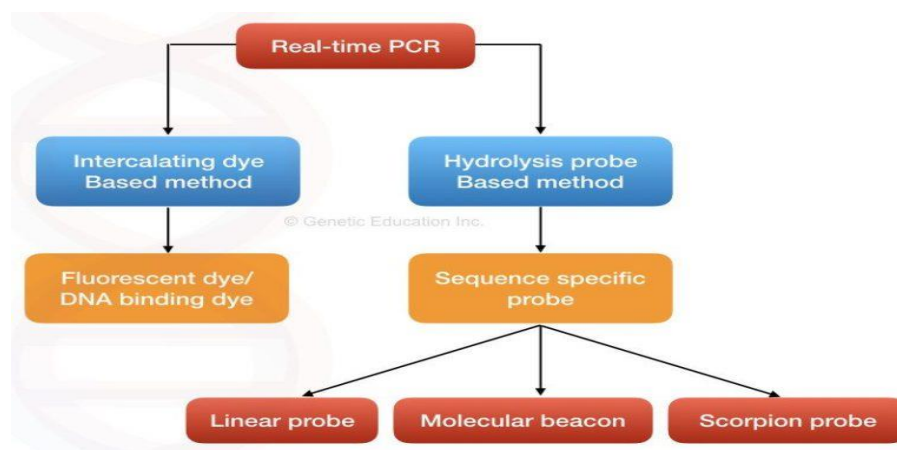


Fig 11.2: qRT-PCR chemistry

DNA binding dye:

For a novice and inexperienced person, the DNA binding dye method is the best technique for real-time detection. The dye has its own fluorescence. Once the dye binds to the double-stranded DNA the fluorescence emitted by the dye increases 100 to 1000 fold than the original signal. However, the original dye fluorescence is taken as the baseline for the detection. The method is rapid, quick, reliable and cost-effective. Also, the chance of error in the experiments is less and the reaction set up is simple and easy to use. The result of the experiment depends on the specificity of the primers used in the PCR reaction. Because even though, the primers are bind non-specifically, the DNA binding dye binds to the non-specific sequence and gives the fluorescent signals. Because the dye detects the double-stranded DNA to binds, even if the dsDNA is non-specific, the dye must bind to it. Therefore the chance of the non-specific detection is high in the SYBR green dye-based method. The SYBR green is one of the most popular dyes used in real-time PCR. The sensitivity of the experiment is limited. Again a question arises in mind.

Is it suitable for the determination of sensitive templates? The answer is yes.

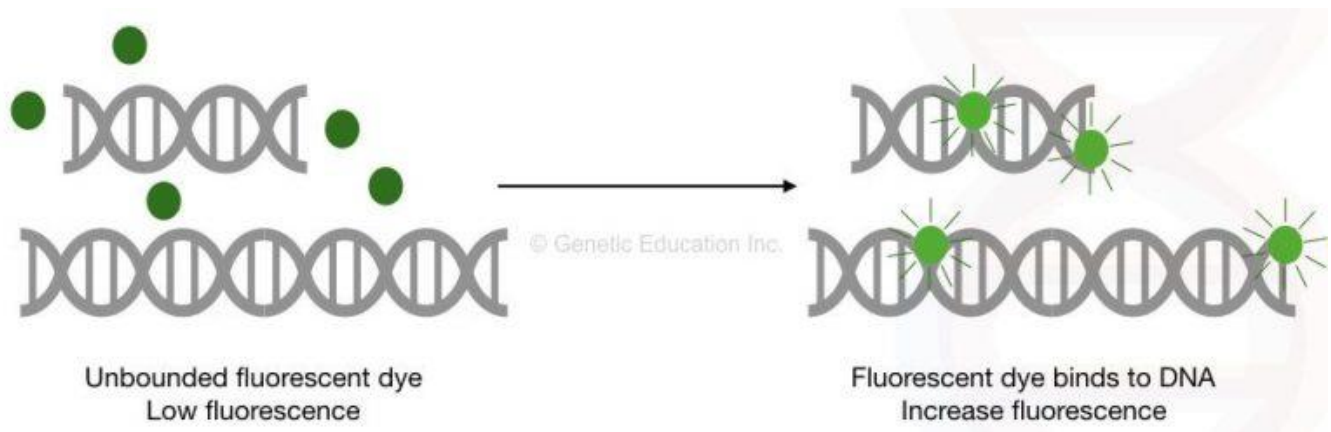


Fig 11.3: Fluorescent dye based detection

Melting curve analysis:

Once the amplification reaction is completed and the fluorescence signals are recorded, the template is melted for the determination of the non-specific bindings. The template is melted using heating, the dye dissociates and the fluorescence signals are reduced. The decreased transition of wide range fluorescence is reported for the specific product while different heat transition recorded for different short non-specific bands. “Larger sequences take more time

and higher temperature for melting while non-specific bands melt at a lower temperature and has different melting temperature curves.” The data are plotted in fluorescence vs melting temperature graph below. The fluorescence vs melting temperature graph is also called a dissociation curve and the method is called a dissociation curve analysis.

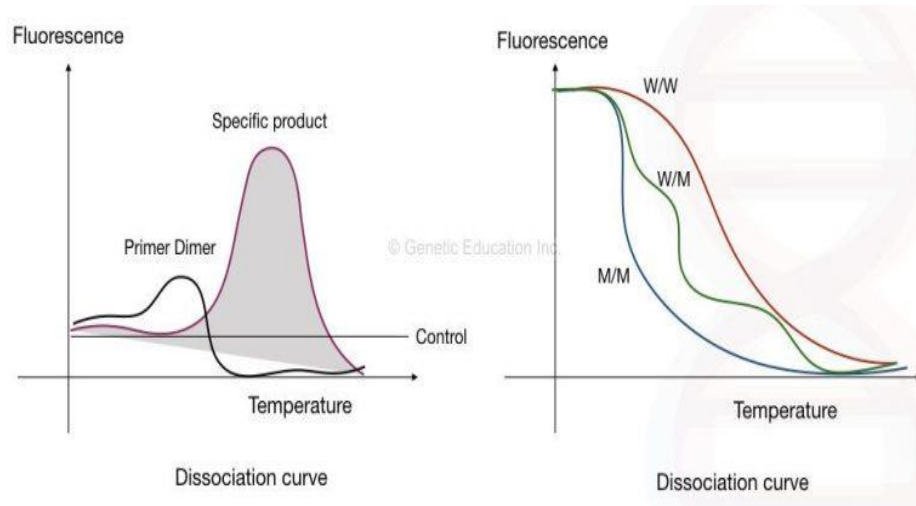


Fig 11.4: qRT-PCR dissociation curve

Image: The image shows the dissociation curve for specific product and primer dimers while another image shows different dissociation curves for two homozygous and a heterozygous. SYBR green and EvaGreen are two main dye used in the quantitative real-time PCR. The experiments are used in the validation of the assays such as DNA microarray.

Linear probe:

Linear probes are the TaqMan probe, relies on the activity of Taq DNA polymerase. The probes are the labelled short single-stranded sequence-specific DNA molecules which are radio or fluorescent labelled. Here the probe is labelled with the fluorescent dye called a reporter molecule, situated at the 3' end. The other 5' end has the quencher dye which is in the close proximity to the reporter dye and quenches the fluorescence of the reporter dye. Now, in the probe base method, not only the probe but the Taq DNA polymerase plays an important role. The Taq DNA polymerase used in the real-time PCR has the 5' to 3' exonuclease activity, which removes the probe by extending the DNA. The main advantage of the probe-based method is that we can use multiple probes for multiple template DNA sequences. This means we can amplify multiple templates in a single reaction efficiently. TAMRA and Black

Hole Quencher are two widely used quencher dyes. While FAM is the most popular reporter dye.

