

MOLECULAR BIOLOGY LABORATORY

Department of Microbiology

LAB 1: Safety and Biosafety

Dr. Afra Ali & Eman jasim

Biosafety

Biosafety is about the intrinsic hazards of living organisms and how to handle them safely.

Before working with pathogens or genetically modified organisms (GMOs), possible hazards should be considered and proportionate measures taken to minimize risks for human health and the environment.

Risk Groups

Risk Group I: low individual and community risk; a microorganism unlikely to cause human disease or animal disease of veterinary importance.

Risk Group II: moderate individual risk and limited community risk; a pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers.

Risk Group III: high individual risk and low community risk; a pathogen that usually produces serious human disease but does not ordinarily spread from one infected individual to another.

Risk Group IV: high individual and community risk; a pathogen that usually produces serious human or animal disease and may be readily transmitted from one individual to another.

Hazards

What are the hazards?

1. Pathogenicity of an organism.
2. Toxicity: Toxicity means poisoning.
3. Allergenicity: a non-toxic, immune-system-mediated undesired reaction of the body to a substance or agent.
4. Disturbance of ecological balances, Disturbance of an ecological balance may happen when a GMO possessing a certain characteristic is accidentally spread to the environment, or when genetic material originating from that organism spreads to other organisms in the environment.
5. Other harmful effects: Sometimes there are other unwanted effects that urge one to be even more cautious when handling biological material. It is not possible to give an exhaustive list of these effects.

The Seven Basic Rules of Biosafety

1. Do not mouth pipette.

2. Manipulate infectious fluids carefully to avoid spills and production of aerosols and droplets.
3. Restrict use of needles and syringes to procedures for which there are no alternatives; use sharps carefully and dispose of them in leak- and puncture-resistant containers.
4. Use protective laboratory coats and gloves.
5. Wash hands after laboratory activities, after removing gloves, and immediately after contact with infectious materials.
6. Decontaminate work surfaces before and after use and immediately after spills.
7. Do not eat, drink, store food, or smoke in the laboratory.

Decontamination

Two common modes of action are:

- 1-destroying the lipid membrane of a microorganism, causing leakage of cell material
- 2- destroying proteins and enzymes necessary for survival.

Ethanol, quaternary ammonium salts and surfactants exploit the first mode.

Strong oxidizing agents such as chlorine or hydrogen peroxide exploit the second mode.

Phenolics such as Lysol work by destroying both proteins and the lipid membrane. Natural Routes of Infection

The effectiveness of different decontaminants

	Fungi	bacteria	mycobacteria	spores	lipid viruses	non-lipid viruses
Ethanol	-	+++	+++	-	+	V
Hypochlorite	+	+++	++	++	+	+
Formaldehyde	+++	+++	+++	+++*	+	+
Peroxide	+	+++	++	++	+	+

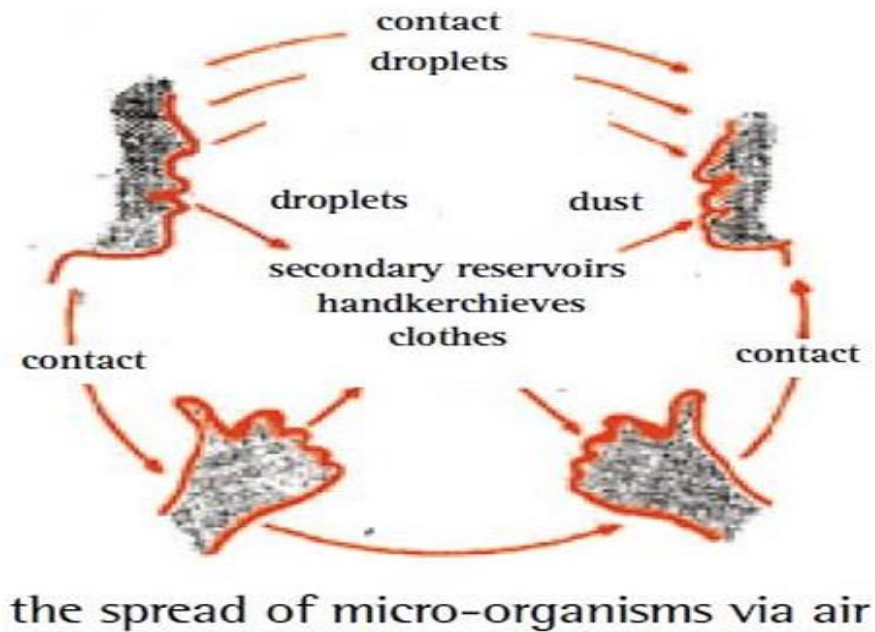
* above 40 °C

V = variable

Pathogens have their own routes of infection by which they spread from one host organism to another. Depending on the laboratory work, these routes may occur in the laboratory. Airborne organisms may involve very small droplets; infection may also result from direct contact with hands, handkerchiefs or clothes.

to another. The table below lists a number of important routes of infection:

Route of infection	example
Skin contact	fungi
Through air or aerosols	flu
Through pricking (insects or needles)	malaria Yellow fever
Blood-blood contact	HIV-virus Hepatitis B
Through wounds	staphylococci
Through faecal material	typhoid bacteria Poliovirus



Biological Waste

Biological waste should be distinguished as waste that has been inactivated before disposal and waste that has not been inactivated. The latter is treated as hazardous medical waste.

Biological Waste Includes :

- 1 -Genetically modified and/or pathogenic biological material such as cell cultures, microorganism cultures, tissues and blood.
- 2 -Organic laboratory waste such as gels.
- 3-Biologically contaminated materials such as gloves, tissues, disposable culture flasks and pipettes.

4 -Materials that are not necessarily contaminated, but cannot be thrown into an ordinary waste disposal bag because they have sharp edges or look dirty (bones, blood, etc.).

Biological waste does not include:

Radioactive contaminated material is not included and should be dealt with separately.

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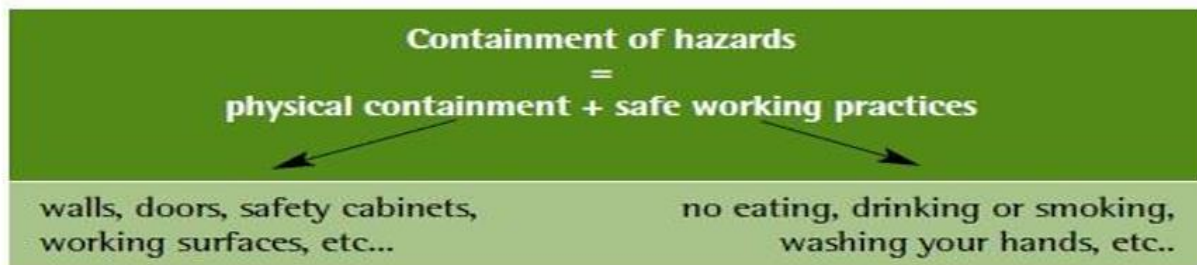
LAB 2: Instruments and Equipment

Dr. Afra Ali & Eman jasim

Containment and Safety Cabinets

Containment is a combination of infrastructure and working practices.

