Core Techniques in Molecular Biology: A Practical Guide

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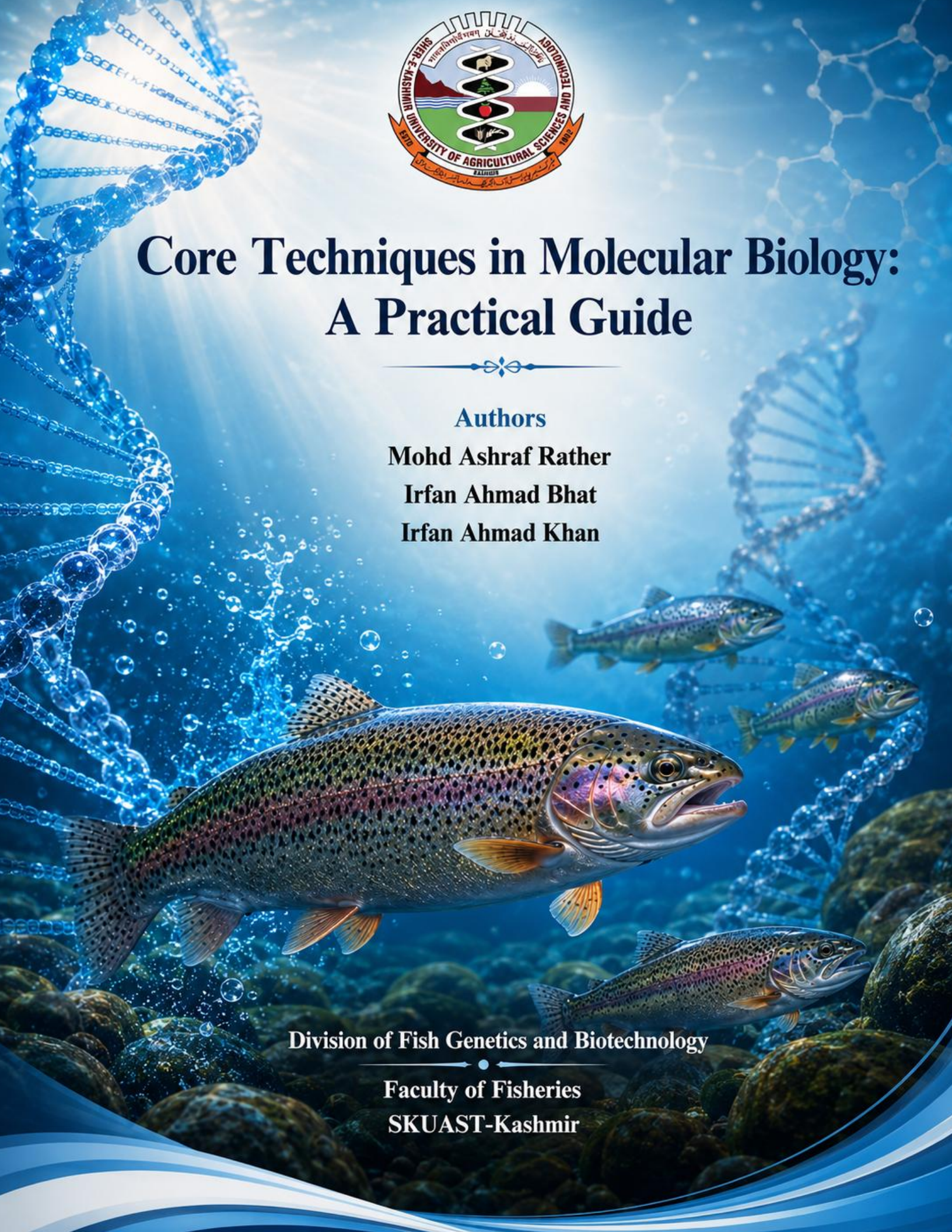

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Preface

Molecular biology has revolutionized the way we understand life at the molecular level, providing powerful tools to study DNA, RNA, proteins, and their interactions. **Core Techniques in Molecular Biology: A Practical Guide** is a comprehensive laboratory resource designed to help students, researchers, scientists, and biotechnology professionals develop the practical skills needed to perform modern molecular biology experiments with confidence and precision. By combining fundamental concepts with step-by-step laboratory protocols, the guide bridges the gap between classroom learning and real-world research.