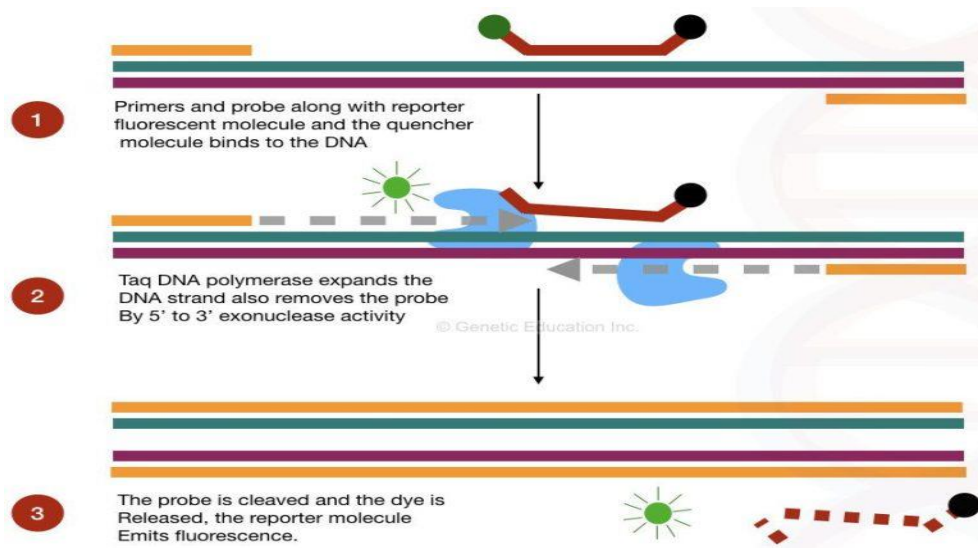


Fig 11.5: Probe based detection

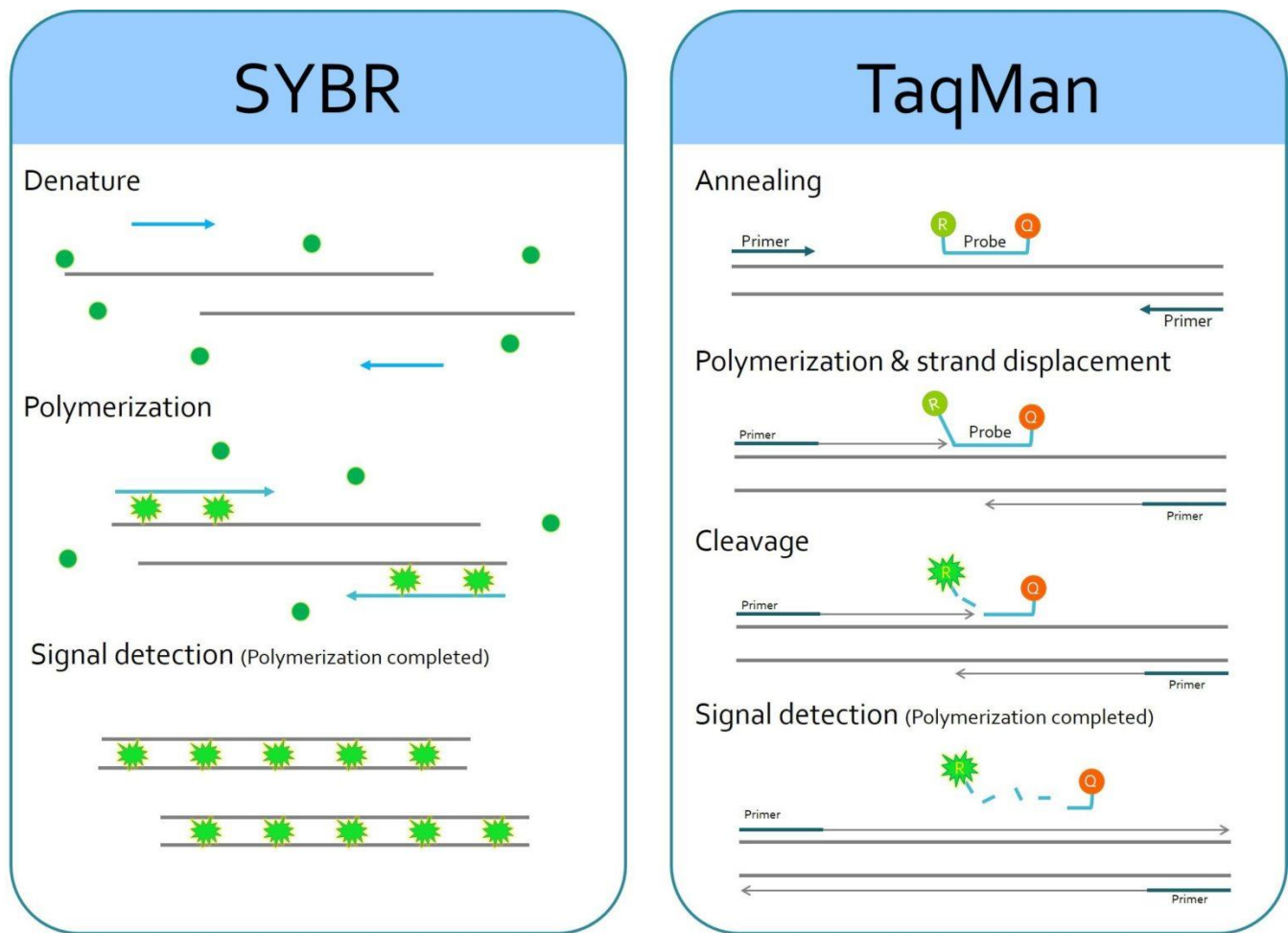


Fig 11.6: Difference between dye and probe based detection

Table 11.1: Common terminologies

Ct value	The point at which the fluorescent is measurable.
Baseline	Point of the initial amplification where the fluorescent is nearly zero. No template zone.
Threshold	A threshold is a set of single which distinguish amplification signals from the background signals
Background signals	Signals of unamplified DNA
Exponential phase	The phase at which the reported amplification at its highest peak.

Steps and procedure of real-time PCR:

The quantification is achieved by amplifying and monitoring the DNA or RNA present into the sample. For the quantification of the gene expression, the RNA is quantified into the real-time PCR. If DNA is present into the sample in higher quantity, amplification and quantification start at the early stage of the reaction otherwise, the amplification starts in late stage. As like the conventional PCR, there are three main steps in real-time PCR;

- **Denaturation**
- **Annealing**
- **Extension**

Denaturation occurs at 94°C where the double-stranded DNA is denatured and two single-stranded DNA is generated. The DNA is melted. This single-stranded DNA is the sight of the annealing for the primers in the later step of the amplification.

Annealing occurs at 55°C to 66°C in which the sequence-specific primer binds to the single-stranded DNA. Along with it, the fluorescent dye or the probe binds to the DNA sequence too.

Extension occurs at 72°C at which the Taq DNA polymerase activated highest. In this step, the Taq adds dNTPs to the growing DNA strand.

Reagents Required:

- PCR master mix including Taq polymerase, dNTP, MgCl₂ and buffer (in case of one-step reverse-transcriptase PCR, the master mix also contains the Reverse Transcriptase enzyme for initial step of cDNA synthesis from RNA template)
- PCR primers
- Labeled Probe or DNA binding dyes
- PCR grade Water
- DNA/RNA standards (for quantification assays)
- Negative and Positive Controls

Equipment Required:

- Real-Time Thermal Cycler with analysis software
- Vortex Mixture
- Microcentrifuge
- Pipettes
- PCR safety cabinet

Consumables:

- Optically clear PCR tubes/strips/plates according to equipment compatibility
- Filtered pipette tips
- 1.5 ml centrifuge tubes

Procedure:

1. Label the PCR tubes for samples and controls. In case of quantification experiments, tube will also be labeled for standards.
2. Thaw the PCR reagents and prepare PCR reaction mix. A generalized recipe of real-time PCR is given in the following table. The amount of ingredients may vary according to the desired protocol and manufacturer's instructions. Calculate the volume of total reaction mix required for the whole batch including samples, controls and standards.
3. Mix the reagent by gentle vortexing followed by short spin.
4. Aliquot the reaction mix in the individual PCR reaction tubes/well.
5. Add the template i.e; sample/ control/ standards in the appropriate labeled tube. The volume of template varies according to the protocol in use. In the above example, 5 μ l DNA templates will be added to each tube so that 20 μ l total reaction volume is achieved. Whereas, in case of RNA, 6 μ l template samples will be added to achieve 15 μ l total reaction volume.
6. 10. Open the Real-PCR machine's software and edit run parameters e.g. Sample IDs, plate map, sample volume, detection wavelengths and cycling conditions according to desired protocol. A generalized cycling protocol for TaqMan assay is given below.