Containment: A combination of infrastructure and working practices:



A safety cabinet is an important type of physical containment and creates a safe working space within the laboratory.

Biosafety Cabinet (BSC)

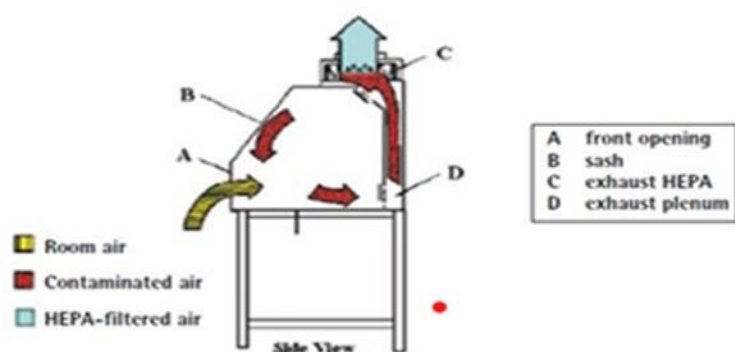
A BSC is an enclosed, ventilated laboratory workspace for safely working with materials contaminated with or potentially contaminated with pathogens requiring a defined biosafety level.

Class I: provides safety to the person and environment only.

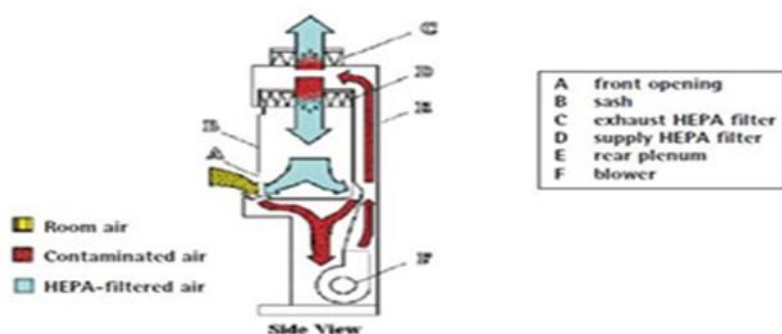
Class II: provides safety to the person, environment and samples.

Class III: provides safety to the person, environment and samples; generally installed in maximum-containment laboratories and designed for work with more dangerous pathogens such as viruses.

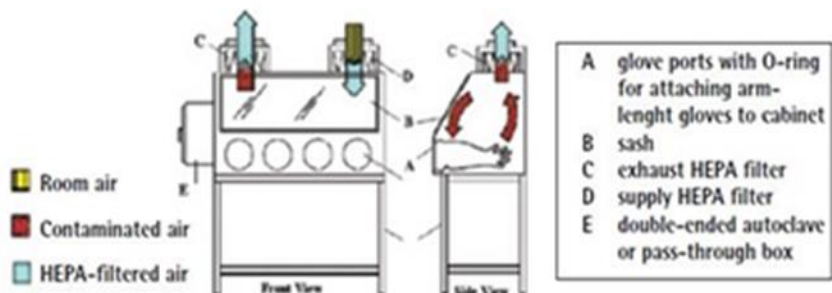
Class I safety cabinets

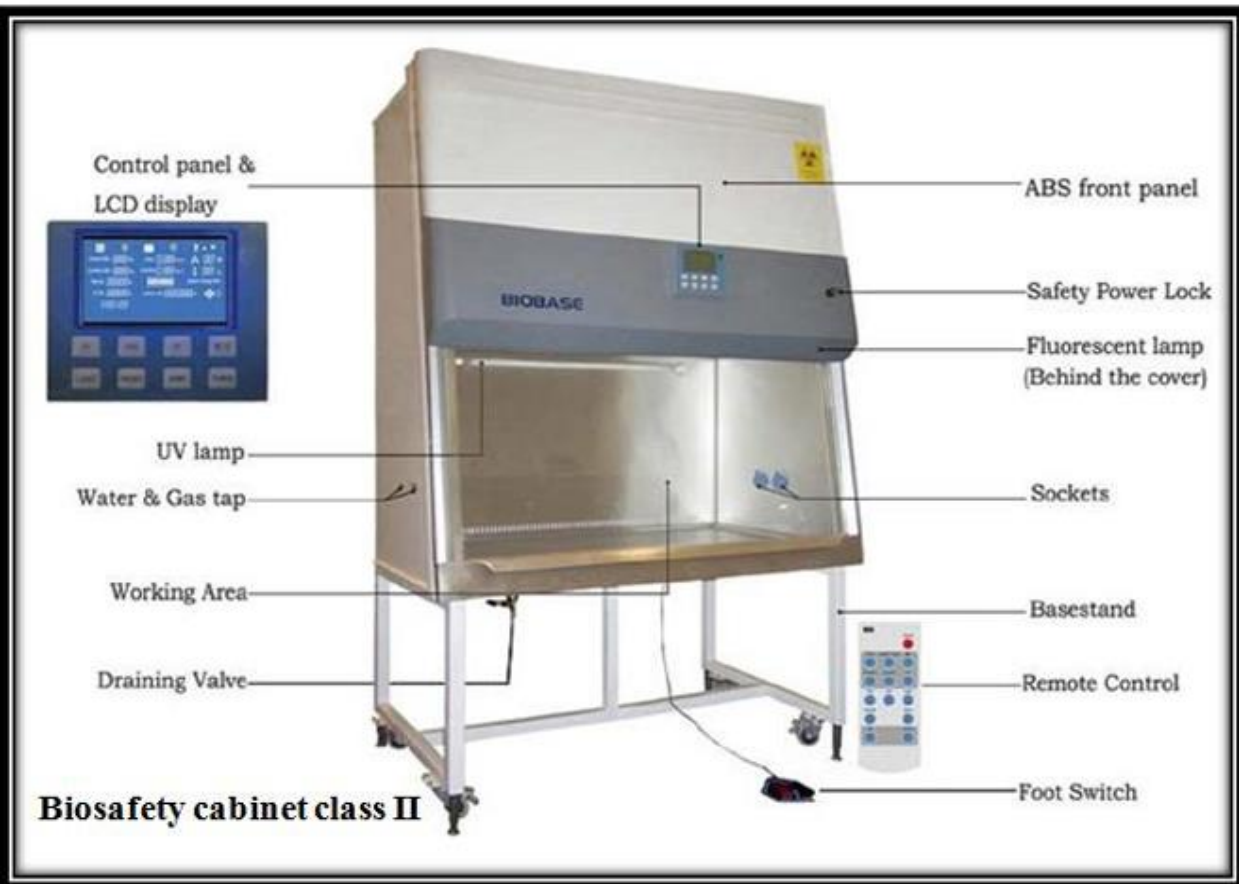


Class II safety cabinets



Class III safety cabinets





Main Instruments

Instrument	description	Picture
PCR work station	enclosed, sterilized space work.	