The guide introduces the essential techniques that form the backbone of molecular biology research, including DNA and RNA extraction, polymerase chain reaction (PCR), agarose gel electrophoresis, restriction enzyme digestion, plasmid isolation, molecular cloning, DNA sequencing, quantitative real-time PCR (qPCR), protein extraction, SDS-PAGE, Western blotting, and basic bioinformatics analysis. Each technique is presented with its scientific principles, required reagents and equipment, experimental procedures, expected results, troubleshooting tips, and safety precautions, enabling users to perform experiments accurately and reproducibly.

In addition to laboratory methods, the guide emphasizes Good Laboratory Practices (GLP)**,** quality control, sample handling, contamination prevention, proper documentation, and ethical research conduct. It also introduces modern computational tools for sequence analysis, primer design, gene annotation, phylogenetic analysis, and genomic data interpretation, reflecting the growing importance of bioinformatics in life science research.

This practical guide is highly valuable for undergraduate and postgraduate students**,** as it strengthens their laboratory skills and builds a strong foundation in molecular biology. Research scholars and Ph.D. students can use it as a reliable reference while designing experiments, optimizing protocols, and interpreting molecular data. Scientists and faculty members will find it useful for training students, standardizing laboratory procedures, and conducting advanced research in genetics, genomics, biotechnology, microbiology, fisheries, agriculture, veterinary sciences, environmental sciences, and biomedical research. The guide is equally beneficial for professionals working in diagnostic laboratories, pharmaceutical industries, forensic laboratories, and biotechnology companies, where molecular techniques play a central role in research, quality assurance, disease diagnosis, and product development.

Overall, Core Techniques in Molecular Biology: Practical Guide serves as an indispensable reference that combines theoretical understanding with practical laboratory expertise. By mastering the techniques described in this guide, learners gain the confidence and technical competence required to conduct high-quality molecular research, contribute to scientific innovation, and address emerging challenges in healthcare, agriculture, aquaculture, environmental conservation, and biotechnology.

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Introduction to Basic Chemical Calculations for Reagent Preparation

Introduction

Reagents are a basic requirement for most of the molecular biology protocols. Different types of reagents such as TAE buffer, Tris base solution, 70% ethanol, agarose solution, etc are used for a different purpose in molecular biology laboratory. These reagents form the backbone and dictate the principal of molecular techniques. Further reagents preparation requires basic information on different types of concentration unit and also the chemical formulas. This chapter is particularly incorporated to throw light on all those preliminary aspects which will be helpful in various types of reagent preparation.

Concentration

Concentration in simple language stated as the amount of solute per unit volume. Concentrations are presented in different forms such as percentage, normality, molarity, molality, etc. A basic idea related to the chemical formula of different types of concentrations used in molecular biology will be helpful in preparing reagents. Following sections will elaborate on the different types of concentrations unit used in molecular biology.

Percentage (%) solutions

Many chemical reagents are prepared as a percentage solution (agarose solution, SDS solution etc.). The simple meaning of percentage is per 100. Therefore, 20% means 20 per 100 or 20 out of every 100. Depending on the physical state of solute percentage concentration can be expressed as a weight per volume percentage (% w/v) or a volume per volume percentage (% v/v). A % w/v solution refers to the weight of solute in grams in a total volume of 100 ml of solution. A % v/v refers to the volume of liquid solute in ml in a final volume of 100 mL solution.

$$\text{Volume percent} = \frac{\text{volume of solute}}{\text{volume of solution}} \times 100\%$$

$$\text{Weight percent} = \frac{\text{weight of solute}}{\text{weight of solution}} \times 100\%$$

Example – 1.5% agarose solution preparation for agarose gel electrophoresis, 10% SDS solution, 70% ethanol solution etc.

Normality

It is defined as the number of mole equivalents per liter of solution:

Normality = number of mole equivalents/1 L of solution

Normality relates the amount of solute to the total volume of the solution; however, normality is specifically used for acids and bases. The mole equivalents of an acid or base are calculated by determining the number of H^+ or OH^- ions per molecule.

Molarity (M)

It is defined as the number of **moles** of solute per **liter of solution**.

Molarity = moles of solute/liters of solution

Molarity tells us the number of moles of solute in exactly one liter of a solution. (Note that molarity is spelled with an "r" and is represented by a capital M.)

We need two types of information to calculate the molarity of a solute in a solution:

- The moles of solute present in the solution.
- The volume of solution (in liters) containing the solute.