Table 11.2: Preparation of Real-Time PCR Reaction Mix for cDNA template

Reagent	Volume per Reaction
2x PCR Master Mix	10 μ l
Forward Primer (100 μ M stock)	0.2 μ l
Reverse Primer (100 μ M stock)	0.2 μ l
TaqMan Probe	0.1 μ l
DEPC H ₂ O	4.5 μ l
Template	1 μ l
Total	15 μ l

Overview of real-time PCR

This section provides an overview of the steps involved in performing real-time PCR. Real-time PCR is a variation of the standard PCR technique that is commonly used to quantify DNA or RNA in a sample.

Using sequence-specific primers, the number of copies of a particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing the thermal cycling needed for the PCR reaction.

Real-time PCR steps

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

1. Denaturation: High-temperature incubation is used to “melt” double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

2. Annealing: During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (T_m) of the primers (typically 5°C below the T_m of the primer).

3. Extension: At 70–72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step, using 60°C as the temperature.

Two-step qRT-PCR

Two-step quantitative reverse transcriptase PCR (qRT-PCR) starts with the reverse transcription of either total RNA or poly (A) RNA into cDNA using a reverse transcriptase (RT). This first-strand cDNA synthesis reaction can be primed using random primers, oligo (dT), or gene-specific primers (GSPs). To give an equal representation of all targets in real-time PCR applications and to avoid the 3 bias of oligo (dT) primers, many researchers use random primers or a mixture of oligo(dT) and random primers. The temperature used for cDNA synthesis depends on the RT enzyme chosen. After reverse transcription, approximately 10% of the cDNA is transferred to a separate tube for the real-time PCR reaction.

One-step qRT-PCR

One-step qRT-PCR combines the first-strand cDNA synthesis reaction and real-time PCR reaction in

the same tube, simplifying reaction setup and reducing the possibility of contamination. Gene-specific primers (GSP) are required. This is because using oligo (dT) or random primers will generate nonspecific products in the one-step procedure and reduce the amount of product of interest.

Practical no: 12

DNA fingerprinting

A laboratory genetic technique or method to identify individuals using bodily samples such as blood, saliva, or hair is referred to as DNA fingerprinting. Or we can define DNA fingerprinting as, A DNA test to establish a link or relation between two person or living organisms by analyzing their STR and VNTRs is known as DNA fingerprinting.

DNA profiling, DNA testing, DNA analysis, Genetic profile, DNA identification, genetic fingerprinting, and genetic analysis are some of the popular names used for DNA fingerprinting. The present method is employed usually in criminal verification and crime scene investigation. However, it is also applicable to establishing a relationship between two persons and to know someone's identity. The testing method is practiced not only for humans but also for any organisms present on earth. Since 1984, it has been a gold-standard method for personal verification. Because of the unique DNA pattern, two persons differ biologically. And this mechanism is the basis of the present method.

DNA is our blueprint, basis of life, encodes proteins, and regulates gene expression. It is made up of sugar, phosphate, and nitrogenous bases. DNAs are located on chromosomes. The whole set of DNA or chromosomes are known as the genome. Interestingly, there are several regions in our genome that are unique and hypervariable. Every person or organism has different patterns for those regions using which a DNA fingerprinting is performed.

What is DNA fingerprinting?

The technique of DNA fingerprinting changed the era of identification, characterization, and classification of organisms.

Notably, approximately 99% of our DNA (deoxyribose nucleic acid) is similar. A 0.1% difference is sufficient to make someone so unique. We are using this 0.1% portion for DNA profiling which is often known as DNA fingerprinting. There is a reason, why only DNA is used. In all bodily parts, tissues, and cells (except germ cells) our DNA is the same. Even, after the death of the individual, it remains the same. It can remain stable even after 1000 years. As we know, 97% of our genome is non-coding, repetitive, and junk, protein formation is regulated by only 3% portion, we know it as genes. Moreover, repetitive nature and polymorphic properties of junk DNA are utilized to perform DNA fingerprinting.

The number of repeats and the sequence structure of those regions varies between individuals and organisms based on that the entire DNA profile or the DNA print can be prepared.

A comprehensive overview of the whole method is given below. The DNA is isolated from the bodily sample, digested or amplified using restriction digestion or PCR, respectively. Using either agarose gel electrophoresis or sequencing, the DNA fragments are separated and identified. Though the PCR based method for DNA fingerprinting is accurate and too fast but after the discovery of real-time PCR and DNA sequencing, it becomes even more powerful. The molecular tools available nowadays create 100% accurate patterns of a person's DNA.

Satellites DNA:

In Genetics, the satellites are repetitive DNA regions, located on telomeres and centromeres and abnormal repeats halt DNA replication. It clearly indicates that satellites help to do proper replication. Mutation in those sequences causes the end replication errors or problems. Note one thing here, these satellite DNAs are non-coding. Mainly two types of satellite regions are present in the human genome based on their repeat sequence nature: minisatellite and microsatellites. Minisatellite region contains repeated DNA sequences of 10 to 60 bp. 5 to 50 repeats of it are present in our genome. For example, VNTRs. Minisatellites are highly variable (polymorphic), unique, and GC-rich sequences. As we said above, It is found mostly in telomeric regions (90% sequences). Contrary, microsatellites are smaller than minisatellites. It's 1-6 bp long and repeated 5 to 10 times in a genome. For example, STR and SSR. Read more on STR- short tandem repeats. These regions are also hypervariable, non-coding and telomeric, likewise the VNTRs. We have covered the entire article on VNTR and STR in genetic marker, for a more detailed understanding of genetic marker read the article: Genetic markers.

The role of a non-coding region:

Gene exists in nature because it can encode various proteins. Through the collective efforts of replication, transcription and translation, DNA, mRNA, and proteins form, respectively. But the DNA other than genes aren't able to construct any protein, however, as per recent findings, non-coding DNA regulates gene expression. Cell, tissue, and organs specific gene expression is regulated by the loosely packed, junk, and non-coding DNA. Read more: Gene regulation.

VNTRs and STRs:

“Tandem repeats are the sequences which are located one after another into the genome.”

It varies from individual to individual. For example, if a person “A” has 45 VNTRs (with 20bp) and 9 STRs (with 5bp), The possibility of having this same number of repeats for this specific VNTR and STR in another individual is almost negligible. Nonetheless, in the case of monozygotic twins, it is quite possible. Monozygotic twins are developed from a single type of embryo (due to the splitting of an embryo), so the chances of having the same VNTR and STR profile are higher. Still, monozygotic twins aren’t similar biologically. Read this article to know more: Do Identical Twins Have The Same DNA? For normal individuals, the chance of having the same DNA profile is 1 in 10,000,000,000,000(the total world population is 7,600,000,000, imagine the possibility).