Micropipettes in different size	To transfer the appropriate amount of volumes	
Different size of eppendorf tubes	To keep and holder samples in experiment	

Vortex shaker	To mix the reaction continent	
Cool centrifuge	To participate cell continent	
Conventional PCR	To amplified DNA molecules (quality identification)	

Real-time PCR	To amplified DNA molecules and measuring gene expression(quality and quantity identification)	
Nanodrop	To measuring nucleic acid concentration and purity	
Gel electrophoresis	To separate nucleic acid and protein with molecular weigh less than 10000 bp	

Pulse gel electrophoresis	To separate nucleic acid and protein with molecular weigh more than 10000 bp	
UV-Transilluminator	To detect nucleic acid and protein were separated in gel electrophoresis run	
DNA sequencer	To determine sequence of nucleotide in DNA strand	
Deep freeze	To keep biological material in low temperature	

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LAB 3: Preparation of Buffers and Solutions

Dr. Afra Ali & Eman jasim

Buffer

A buffer is a solution which tends to maintain a constant pH when excess acid or base is added.

Buffers contain mixtures of weak acids and bases that make them relatively resistant to pH change.

1. Molecular Weight

Molecular weight is simply the weight of all the atoms in a substance. A molecule is a stable group of two or more atoms and is electrically neutral.

Example: H_2O : $\text{M.Wt} = (1 \times 2) + (1 \times 16) = 18 \text{ grams/mole}$.

Example: C_8H_{10} : $(8 \times 12.01) + (10 \times 1.01) = 106.18 \text{ grams/mole}$.

Moles: $\text{Moles} = \text{weight} / \text{molecular weight}$.

2. Molarity (M)

Molarity is the number of moles of solute divided by liters of solution.

$$M = \frac{Wt}{M.Wt} \times \frac{1000}{V} \quad \text{OR} \quad \text{Molarity} = \frac{\text{moles of solute}}{\text{Liters of solution}}$$

Example: Determine the molarity when 3.0 moles of sucrose are

dissolved to make 2.0 liters of solution.

$$\frac{3.0 \text{ mol}}{2.0 \text{ liters}} = X = 1.5 \text{ M}$$

Example: Attended solution a 0.2M of sodium chloride (NaCl) and the

volume of 500mL, note that the molecular weight 58.5 for salt???

$$M = \frac{Wt}{M.Wt} \times \frac{1000}{V}$$

$$0.2 = \frac{3.0 \text{ mol}}{2.0 \text{ liters}} \times \frac{1000}{V} \rightarrow Wt = 5.85 \text{ gm}$$

3. Normality (N)

Normality is a measure of concentration equal to the gram equivalent weight per liter of solution.

$$N = \frac{Wt}{Eq.Wt} \times \frac{1000}{V}$$

Example: Attended a solution of Mg (OH)₂ with Normality 0.2N and the volume of 200mL note that weight Molecular = 58.

$$N = \frac{Wt}{Eq.Wt} \times \frac{1000}{V}$$

$$0.2 = \frac{Wt}{58/2} \times \frac{1000}{200} = Wt = 1.16 \text{ gm}$$

Equivalent weight of an acid = molecular weight of acid / number of H groups.

Equivalent weight of a base = molecular weight of base / number of OH groups.

Relationship: $N = n \times M$, where n = number of equivalents.

- Dilution / Equivalence Relationships

$$M_1 V_1 = M_2 V_2$$

$$N_1 V_1 = N_2 V_2$$

$$C_1 V_1 = C_2 V$$

- Percentages

$$W/W (\%) = (\text{weight of solute} / \text{weight of solution}) \times 100.$$

$$W/V (\%) = (\text{weight of solute} / \text{volume of solvent}) \times 100.$$

$$V/V (\%) = (\text{volume of solute} / \text{volume of solution}) \times 100.$$

1- Example: Solution consists of dissolving 10 grams of sodium hydroxide

per 100 grams of water. Calculate the percentage of sodium hydroxide?

Block solution $10+100=110$ grams.

$$W/W = \frac{\text{Wt.of solute}}{\text{Wt.of solution}} \times 100$$

$$W/W = \frac{10}{110} \times 100 \rightarrow W/W = 9.1\%$$

2-Example, when it is said that a solution of sugar in water

Concentration 10% (W/V). This means that 10 gm of sugar dissolved

in 100mL of water

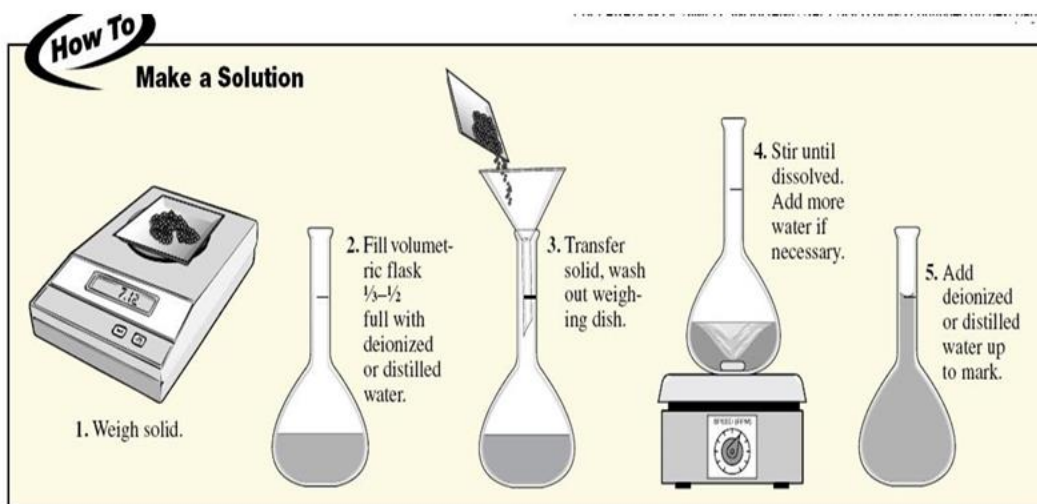
$$\text{Wt/V (\%)} = \frac{\text{Wt. of solute}}{\text{Volum of solvent}} \times 100$$

3- Example, when it is said that a certain solution consists of alcohol and

water and the concentration of alcohol 40%(v/v), this means that in every

100mL of solution is that there is alcohol 40mL and 60mL water.

$$\text{V/V} = \frac{\text{Volum of solute}}{\text{Volum of solvent}} \times 100$$



Add 5mL of Triton X-100 + 186gm of EDTA + 1.25mL of Tris- HCL to **200mL** of DW, adjust
pH to 8, then complete the volume to 250mL.

1. Prepare (250mL) of a solution consists of (5% glucose), (5M EDTA), (30mM) Tris-HCL. Adjust pH to (8). M.
Wt. of Tris-HCL = 121, stock solution of EDTA = (7M) .