To calculate molarity, we use the equation:

$$\textbf{Molarity} = \frac{\textbf{moles of solute}}{\textbf{Volume of solution in liters}}$$

Molality (m)

It is defined as the number of **moles** of solute per **kilogram of solvent**.

Molality = moles of solute/kilograms of solvent

Molality, **m**, tells us the number of moles of solute dissolved in exactly one kilogram of **solvent**. We need two pieces of information to calculate the molality of a solute in a solution:

- The moles of solute present in the solution.
- The mass of solvent (in kilograms) in the solution.

To calculate molality we use the equation:

$$\textbf{Molality} = \frac{\textbf{moles of solute}}{\textbf{mass of solvent in kilograms}}$$

Although their spellings are somewhat similar, molarity and molality cannot be interchanged. **Molarity** is a measurement of the moles in the total volume of the solution, whereas **molality** is a measurement of the moles in relationship to the mass of the solvent.

When water is the solvent and the concentration of the solution is low, these differences can be negligible (d = 1.00 g/mL). However, when the density of the solvent is significantly different than 1 or the concentration of the solution is high, these changes become much more evident.

Example:

Compare the molar and molal volumes of 1 mole of a solute dissolved in CCl_4 (d = 1.59/mL). For a **1 Molar** solution, 1 mole of solute is dissolved in CCl_4 until the final volume of solution is 1 L. For a **1 molal** solution, 1 mole of solute is dissolved in 1 kg of CCl_4.
1 kg of CCl_4 × (1,000 g/1 kg) × (mL/1.59 g) = 629 mL CCl_4

Concentration by a factor of X

Sometime concentration of a solution is expressed as a multiple of its standard working concentration which actually represents the strength. Higher the multiple higher will

be concentration and vice a versa. For example, many buffers used for PCR reaction, agarose gel electrophoresis are prepared as a 50X stock solution and 1X working solution.
Example- 50X Stock TAE Buffer {242 g Tris base + 57.1 ml Glacial acetic acid + 100 ml EDTA (0.5 M; pH 8.0). Make volume to 1000 ml.}

Preparation of low dilution reagent (working solution) from high concentration (stock solution)

It is more often used in molecular biology where a low concentration working solution is prepared from a high concentration stock solution. For example, 0.5X TAE buffer is prepared from 50 X TAE buffer stock solutions.

Similar kind of calculation is carried out during the estimation of a PCR reaction component. Following formulae can be used very well for such objective-

$$\mathbf{C_s\ V_r = C_w\ V_f}$$

Where-
C_s- Concentration of Stock solution
V_r- Volume of stock solution required
C_w- Concentration of working solution
V_f- Final volume of working solution

Note: Units of initial and final concentration as well as volume must be same.

Concentration Unit Conversion

Sometime it is required to convert one concentration unit into another. For example molarity of 2 N HCl solution or normality of 2M HCl solution. Similarly percentage to molarity and vice a versa.

Normality Molarity

Normality = *n* × Molarity (where *n* is an integer)

For an **acid solution**, *n* is the number of H^+ ions provided by a formula unit of acid.

Example:

A **5 M** H_2SO_4 solution is the same as a **10 N** H_2SO_4 solution.

For a **basic solution**, *n* is the number of OH- ions provided by a formula unit of base.

Example:

A **4M** $Ca(OH)_2$ solution is the same as an **8 N** $Ca(OH)_2$ solution.

Note: *The normality of a solution is NEVER less than its molarity!*

Molarity Percentage

Molarity is a concentration of moles of solute per 1000 ml solution, it is simple to convert into a percentage value, an expression of a gram amount in 100 ml.

$$\text{Molarity} = \frac{\%\text{ of solution}}{\text{MW of solute}} \times 10$$

Problem: Molarity of 0.85% NaCl solution.

M= 8.5/58.44 = 14.5

Genomic DNA isolation from Animal/Fish tissue using Phenol-Chloroform Method

Introduction-

Genomic DNA is used as starting material (mostly as a template) in most of the molecular techniques such as barcoding, promoter amplification, etc. There are several methodologies available for genomic DNA isolation from animal tissue. Phenol chloroform based method is one of the widely used methods for animal tissue which yield good quality DNA for the downstream process. This unit will throw light on different aspects of phenol chloroform based DNA isolation.