Table 12.1: Difference between VNTR and STR

TR	STR
Variable number of tandem repeats	Short tandem repeats
A type of minisatellite	A type of microsatellite
Consists 10 to 60 bp	Consists 1 to 6 bp
10 to 1000 repeats in a genome	5 to 200 repeats in a genome
Produce heterogeneous array	Produce homogeneous array

Here we have explained one simple example to understand the topic more precisely. Various VNTRs (V1, V2, V3, V4 and V5) are digested with a single type of restriction endonuclease, and the fragments are serrated on the agarose gel. The results are shown in the figure below,

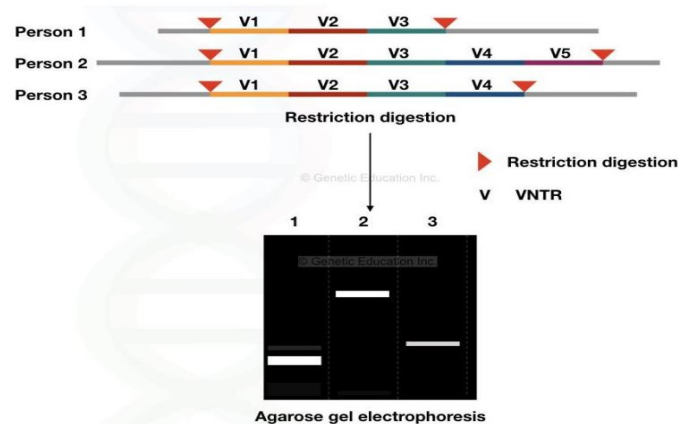


Fig 12.1: View of VNTR

Steps:

Collection of a biological sample- blood, saliva, buccal swab, semen, or solid tissue.

DNA extraction**Restriction digestion or PCR amplification****Agarose gel electrophoresis, capillary electrophoresis or DNA sequencing****Interpreting results****Process of DNA fingerprinting:**

Sample collection, DNA extraction, digestion or amplification and analysis results are major steps.

Step 1: Sample collection:

DNA can be obtained from any bodily sample or fluid. Buccal smear, saliva, blood, amniotic fluid, chorionic villi, skin, hair, body fluid, and other tissues are the major types of samples used. In criminal cases, a buccal swab is taken usually. The buccal swab sample collection method is non-invasive and handy. However, if not maintained properly, a buccal swab can easily be contaminated with bacteria. Further, the Buccal swab DNA yield is very less. A blood sample is a good replacement for a buccal swab sample. We can use a blood sample as well.

Step 2: DNA extraction

We have to first obtain DNA. To perform any genetic applications, DNA extraction is one of the most significant steps. Good quality and quantity DNA increases the possibilities of getting good results.

You can use either of DNA extraction method enlisted below,

Phenol-chloroform DNA extraction method

CTAB DNA extraction method

Proteinase K DNA extraction method

Nevertheless, we strongly recommend using a ready to use DNA extraction kit for DNA fingerprinting.

The purity and quantity of DNA should be ~1.80 and 100ng, respectively to perform the DNA test. Purify the DNA using the DNA purification kit, if needed. After that, quantify the DNA using the UV-Visible spectrophotometer.

DNA fingerprinting techniques:

Step 3: Restriction digestion, amplification or DNA sequencing

Three common methods are used:

RFLP based STR analysis

PCR based analysis

Real-time PCR analysis

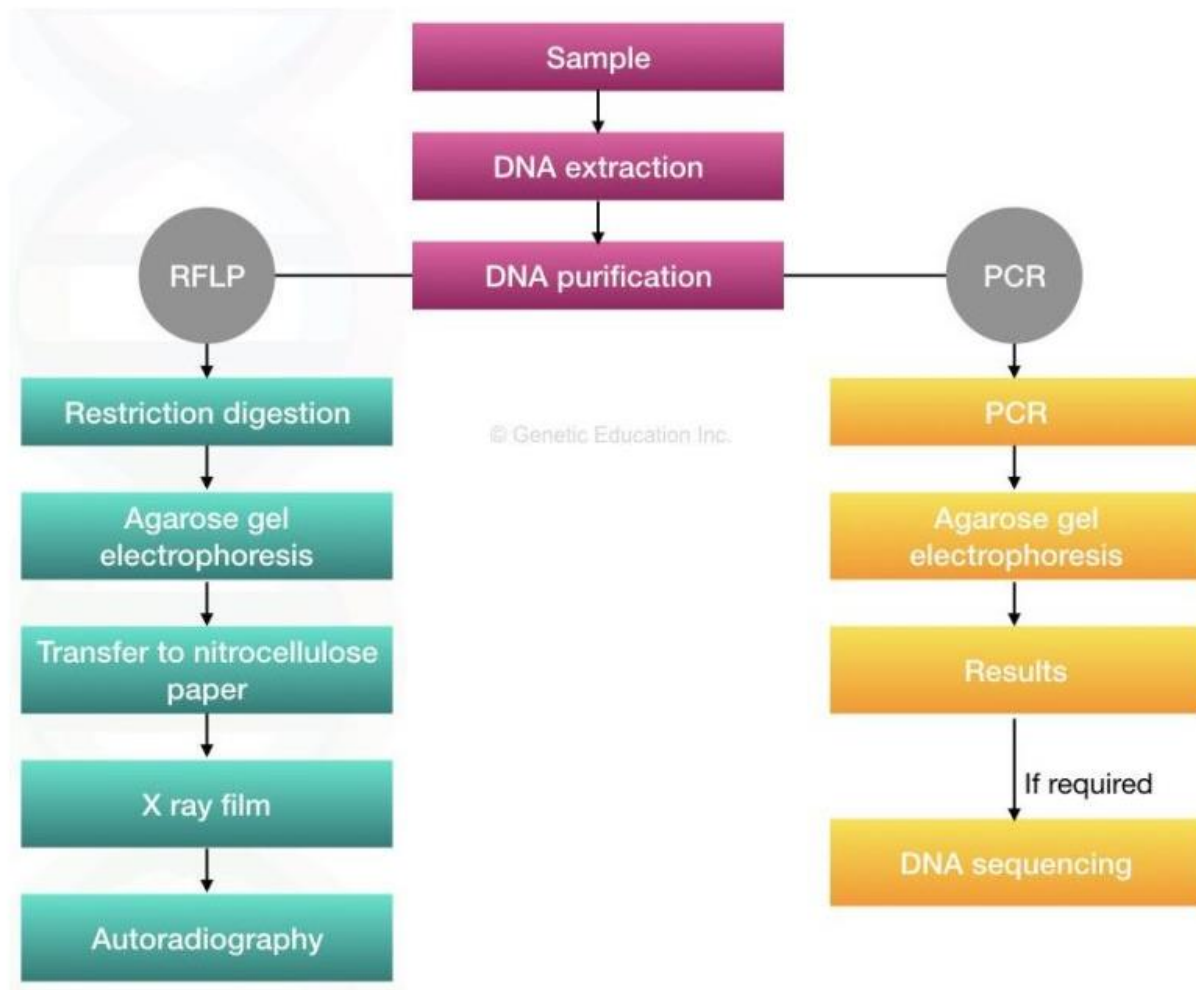


Fig 12.2: Plan of sample processing

Practical no: 13

Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique invented in 1984 by the English scientist Alec Jeffreys during research into hereditary diseases. It is used for the analysis of unique patterns in DNA fragments in order to genetically differentiate between organisms – these patterns are called Variable Number of Tandem Repeats (VNTRs).

Genetic polymorphism is defined as the inherited genetic differences among individuals in over 1% of normal population. The RFLP technique exploits these differences in DNA sequences to recognize and study both intraspecies and interspecies variation.