2. Prepare (250mL) of a solution consists of (0.4M Tris-Hcl), (1mM EDTA), (0.06M) NaCl & 8% SDS. If you know that
stock solution of EDTA = 1M, Molecular weight of Tris-Hcl = 121.

3. Prepare (250mL) of a solution consists of (2% Triton X-100), (2M EDTA), (20mM) Tris-HCL. Adjust pH to (8). M.
Wt. of EDTA = 372, stock solution of Tris-HCL = (4M).

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LAB 4: DNA Extraction - Principles and Method

Dr. Afra Ali & Eman jasim

Introduction

DNA is the blueprint for life; living organisms contain DNA.

DNA isolation is one of the most basic and essential techniques in the study of DNA. DNA extraction and purification are important in biotechnology and forensic science.

The extraction process obtains DNA and removes impurities associated with proteins, fats and other materials.

General DNA Extraction

Most DNA extraction protocols consist of two parts:

- (1) a technique to lyse cells gently and solubilize DNA;
- (2) enzymatic or chemical methods to remove contaminating proteins, RNA or macromolecules.

For plants **four** steps are used to remove and purify DNA:

- 1- Lysis — open cells and expose DNA.
- 2- Precipitation — precipitate DNA.
- 3- Wash — wash the DNA preparation.
- 4- Resuspension — dissolve the DNA for storage/use.

Nucleoprotein Complex and Deproteinization

Nucleic acids in living cells are interconnected with proteins as a **nucleoprotein complex**.

Extraction begins by breaking cell walls or cell membranes carefully to allow DNA and other cellular components to exit without significant damage.

followed by separation of DNA from proteins through deproteinization.

1. Enzymatic Treatments

Proteolytic enzymes such as Proteinase K and Pronase break protein molecules into short peptide chains to facilitate their removal.

2. Chemical Treatments

Chelating agents such as EDTA remove Ca^{2+} and Mg^{2+} , which contribute to stability of protein complexes and nuclear/cellular membranes and are involved in nuclease activity.

Detergents include SDS (ionic), Sarkosyl and Triton X-100 (non-ionic). describes detergents as effective agents for disruption and protein treatment.

Organic solvents include Isoamyl alcohol, Chloroform and Phenol. They act in a **two-phase system**; help denature proteins and remove fat; DNA is recovered in the aqueous phase.

Cold alcohol in the presence of salt such as sodium acetate is used for DNA precipitation. The PDF states that salt helps keep DNA secondary structure without denaturation and inhibits microbial growth.

3. Mechanical Treatment

Centrifugation is used as a mechanical treatment. The type of treatment depends on the tissue source.

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LAB 5: Plant Chromosomal DNA Extraction

Dr. Afra Ali & Eman jasim

Introduction

DNA must be extracted from living cells, purified and stored in a suitable laboratory environment.

Plant extraction is more difficult because plant cells have a solid cell wall mainly composed of cellulose.

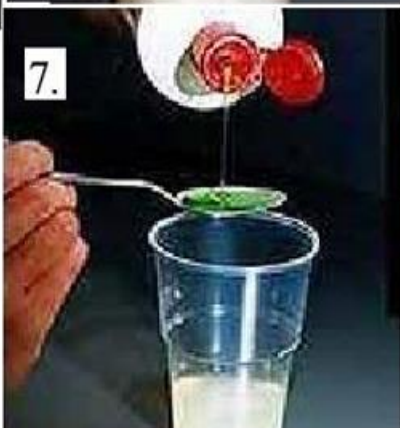
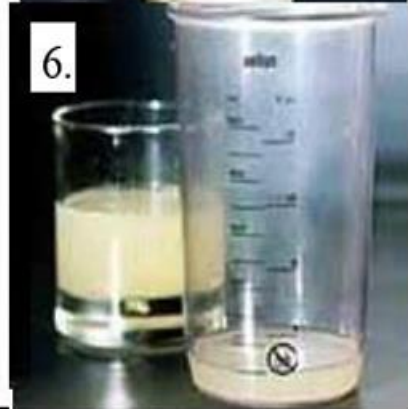
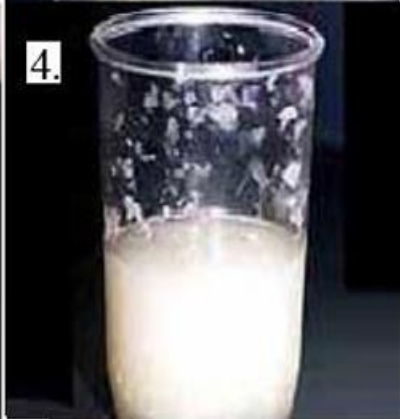
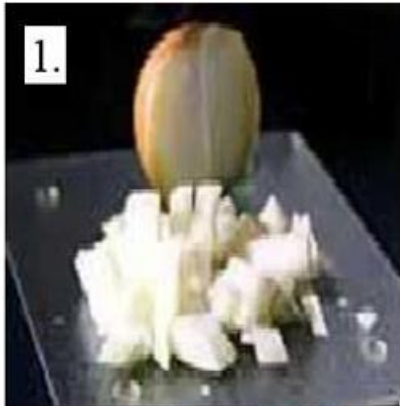
DNA Isolation from Onion

The method for extracting large amounts of DNA from onion and other vegetable sources, using isopropanol, sodium citrate and SDS.

Procedure

1. Chop the onion into small pieces and place them in a blender.
2. Measure about 200 mL water, add one level teaspoon of salt and cool the salty water.
3. Add enough salty water to just cover the onion.
4. Blend for about 10 sec.
5. Pour the onion-salt-water mixture through a strainer and collect the liquid.
6. Measure 50 mL of onion liquid and pour into a glass.

7. Add 2 teaspoons of dishwashing detergent and stir very gently.
8. Measure 100 mL isopropanol and slowly add it to the onion-detergent mixture.
9. Wait for DNA to appear. The DNA is white and rises from the bottom of the glass.
10. Pull the DNA using a wooden stick to gather it.
11. Place the DNA in Eppendorf tubes, add TE buffer and keep it in the deep freezer.





DNA rising from the bottom of the cup to the top.

Importance of Salt

1-Salt helps proteins and carbohydrates precipitate while DNA remains in solution.

2-Cold salt solution provides a favorable environment.

3 -Na⁺ ions neutralize the negative charge of DNA phosphates, allowing DNA molecules to come together.

Purity and Further Treatment

The DNA extracted by this method is not completely pure because histone proteins and RNA may remain associated with it.

Proteolytic enzymes and RNase can be used during the process.

when proteolytic enzymes are unavailable, concentrated **pineapple juice** may be used **because it is rich in protein-digesting enzymes called Bromelain.**

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LAB 6: Bacterial Chromosomal DNA Extraction

Dr. Afra Ali & Eman jasim

Bacterial Chromosomal DNA

The bacterial cell lacks a distinct nucleus and nuclear membrane as in eukaryotic cells.