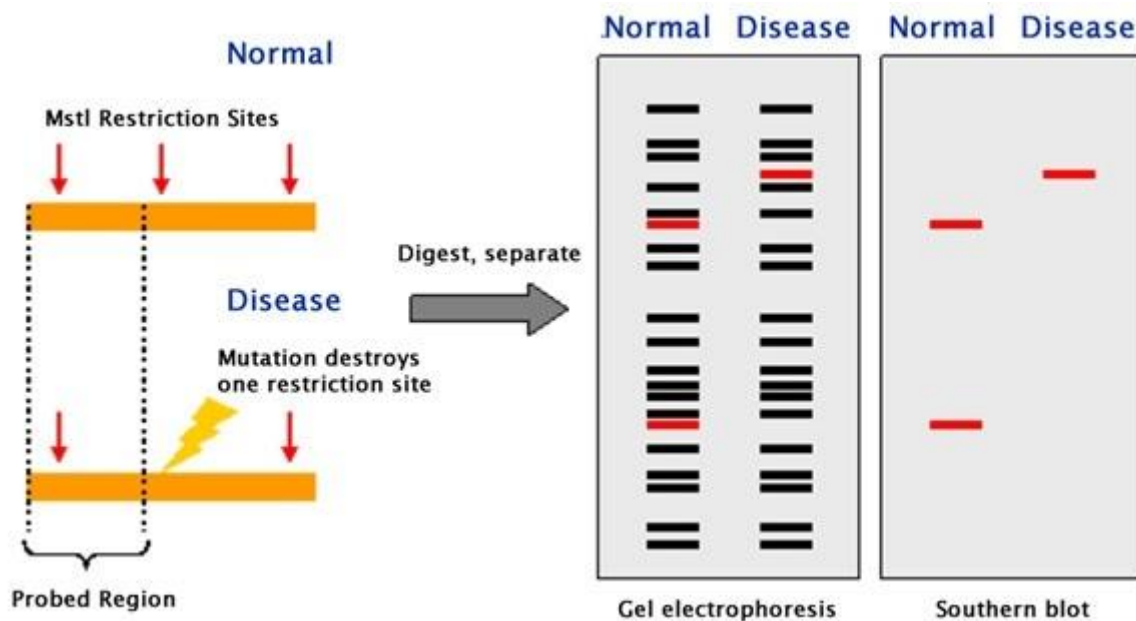


Fig 13.1: View of RFLP procedure

Principle

Restriction endonucleases are enzymes that cut lengthy DNA into short pieces. Each restriction endonuclease targets different nucleotide sequences in a DNA strand and therefore cuts at different sites.

The distance between the cleavage sites of a certain restriction endonuclease differs between individuals. Hence, the length of the DNA fragments produced by a restriction endonuclease will differ across both individual organisms and species.

How does it Work?

RFLP is performed using a series of steps briefly outlined below:

DNA Extraction

To begin with, DNA is extracted from blood, saliva or other samples and purified.

DNA Fragmentation

The purified DNA is digested using restriction endonucleases. The recognition sites of these enzymes are generally 4 to 6 base pairs in length. The shorter the sequence recognized, the greater the number of fragments generated from digestion.

For example, if there is a short sequence of GAGC that occurs repeatedly in a sample of DNA. The restriction endonuclease that recognizes the GAGC sequence cuts the DNA at every repetition of the GAGC pattern.

If one sample repeats the GAGC sequence 4 times whilst another sample repeats it 2 times, the length of the fragments generated by the enzyme for the two samples will be different.

Gel Electrophoresis

The restriction fragments produced during DNA fragmentation are analyzed using gel electrophoresis.

The fragments are negatively charged and can be easily separated by electrophoresis, which separates molecules based on their size and charge. The fragmented DNA samples are placed in the chamber containing the electrophoretic gel and two electrodes.

When an electric field is applied, the fragments migrate towards the positive electrode. Smaller fragments move faster through the gel leaving the larger ones behind and thus the DNA samples are separated into distinct bands on the gel.

Visualization of Bands

The gel is treated with luminescent dyes in order to make the DNA bands visible.

Applications of RFLP

RFLP has been used for several genetic analysis applications since its invention.

Some of these key applications of RFLP are listed below:

1. To determine the status of genetic diseases such as Cystic Fibrosis in an individual.
2. To determine or confirm the source of a DNA sample such as in paternity tests or criminal investigations.
3. In genetic mapping to determine recombination rates that show the genetic distance

between the loci.

4. To identify a carrier of a disease-causing mutation in a family.

Disadvantages of RFLP

1. Since its invention, RFLP has been a widely used genome analysis techniques employed in forensic science, medicine, and genetic studies. However, it has become almost obsolete with the advent of relatively simple and less expensive DNA profiling technologies such as the polymerase chain reaction (PCR).
2. The RFLP procedure requires numerous steps and takes weeks to yield results, while techniques such as PCR can amplify target DNA sequences in a mere few hours.

Practical no: 14

Cloning and Preparation of Chemically Competent Cells

Materials and Reagents

1. Luria-Bertani broth (LB) medium: Bacto-tryptone (BD Biosciences), yeast extract (BD Biosciences)
2. Antibiotics (Sigma-Aldrich/Thermo Fisher Scientific)
3. QIAGEN Plasmid Purification Handbook (QIAGEN)
4. SeaKem® LE Agarose (Cambrex)
5. Plasmid Prep Kit (QIAGEN /Fermentas)
6. PCR Clean-up kit (QIAGEN /Fermentas)
7. Restriction enzymes (New England Biolabs)
8. Alkaline Phosphatase: Calf intestinal alkaline phosphatase (CIP) or Shrimp Alkaline Phosphatase (SAP)
9. Ligase enzyme
10. DNA ladder
11. NaCl
12. LB broth media
13. Ligation reaction

Protocol

Preparing transformation competent E. Coli using CaCl₂

1. It is very important that sterile technique be strictly observed through the prep. All centrifuge bottles, tubes, solutions, pipets, etc. must be sterile. Any contamination during the prep will result in problems for everyone in the lab who uses the competent cells for cloning, thereby probably decreasing your popularity.
2. Inoculate 1 ml of LB with cells from a frozen glycerol stock and incubate overnight at 37°C.
3. Transfer the entire 1 ml into a 1 L erlenmeyer flask containing 500 ml LB and incubate at 37°C.
4. Monitor growth starting at about 2 hours by measuring OD of an aliquot. Let cells grow until the OD₅₅₀ = 0.45 to 0.6. This should take approximately 4 hours.

5. Transfer the culture to 2 x 500 ml centrifuge bottles and chill on ice to 4°C.
6. Centrifuge at 6,000 g for 5 min at 4°C.
7. Pour off supernatant and pipette off remaining supernatant.
8. Resuspend each pellet in 125 ml ice cold 50mM CaCl₂. Combine into a single 500 ml centrifuge bottle.
9. Centrifuge at 6,000 g for 5 min at 4°C.
10. Pour off supernatant and resuspend in 21.5 ml ice cold 50 mM CaCl₂
11. Add 3.5 ml sterile glycerol (100%) and mix gently
12. Prepare 500 µl aliquots in sterile microfuge tubes and snap freeze in liquid nitrogen
13. Store in -80°C freezer. Use within 6 months if possible

Transforming chemically competent E. Coli using heat shock

1. E. Coli cells are stored at -80°C in aliquots of 500 µl. Thaw one vial on ice. Generally need 100 µl for each transformation. It is best not to refreeze whatever is leftover, just throw it away.
2. For each transformation, pipet 100 µl of competent cells into a sterile 1.5 ml microfuge tube.
3. Add DNA (5-10 µl of PCR product or 1 µl plasmid) to each tube and incubate on ice for 20-25 mins (can be as short as 5 min). This allows the DNA to adsorb to cell surfaces.
4. Heat shock the cells/DNA to allow uptake of DNA into cells. Place tubes in 42° C water bath for 45 seconds. Variation: place all tubes at 37°C for 2 mins. Return cells to ice.
5. Add 1 ml of LB or SOC to each tube and incubate at 37°C for 45-60 mins in a shaking incubator to let the cells recover from the heat shock and start growing again. Liquid should appear cloudy at this point.
6. Streak the cells onto agar plates with the correct antibiotic. If you are using blue/white selection remember to coat the plates with 40 µl of 2% X-gal in DMSO at least one hour before streaking the bacteria. I usually streak two plates for each transformation. On one plate streak 50 µl of the 1 ml cell slurry. Spin the remaining 950 µl by placing in a table-top centrifuge and bringing the speed up to 12,000 g for one or two seconds. This should pellet the cells. Pour off the majority of LB/SOC, then resuspend the pellet

in the remaining ~100 μ l and spread this on the second plate.

7. Incubate plates at 37°C overnight.
8. Pick colonies the next day

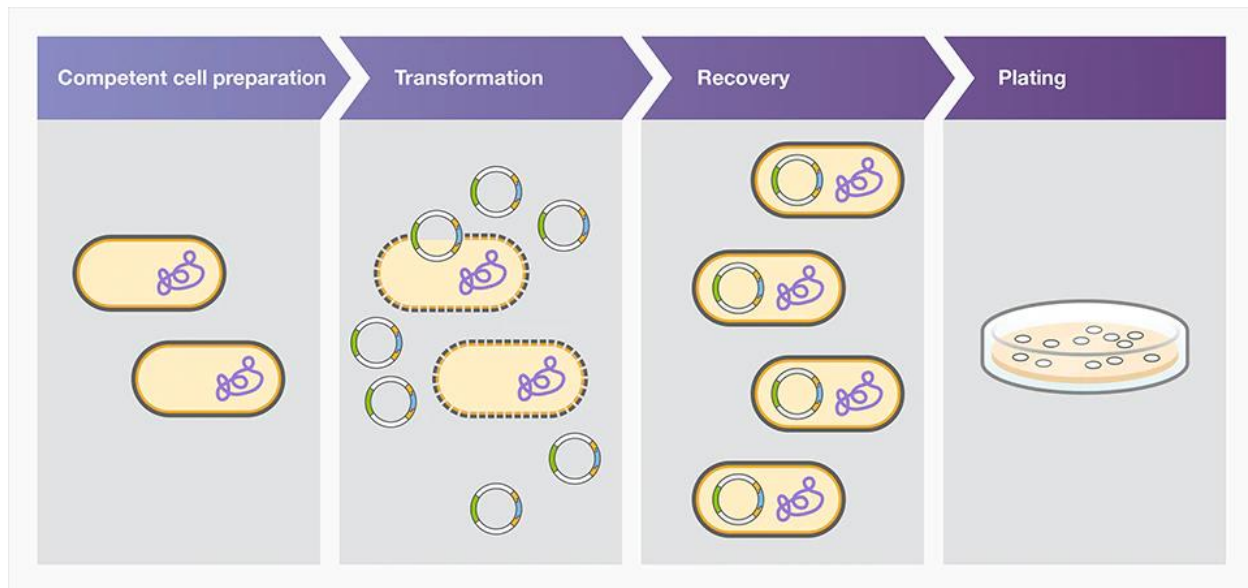


Fig 14.1: Bacterial transformation

Practical no: 15

Isolation of bacterial Plasmid

While genomic DNA extraction is pretty straightforward, extracting plasmid DNA can be a little more complicated since you should be able to identify and use the appropriate lysis method to successfully separate the plasmid DNA from the gDNA. Basically, a milder treatment (i.e. alkaline lysis) is required when extracting plasmid DNA. Here's how you go about extracting them.

1. Cell Cultivation

The procedure starts with the cultivation of bacterial cells in varying amounts of growth medium. When sufficient growth is achieved, you can remove the cells from the medium through centrifugation.

2. Resuspension and Cell Lysis

A cell pellet from the saturated culture is resuspended in an isotonic solution containing Tris, EDTA (to disrupt the cell wall and prevent DNases from damaging the plasmid), glucose (to prevent the cells from bursting) and RNase A (to degrade cellular RNA during cell lysis). An alkaline solution containing sodium dodecyl sulfate (SDS) is then added to facilitate cell lysis and the complete denaturation of both genomic and plasmid DNA along with all the proteins in the solution.

3. Neutralization, Cleaning and Concentration

A potassium acetate solution is then used to neutralize the sample and separate the plasmid DNA from the gDNA. The smaller plasmid DNA tends to renature easily while the larger, more complicated gDNA remains denatured and precipitates out of the solution.

Upon centrifugation, gDNA will form a pellet while plasmid DNA remains soluble. The plasmid DNA remaining in the supernatant can then be precipitated with ethanol or purified using a phenol-chloroform mixture or spin filter technology.

Some Tips to Consider for Plasmid DNA Extraction:

- The cell lysis step should be done quickly since overdoing it can irreversibly denature the plasmid.
- While the resuspension and lysis buffers should be mixed thoroughly, make sure you

do not vortex or mix it vigorously to prevent the DNA from breaking into smaller fragments. If they are small enough, broken gDNA can reanneal and remain in the solution.

- Wear gloves and appropriate eye protection when working with harsh chemicals such as NaOH and SDS.

E. coli Plasmid DNA MiniPrep Protocol

Introduction

The isolation of plasmid DNA from E. coli using an alkaline lysis is a well-established method. E. coli with plasmid is cultured in media with antibiotics to a high cell density, harvested, and then lysed with a SDS/NaOH solution. Rapid acidification using concentrated potassium acetate causes the precipitation of protein and chromosomal DNA. Plasmid DNA, which is supercoiled, remains in solution and can be captured on a silica spin column. The plasmid DNA is washed with an ethanol solution and then eluted in water or TE buffer.

Material

4. Microfuge tubes
5. Resuspension buffer (50 mM Tris HCl, pH 8, 10 mM EDTA, 100 µg/ml RNase A)
6. Lysis buffer (0.2 N NaOH, 1% SDS)
7. Neutralization buffer (3/5 M Potassium acetate, pH 6)
8. Spin columns (Product Number SSC 100-01)
9. Isopropanol
10. Wash buffer (70% Ethanol)
11. Elution buffer (water or TE buffer- 10 mM Tris, pH 8, 1 mM EDTA)

Equipment

1. Vortexer
2. Centrifuge

Protocol

1. Culture E. coli with plasmid in LB media with antibiotic selective pressure, overnight on a shaker at 37°C.
2. Pellet 1.5 ml of bacterial culture in a microfuge tube by centrifuging for 2 minutes at 10,000 rpm.
3. Decant the supernatant and add 200 µl of the resuspension buffer. In order to resuspend

the pellet you may have to vortex.

4. Add 250 μ l of the lysis buffer, invert the tube 10 times to mix thoroughly. The solution should become clear and viscous.
5. Add 350 μ l of the neutralization buffer, invert the tube 10 times or until a precipitate forms. The precipitate is a mixture of protein and chromosomal DNA.
6. Centrifuge the tube for 10 minutes at 10,000 rpm. Transfer the supernatant to a microfuge tube and add 0.7 isopropanol. Incubate at -20°C for 15 minutes.
7. Transfer the solution to a spin column.
8. Centrifuge the spin column for 1 minute at 7,000 rpm. Discard the flow through.
9. Add 400 μ l of the wash buffer and centrifuge for 1 minute at 7,000 rpm. Discard the flow through. Repeat this step.
10. Centrifuge for an additional 2 minutes at 10,000 rpm to remove residual wash buffer.
11. Transfer the column to a clean microfuge tube. Add 50 μ l of elution buffer and centrifuge for 1 minute at 10,000 rpm.

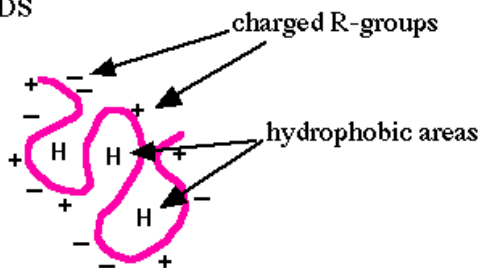
Practical no: 16

Analysis of proteins by SDS-PAGE

SDS-PAGE

The purpose of SDS-PAGE is to separate proteins according to their size, and no other physical feature. In order to understand how this works, we have to understand two halves of the name: SDS and PAGE. In this Technique we are trying to separate many different protein molecules of different shapes and sizes, we first want to denature so that the proteins no longer have any secondary, tertiary or quaternary structure (i.e. we want them to retain only their primary amino acid structure). Consider two proteins that are each 500 amino acids long but one is shaped like a closed umbrella while the other one looks like an open umbrella. If you tried to run down the street with both of these molecules under your arms, which one would be more likely to slow you down, even though they weigh exactly the same? This analogy illustrates mass and the 3D structure of a molecule will determine how well it can move through an environment. We use SDS to denature all proteins to the same linear shape.