The nuclear material, DNA, is called the nucleoid and is located close to the center of the cell.

The genome is described in the PDF as a single circular chromosome of about 1 mm, with no loose end and associated genes, forming a supercoiled molecule.

Procedure

1. Grow bacteria in 3–4 mL Brain Heart Infusion broth overnight at 37°C.
2. Centrifuge 1–1.5 mL bacterial culture in an Eppendorf tube for 5 min and remove the supernatant.

3. Suspend the sediment in 1.5 mL lysis buffer: 400 mM Tris-HCl, 60 mM EDTA, 150 mM NaCl, 1% SDS, pH 8; leave at room temperature for 10 min.
4. Add 150 μ L potassium acetate solution: 5 M potassium acetate 60 mL + glacial acetic acid 11.5 mL + distilled water 28.5 mL.
5. Shake the tubes by vortex for 10 sec.
6. Centrifuge for 1 min at 10,000 $\times$ g.
7. Transfer the upper aqueous phase and add a similar volume of phenol:chloroform (1:5), pH 8; shake well.
8. Centrifuge for 1 min at 10,000 $\times$ g.
9. Transfer the supernatant and add a similar volume of isopropanol; shake well.
10. Centrifuge for 2 min at 10,000 $\times$ g and discard the supernatant.
11. Wash the sediment with 1 mL of 70% ethanol.
12. Centrifuge for 1 min at 10,000 $\times$ g, discard the supernatant and dry the sediment.
13. Dissolve the sediment in 10–20 μ L TE buffer and keep frozen at -20°C .

Rapid Boiling DNA Extraction

1. Grow bacteria overnight at 37°C on Brain Heart Infusion agar.

2. Suspend a few bacterial colonies in 1 mL distilled water in an Eppendorf tube and vortex.
3. Boil in a water bath for 10 min and centrifuge directly at 13,000 rpm for 5 min.
4. Transfer the supernatant to another clean Eppendorf tube, centrifuge quickly at 6,000 rpm for 2 min, remove the supernatant and dissolve the pellet with TE buffer.

Benefits of Additives

TE buffer / EDTA: captures Mg^{2+} and Ca^{2+} , helps disrupt the bacterial cell wall, stops nuclease activity such as DNase, helps disengage the nucleoprotein complex and dissolves the DNA sample.

Sarkosyl or SDS: ionic detergents used for protein denaturation and removal of fat from cell walls.

Chloroform:Phenol: denatures proteins; the PDF also states that it removes remaining phenol atoms and inhibits RNase activity.

Isopropanol: precipitates DNA.

70% ethanol: washes the DNA sample.

Break cells to open and expose DNA; remove membrane lipids by adding detergent; precipitate DNA with alcohol such as ethanol or isopropanol; centrifugation produces a pellet.

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LAB 7: Plasmid DNA Isolation and Extraction

Dr. Afra Ali & Eman jasim

What is Plasmid DNA?

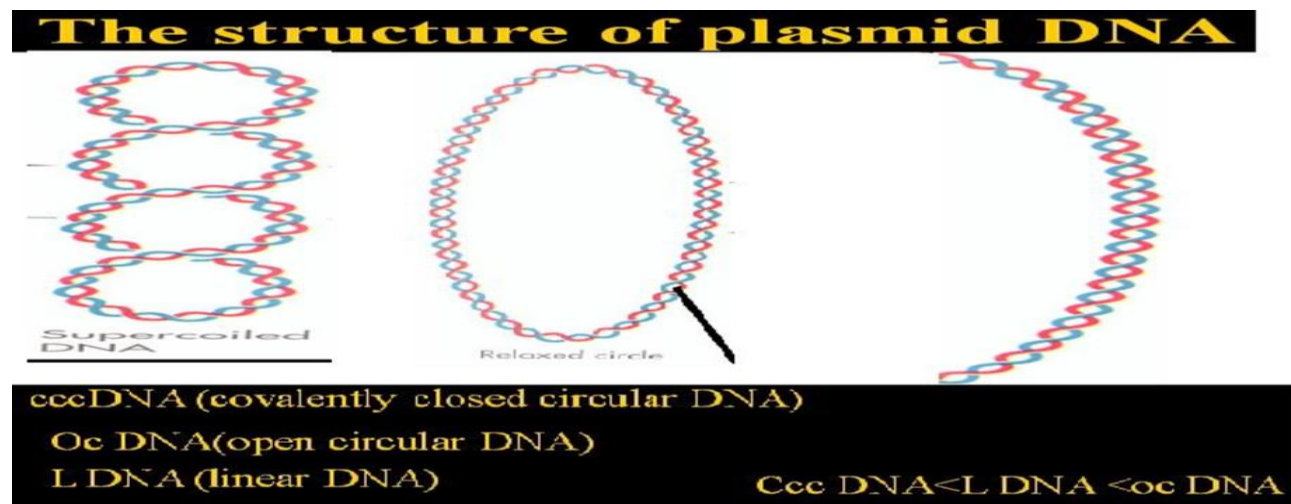
Plasmids are autonomously replicating, extra-chromosomal circular DNA molecules, distinct from normal chromosomal DNA and nonessential for cell survival under nonselective conditions.

Bacterial plasmids are closed circular double-stranded DNA molecules ranging from 1 to >200 kb. They are additional genetic units inherited and replicated independently of the bacterial chromosome.

Plasmids may contain genes advantageous to the host, including genes for antibiotic resistance, resistance to environmental toxins or toxin production.

The term plasmid was introduced by Joshua Lederberg in 1952.

Plasmids are much smaller than the bacterial chromosome. They can be strongly wrapped around themselves, forming a supercoiled **or covalently closed circular (CCC) plasmid**.



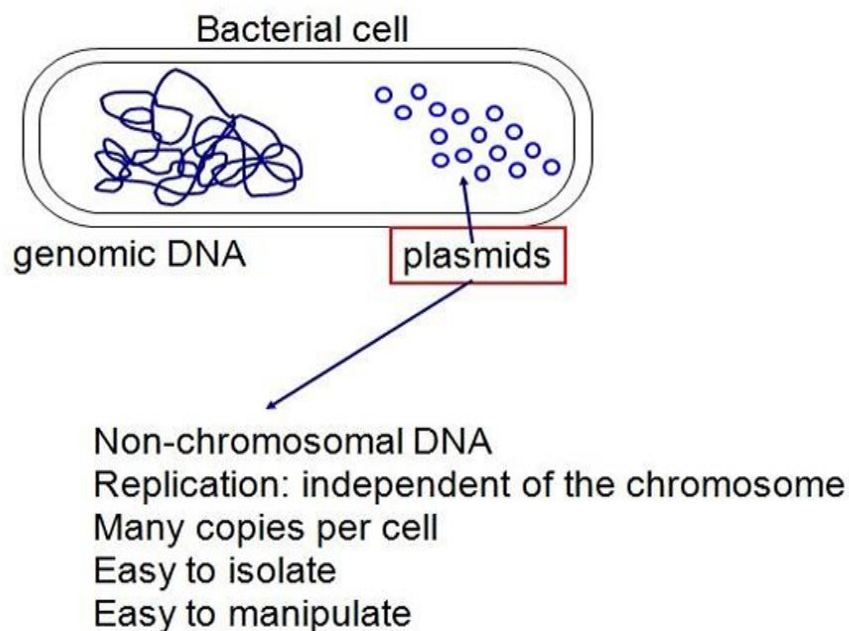
Types of Bacterial Plasmids

1. **Fertility (F) plasmids**: capable of conjugation or mating.
2. **Resistance (R) plasmids**: contain antibiotic or drug resistance genes; also called R-factors.