BEFORE SDS



AFTER SDS



SDS (sodium dodecyl sulfate) is a detergent (soap) that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubilized by the detergent, plus all the proteins will be covered with many negative charges. The end result has two important features:

- 1) All proteins retain only their primary structure
- 2) All proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.

PAGE

If the proteins are denatured and put into an electric field, they will all move towards the positive pole at the same rate, with no separation by size. So we need to put the proteins into an environment that will allow different sized proteins to move at different rates. The environment of choice is polyacrylamide, which is a polymer of acrylamide monomers. When this polymer is formed, it turns into a gel and we will use electricity to pull the proteins through the gel so the entire process is called **polyacrylamide gel electrophoresis** (PAGE). A polyacrylamide gel is not solid but is made of a labyrinth of tunnels through a meshwork of fibers.

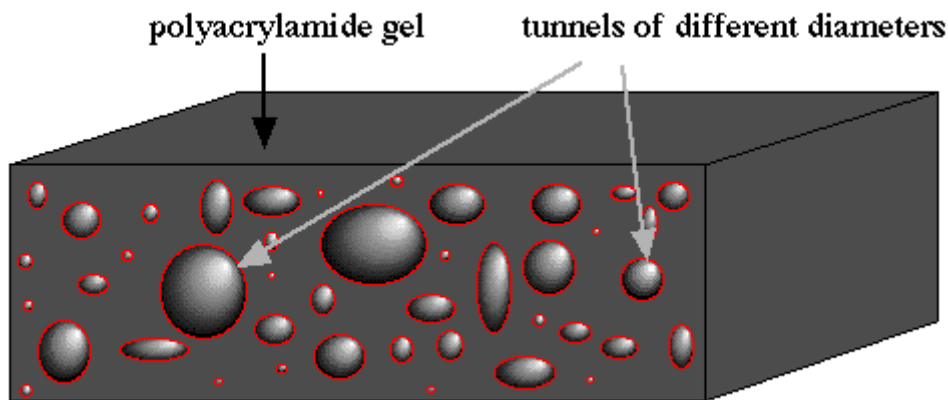


Fig 16.2. This picture shows a slab of polyacrylamide (dark gray) with tunnels (different sized red rings with shading to depict depth) exposed on the edge. Notice that there are many different sizes of tunnels scattered randomly throughout the gel.

Figure of Running Gel

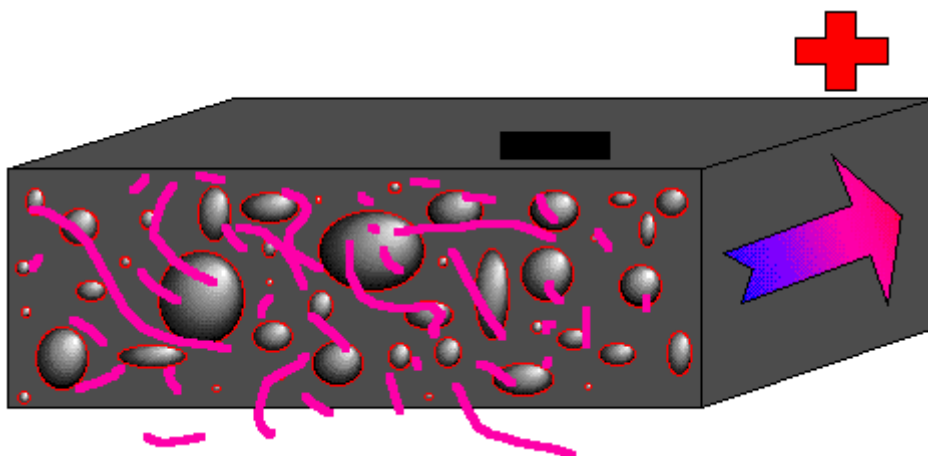


Fig 16.3. Showing a mixture of denatured proteins (pink lines of different lengths) beginning their

journey through a polyacrylamide gel (gray slab with tunnels). An electric field is established with the positive pole (red plus) at the far end and the negative pole (black minus) at the closer end. Since all the proteins have strong negative charges, they will all move in the direction the arrow is pointing (run to red).

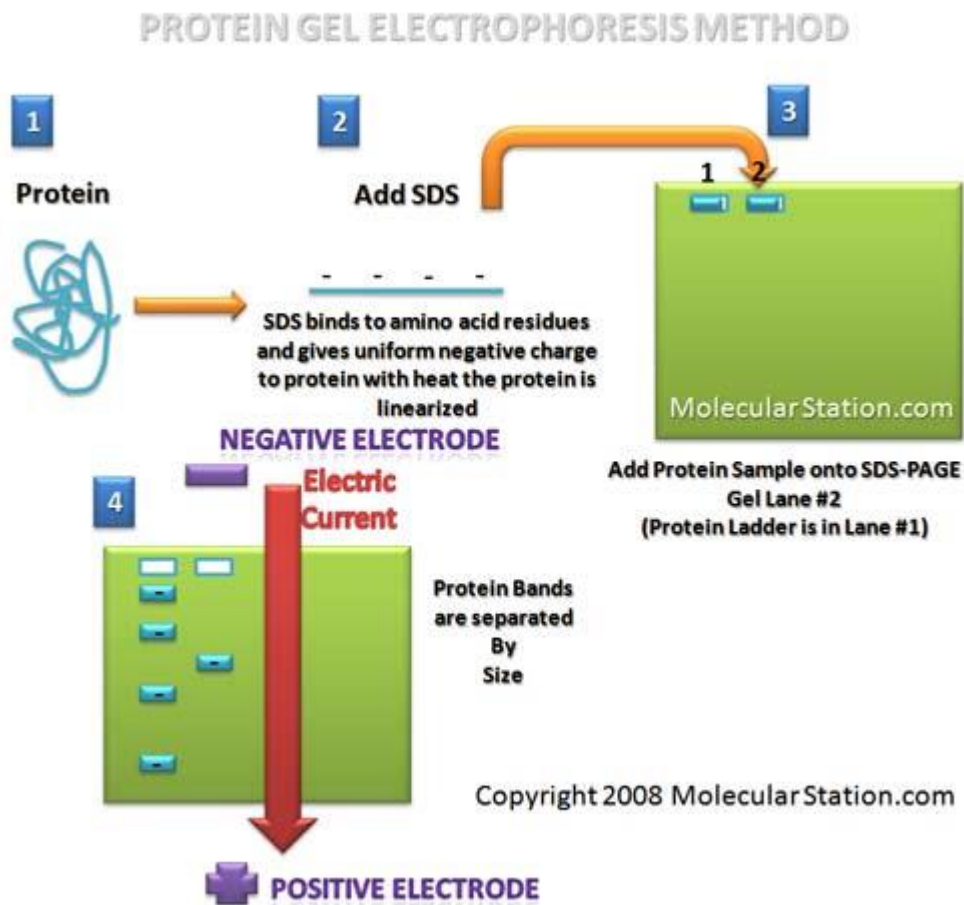


Fig 16.4. Hypothetical Figure of Gel

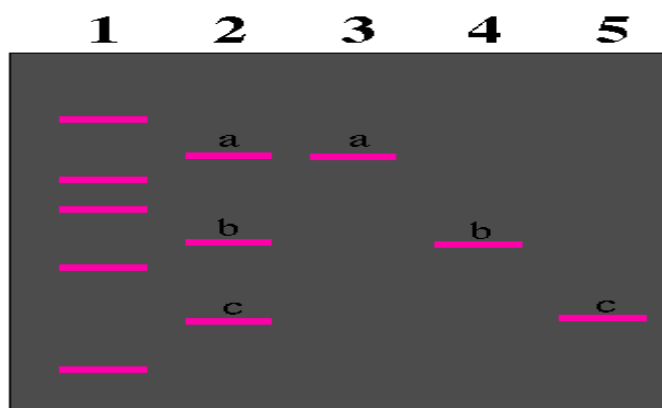


Fig 16.5. Top view of an SDS PAGE after the current has been on for a while (positive pole at the bottom) and then turned off. The gel (gray box) has five numbered lanes where five different samples of proteins (many copies of each kind of protein) were applied to the gel. (Lane 1, molecular weight standards of known sizes; Lane 2, a mixture of three proteins of different sizes with **a** being the largest and **c** being the smallest protein; Lane 3, protein **a** by itself; Lane 4, protein **b** by itself; Lane 5 protein **c** by itself.) Notice that each group of the three proteins migrated the same distance in the gel whether they were with other proteins (lane 2) or not (lanes 3-5). The molecular weight standards are used to measure the relative sizes of the unknown proteins (**a**, **b**, and **c**).