3. Col-plasmids: contain genes coding for colicins, proteins that can kill other bacteria.

4. Degradative plasmids: enable digestion of unusual substances such as toluene or salicylic acid.

5. Virulence plasmids: convert the bacterium into a pathogen.



Boiling Method

The **boiling lysis** method is quick and recommended **for small plasmids up to 10 kb.**

Plasmids larger than 10 kb should be isolated by other methods such as alkaline lysis.

The quality is not as good as plasmid isolated by alkaline lysis, but is adequate for restriction digestion analysis.

Bacterial cells receive brief heat treatment in a boiling water bath in the presence of lysozyme and Triton X-100.

Because of its small size, plasmid DNA comes out of the bacterial cell whereas genomic DNA remains trapped inside. High-speed centrifugation separates plasmid DNA from cell debris. Plasmid DNA is recovered by ethanol or isopropanol precipitation.

Modified Alkaline Lysis Method

Experiment: *E. coli* carries an Ampicillin-resistance marker on plasmid RY121, encoding β -lactamase.

1. Inoculate bacteria onto selective Brain Heart Infusion agar plates containing Ampicillin; incubate overnight at 37°C in the shaker and harvest.
2. Put 2 mL TE buffer in an Eppendorf tube, transfer a loopful of bacterial growth, centrifuge at 11,000 rpm for 10 min, and resuspend the pellet in 40 μ L TE buffer.
3. Prepare fresh lysis buffer, add 600 μ L to the cell suspension and mix gently.
4. Incubate at 37°C for 20–30 min.

5. Add 2 M Tris-HCl (pH 7), 30 μ L for neutralization, and mix.
6. Add 5 M NaCl, about 240 μ L, for precipitation of chromosomal DNA and protein; incubate on ice for 4 h and centrifuge at 11,000 rpm for 10 min.
7. Transfer the supernatant to another tube, add double volume of absolute ethanol, incubate on ice overnight and centrifuge at 11,000 rpm for 10 min.
8. Remove the supernatant and dry the pellet; add 1 mL 70% ethanol, centrifuge at 11,000 rpm for 10 min; remove supernatant, dry the pellet and dissolve in 20 μ L TE2 buffer; store at -20°C .

Benefits of Additives

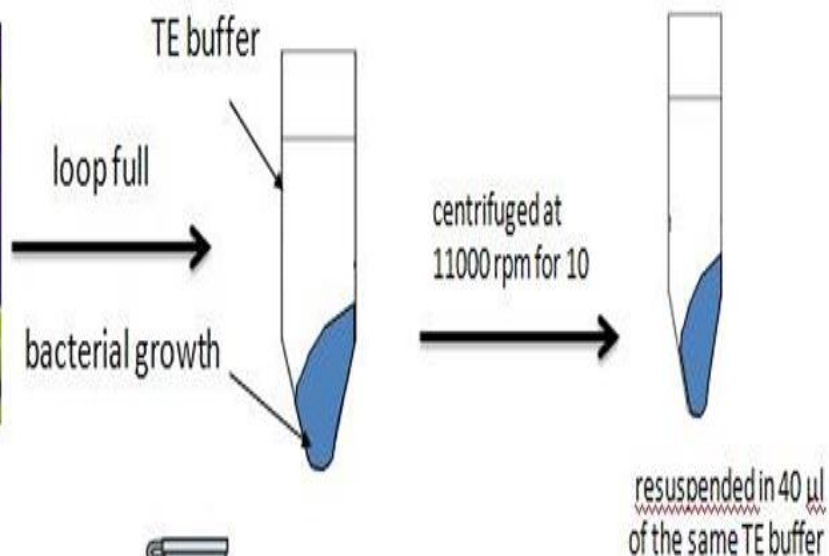
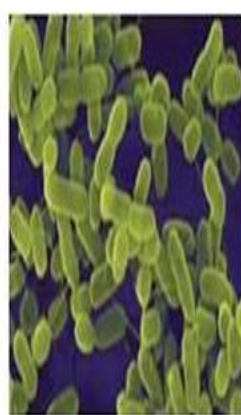
EDTA: removes Ca^{2+} and Mg^{2+} and inhibits nuclease activity.

SDS: denatures proteins and removes fat from cell walls.

NaCl: ionizes to $\text{Na}^{+}/\text{Cl}^{-}$ and associates with proteins and sedimentation.

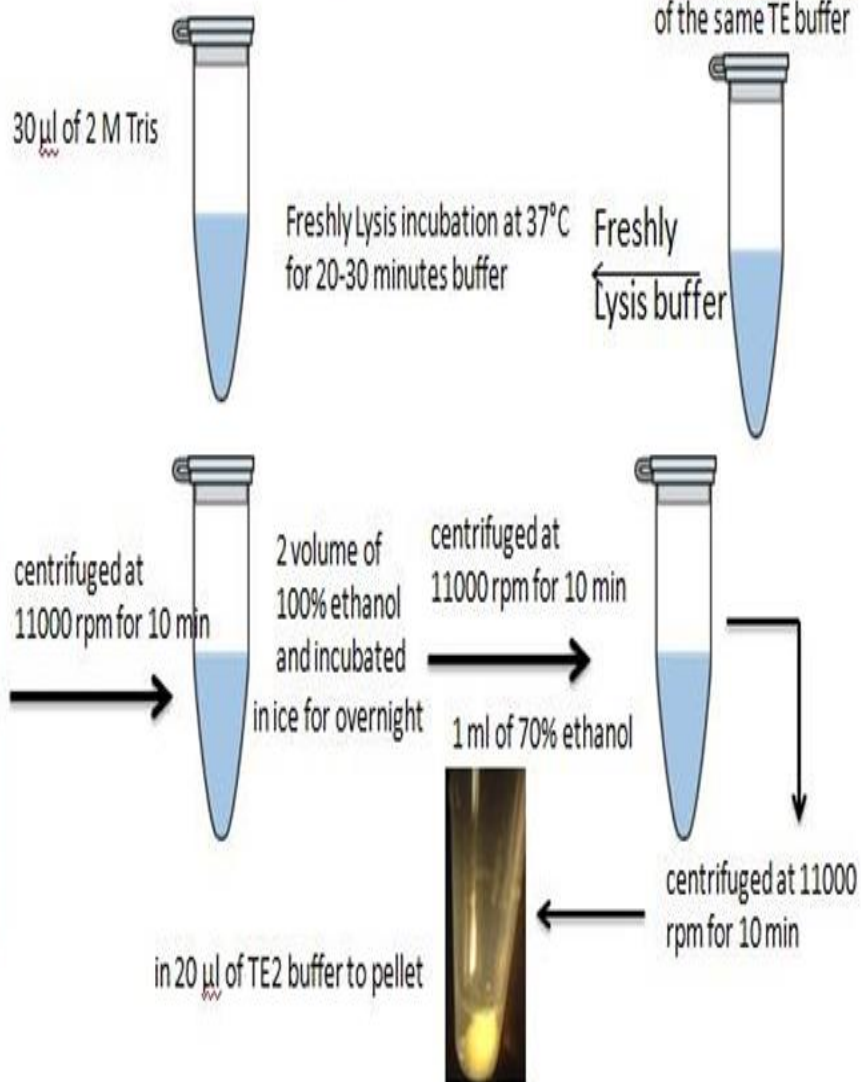
100% ethanol: precipitates DNA.

70% ethanol: washes the DNA sample.



240 μ l of 5 M NaCl + 30 μ l of 2 M Tris

incubate on ice for 4 hrs



MOLECULAR BIOLOGY LABORATORY

Department of Microbiology

LAB 8: Polymerase Chain Reaction (PCR)

Dr. Afra Ali & Eman jasim

What is PCR?

Polymerase Chain Reaction (PCR) is a molecular-biology technology used to amplify a single or a few copies of a DNA fragment by several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence in vitro.

that PCR was invented by Kary Mullis in 1984 in California and became an important technique in medical and biological research.

PCR allows analysis of DNA fragments present in minute quantities, such as DNA from blood at a crime scene or trace DNA from fossils.

Taq Polymerase

The technique was made possible by Taq polymerase, the DNA polymerase used by *Thermus aquaticus*, discovered in Yellowstone National Park hot springs.

Taq polymerase is stable at the high temperatures needed for amplification, whereas other DNA polymerases become denatured.

Why “Polymerase”? It is called “polymerase” **because the only enzyme**

used in this reaction is DNA polymerase.

Applications of PCR:

- 1- Medicine: The PCR technique enables early diagnosis of malignant diseases.
- 2- Classification of organisms.
- 3- Mutation detection.
- 4- Detection of pathogens.
- 5- Gene therapy.
- 6- Finger print.
- 7- Forensic science: PCR is very important for the identification of criminal
- 8- It is also used in diagnosis of retroviral infection, bacterial infections, cancers, sex determination of embryos.
- 9- Evolutionary studies: It plays an important role in phylogenetic analysis.

PCR Contamination

Contamination is a major consideration in PCR. Even a very small amount of target DNA contamination may be amplified and produce a false-positive identification.

The gives a forensic example in which cross-contamination between a crime-scene sample and a positive control could result in erroneous incrimination.

Requirements of PCR Technology

- 1. Thermal cycler:** heats and cools reaction tubes to achieve the temperatures required at each step.
- 2. DNA template:** contains the target DNA region. The PDF gives examples including DNA from blood, skin, feathers or old PCR products and states a standard concentration of 25 ng/μL.
- 3. Pair of primers:** short artificial DNA fragments containing sequences

complementary to the target region, that are complementary to the 3'

(three prime) ends of each of the sense(forward) and the 5' end of the

anti-sense (reverses) strand of the DNA target, usually the length of 18-30 nt.

4- Master Mix which Contains:

- 1 -Taq DNA polymerase** the enzyme that puts the free nucleotides together.

It starts at the 3'end of the primer, and uses the complementary DNA strain

as a template.

2- Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), Free nucleotides (G, A, T,C) of which the artificial DNA copies are made, the building-blocks from which the DNA polymerase synthesizes a new DNA strand.

3- Buffer solution, maintains pH and ionic strength of the reaction solution suitable for the activity of the enzyme.

4- Mg^{++} ions - cofactor of the enzyme.

5. Nuclease-free water must be present for the reaction to work.

PCR Procedure

PCR typically consists of 25–40 repeated cycles. Each cycle includes template denaturation, primer annealing and primer extension.

1-Initialization step: 94–96°C (or 98°C for extremely thermostable polymerases) , held for 1–9 min; the PDF states it is required for polymerases requiring heat activation in hot-start PCR.

2-Denaturation step: 94–98°C for 30 sec–1 min; disrupts hydrogen bonds between complementary bases and produces single-stranded DNA.

3-Annealing step: 50–65°C for 30 sec–1 min; primers anneal to single-stranded template. The annealing temperature is typically 3–5°C below primer T_m.

4-Extension/Elongation step: commonly 72°C with Taq polymerase. DNA polymerase synthesizes a complementary strand by adding dNTPs in the 5' to 3' direction. Under optimum conditions the target amount doubles at each extension step, producing exponential amplification.

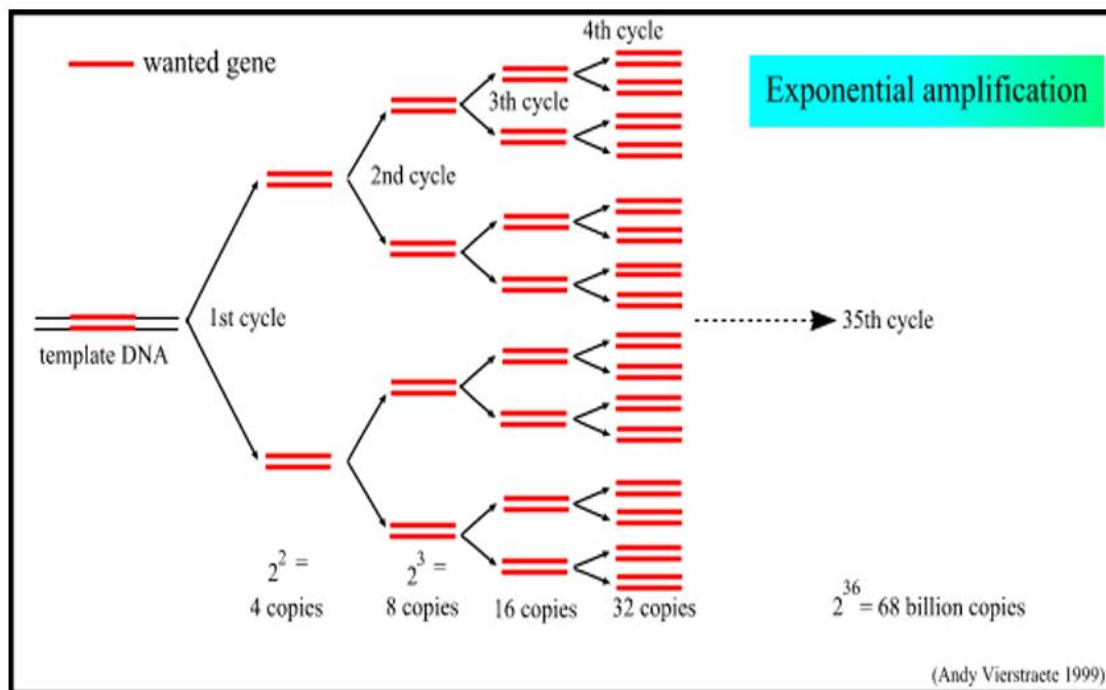
Final elongation: occasionally 70–74°C for 5–15 min after the last cycle to ensure remaining single-stranded DNA is fully extended.