Preparations of Buffer (Discontinuous buffer system)

Separating gel buffer (1.5 M Tris-HCl, pH 8.8)

A 36.6 g Tris base is dissolved in 170 ml of the distilled water and pH is adjusted to 8.8 with diluted HCl. The water is added to a total volume of 200 ml and is stored at 4°C.

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

A 1.21 g of Tris-base is dissolved in 170 ml of the distilled water and pH is adjusted to 6.8 with diluted HCl. The water is added to a total volume of 200 ml and is stored at 4°C.

2X sample buffer (for liquid samples)

The following solutions are dispensed in a 10 ml measuring cylinder:

Glycerol (10%)	1.00 ml
Tris-HCl, pH 6.8 (0.5 M)	1.25 ml
SDS (10%)	2.50 ml
2-mercaptoethanol	0.50 ml
Distilled water q.s.	10.00 ml

The ingredients are mixed well and stored at –20°C in a capped tube.

1X Sample buffer (for solid samples)

The following solutions are dispensed in a 10 ml measuring cylinder:

Glycerol (10%)	0.50 ml
Tris-HCl, pH 6.8 (0.5 M)	1.25 ml
SDS (10%)	2.50 ml
2-mercaptoethanol	0.50 ml
Distilled water q.s.	10.00 ml

The ingredients are mixed well and stored at -20°C in a capped tube.

Running buffer (Electrode buffer)

This was made by dissolving

1 g	Tris-base
43.20 g	Glycine
3.0 liter	Distilled water
3 g	SDS

The final pH was 8.2 and stored at 4°C .

Steps for SDS-PAGE

Vertical gel electrophoresis system (Laemmli, 1970) is used for the separation of polypeptides of different pathogens.

Preparation of gel mould

Two specially cut glass plates are used to mould the gel. The glass plates, spacers and comb are washed in warm detergent solution, rinsed well in tap water and then with de-ionized water. Spacers are placed between the plates forming a sandwich and clamps are applied on each edge of the sandwich, taking care that the spacers remained aligned. After tightening the screws, the glass plate's sandwich is assembled on casting stand with cams through the camming holes on each side. By filling the mould with water, it is confirmed that there is no leakage. After the glass plates have been assembled, the comb is inserted and bottom of the comb is marked on the glass plate. A second line approximately 1 cm from the bottom of the comb is drawn on glass plate.

Casting of gels

Discontinuous SDS-PAGE technique includes a separating gel and a stacking gel. The separating gel solution is poured into the gel mould and overlaid with a layer of water. After polymerization of separating gel, stacking gel is poured and comb is inserted into the gel mould for sample well formation.

Procedures of preparing separating and stocking gels are given below:

Separating gel

Acrylamide stock solution, tris-HCl of pH 8.8, SDS and distilled water are mixed in a 200 ml Erlenmeyer flask. Ammonium persulphate and TEMED are added, swirled very gently to avoid incorporation of air in acrylamide mixture and immediately poured into prepared gel moulds up to

second line mark. The gel surface is layered with water-saturated n-butanol using a Pasteur pipette and is allowed to polymerize for 40 minutes at room temperature.

Stacking gel

The n-butanol is decanted off after the gel has polymerized and is allowed to drain for 2 minutes on tissue paper. Stacking gel is prepared by mixing the acrylamide stock solution, tris-HCl buffer of pH 6.8, SDS and distilled water in 200 ml Erlenmeyer flask. Ammonium persulphate and TEMED are added to gel mixture, swirled gently to remove any air bubbles and then immediately poured into the prepared gel mould, as it is 2 mm far from top edge of mould. The comb is immediately inserted and kept at room temperature for 30 minutes for polymerization.

Sample preparation

For sample preparation, 2x sample buffer (50 μ l) is mixed with purified re-suspended viral suspension (50 μ l) in Eppendorf tube of 1 ml and then 2 μ l of 0.2 per cent bromophenol blue is added. The eppendorf tubes are placed in water bath at boiling temperature for 2 minutes and cooled at room temperature.

Known protein markers are mixed with 1X sample buffer (200 μ l) in 1 ml eppendorf tube and then 3 μ l of 0.2 per cent bromophenol blue is added and kept in water bath at boiling temperature for 2 minutes.

Sample loading

A 30 μ l of each sample is loaded in each well with a syringe bearing a blunt needle long enough to reach the bottom of the well.

Electrophoresis

Electrophoresis is carried out at room temperature at a constant current supply on 100 volts for 8 hours. The system is stopped when bromophenol dye is 1 cm from bottom of separating gel.

Staining

After electrophoresis, gel is removed from the glass plates with the help of scalpel and placed in Coomassie brilliant blue stain solution (Coomassie brilliant blue 0.50 g, Methanol 250 ml, Glacial acetic acid 50 ml and distilled water 225 ml), covered and left for overnight.

De-staining

The dye solution is removed; gel rinsed with tap water and is de-stained with de-staining solution (Methanol 150 ml, glacial acetic acid 35 ml and distilled water 150 ml) for 6 hours by giving two washings with agitation.

Photography

After de-staining the gel is placed on a glass plate and its edges are made clear and smooth by cutting with scalpel. The glass plates bearing gel are placed on top of a light box and photographed.

Molecular weight determination

After the gel has been photographed, the length of the separating gel and the distance traveled by bromophenol blue dye is measured. Rf value for each polypeptide is calculated by formula:

$$Rf = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

A graph is plotted Rf verses log molecular weights of protein marker of known molecular weights to be used as standard for calculation of unknown molecular weights of polypeptides of Newcastle Disease live commercial vaccine viruses.

Table 16.1: Recipe for the preparation of discontinuous buffer separating gel

Sr. No.	Solution	Volume (ml)
1	Acrylamide solution (Stock)	16.2
2	1.5 M Tris-HCl, pH 8.8	10
3	SDS 10%	0.4
4	Ammonium per sulphate (10%)	0.133
5	Distilled water	13.8
6	* TEMED	0.027

* TEMED is added to gel mixture just before pouring the gel.

Table 16.2: Recipe for the preparation of stacking gel (Total volume of 10 ml)

Sr. No.	Solution	Volume (ml)
1	Acrylamide solution (Stock)	1
2	1.5 M Tris-HCl, pH 8.8	2.5
3	SDS 10%	0.1
4	Ammonium per sulphate (10%)	0.05
5	Distilled water	6.33
6	* TEMED	0.02

* TEMED is added to gel mixture just before pouring the gel.

Table 16.3: Acrylamide Stock

Sr. No.	Ingredients	Quantity
1	Acrylamide	300g
2	Bisacrylamide	8g
3	Amberlite MB-I	10g
4	Distilled water q.s.	1L

Mixed well with the help of magnetic stirrer for four hours and filtered through Whatmann Filter No.1 in a brown bottle. The solution is stored in a dark place at 4° C.