PCR Steps

- Initialization step : 94°C 10min
- 35 Cycles :

Denaturation step	94°C 30s	} 1 cycle
Annealing step	55°C 30s	
Extension step	72°C 30s	

- Final elongation : 72°C 10min



Important Laboratory Reminders

- 1- Keep PCR reagents (master mix, primers and DNA template) on ice.
- 2- Keep centrifuge tubes closed to prevent contamination.
- 3- Gloves are recommended to reduce contamination.
- 4- Do not flame centrifuge tubes because they might melt.
- 5- Keep tubes closed and avoid cross-contamination with micropipette tips.
- 6- Use the correct micropipette and tip for the required volume.
- 7- Avoid contaminating micropipettes by slowly releasing the plunger.
- 8- Dirty pipette tips should be autoclaved and discarded in the designated containers

9- Balance the microcentrifuge by placing another tube across from the sample with approximately the same amount of liquid.

Seeing PCR Results

The most common way to see PCR results is by running gel electrophoresis. The PCR product is loaded into agarose gel, where DNA fragments are separated according to size using electricity.

Calculating Primer T_m

$$T_m = 4(G + C) + 2(A + T)$$

Here G, C, A and T are the numbers of the respective nucleotides.

MOLECULAR BIOLOGY LABORATORY

Department of Microbiology

LAB 9: Gel Electrophoresis

Dr. Afra Ali & Eman jasim

Definition and Principle

Gel electrophoresis is widely used for analysis of nucleic acids and proteins. Agarose gel electrophoresis is routinely used for preparation and analysis of DNA.

It separates molecules on the basis of charge and size. Charged macromolecules migrate through a gel in an electrical field; the gel acts as a sieve according to size and shape.

DNA is polyanionic at natural pH. **Because** phosphate groups give nucleic acids a negative charge, DNA migrates away from the negative electrode (cathode) toward the positive electrode (anode).

Types of Gels

1-Agarose: a complex sugar chain from red seaweed with a large pore size, useful for separating large molecules quickly.

2-Polyacrylamide: has a small pore size, useful for separating small molecules slowly and is used for fragments less than about 500 bp. polyacrylamide is a neurotoxin.

Materials used in electrophoresis

1. TBE, TAE or TPE buffer.

2. Agarose.

3. 6X loading buffer:

0.25% Bromophenol blue

0.25% xylol

40% sucrose.

4. Fluorescent dye such as Ethidium bromide or another dye.

Factors Affecting DNA Mobility

1. Agarose concentration: Higher agarose concentrations facilitate separation of small DNA; lower concentrations allow resolution of larger DNA.

2. Voltage: applied, for best resolution of fragments larger than about 2 kb is

attained by applying no more than 5 volts per cm to gel. The cm value is distance between two electrodes, not the length of the gel.

3. Electrophoresis buffer. have been recommended for electrophoresis of DNA:

TAE (Tris-acetate-EDTA) , TB (Tris-borate-EDTA) and TPE (Trisphosphate-EDTA). DNA fragments will migrate at somewhat different rates in **these two buffers** due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity – If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel – If you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in gel to melt it.

4. Effect of Ethidium bromide. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, and use in final cons. 0.5 μg \text{mL} . Because ethidium is a DNA intercalating agent, it is a powerful mutagen. Incorporation of ethidium in the DNA of living organisms (i.e. you and us) can cause (unwanted) mutations.

TAE, TBE and TPE provide pH and ions for conductivity. Water instead of buffer gives essentially no DNA migration. Concentrated buffer such as 10X can generate enough heat to melt the gel.

DNA Stains

Ethidium bromide: fluorescent dye that intercalates between nucleic-acid bases and allows detection of DNA fragments; the

PDF notes a final concentration of 0.5 µg/mL and identifies it as a powerful mutagen.

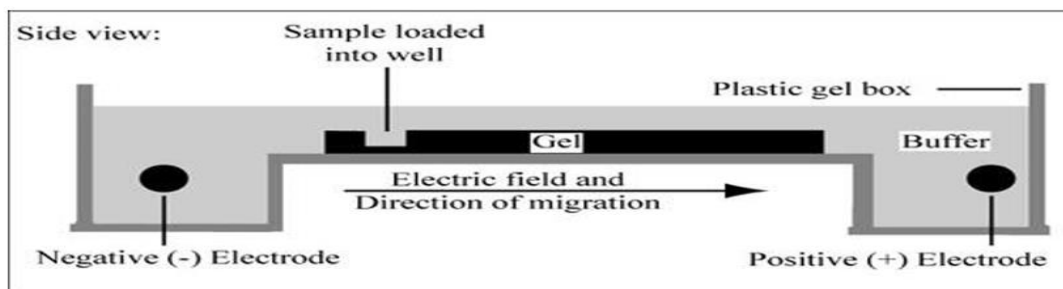
Methylene blue: dark blue and less sensitive than EtBr when viewed with white light.

Silver stain.

SYBR Green: described as more expensive.

System Components

1. Power supply.
2. Cell.
3. Tray or gel bed.
4. Comb.
5. Ultraviolet cabinet.



Procedure

1. Prepare a 1% agarose gel by dissolving agarose in 1X TBE and adding 0.5 µg/mL EtBr.

2. Seal the tray, place combs, cool the solution to about 60°C and carefully pour the melted agarose; avoid air bubbles.
3. Submerge combs in the melted agarose.
4. Allow the gel to cool for about 30–45 min; remove combs and tape.
5. Place the gel in the electrophoresis tray and add enough buffer to cover the gel by at least 1 mm; fill the wells with buffer.
6. Mix DNA samples with loading buffer: 3–5 μL 6X loading buffer with 7 μL DNA sample.
7. Load samples carefully without puncturing the gel. Include a DNA ladder of known sizes.
8. Close the chamber and connect the leads correctly. DNA migrates from cathode (black) toward anode (red).
9. Confirm that the gel runs in the correct direction; Bromophenol blue runs in the same direction as DNA.
10. After electrophoresis, place the gel on the transilluminator and observe distinct bands.
11. Determine fragment size by comparison with a DNA ladder containing DNA fragments of known length.

DNA Ladder

A DNA ladder consists of DNA fragments of known sizes and is used to determine the size of an unknown DNA sample. When run on agarose gel, regularly spaced fragments appear like a ladder.