Reagents –

1. **Stock EDTA solution (250mM, pH 8.0)**
   Dissolve 4.6 g of EDTA $2H_2O$ in 25 ml of double distilled water. Adjust pH to 8.0 with 1 N NaOH and make final volume to 50 ml using double distilled water and autoclave it.
2. **Tris buffer stock solution (1M, pH 8.0)**
   Dissolve 12.114 g of Tris in 50 ml of double distilled water. Adjust pH to 8.0 with 1 N HCl. Make the volume to 100 ml with double distilled water and autoclave.
3. **Lysis buffer (TEN buffer; 50 mM Tris HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl)**
   Mix 6.25 ml of 1M Tris, 5 ml EDTA (250 mM) and 0.73 g NaCl in 100 ml double distilled water and make the final volume up to 125 ml by adding double distilled water and autoclave.
4. **TE buffer (10 mM Tris and 1 mM EDTA, pH: 8.0)**
   Add 1.25 ml of 1M Tris to 0.5 ml EDTA (250 mM) and make the volume up to 125 ml by adding double distilled water and autoclave.
5. **10% SDS**
   Dissolve 10 g of SDS in 100 ml of autoclaved double distilled water.

6. **70% ethanol**

   Mix 70 ml ethanol with 30 ml of autoclaved double distilled water.

7. **Chloroform: isoamyl alcohol (24:1)**

   Mix 96 ml of chloroform with 4 ml of isoamyl alcohol.

8. **Proteinase K**

   20 mg/ml in autoclaved double distilled water, store at -20°C.

9. **Tris saturated phenol**

Principle

The process of genomic DNA isolation involves the removal of everything except DNA such as RNA, protein, lipid, etc. All these starts with the disruption of the cell membrane and nuclear membrane and follow the different event. The entire principle can be explained using four steps as explained following-

1. **Cell Lysis**

It involves disruption of the membrane using lysis buffer which contains tris HCl, EDTA and SDS. SDS is an anionic detergent which binds with membrane protein and denatures it and hence breaks the integrity of lipoprotein membrane (outer and nuclear membrane both) results in membrane disruption. Tris helps in maintaining alkaline pH which is mandatory for DNA isolation. Whereas EDTA acts as a chelating agent that removes the divalent cations such as Mg^{+2} and Ca^{+2} which is required as a cofactor for DNase hence DNA is protected from DNase in the absence of divalent cations. Further SDS also helps in destabilizing another protein present in the cytoplasm and associated with DNA. This step yield cell solution which contains DNA in solution.

2. **Enzyme treatment**

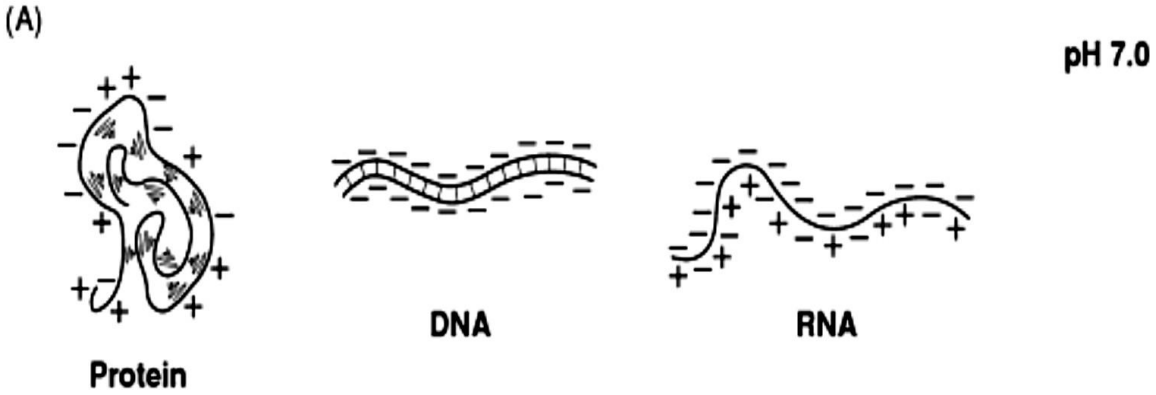
This treatment is provided along with cell lysis hence enzymes are mixed along with lysis buffer. Two enzymes, proteinase K and RNase are used. Proteinase K is a protease extracted from fungus *Engyodontium* album and shows its activity even at 55^0C in the presence of 0.5% SDS under a wide range of pH of 4-12 with an optimum at 8. Hence proteinase K degrades

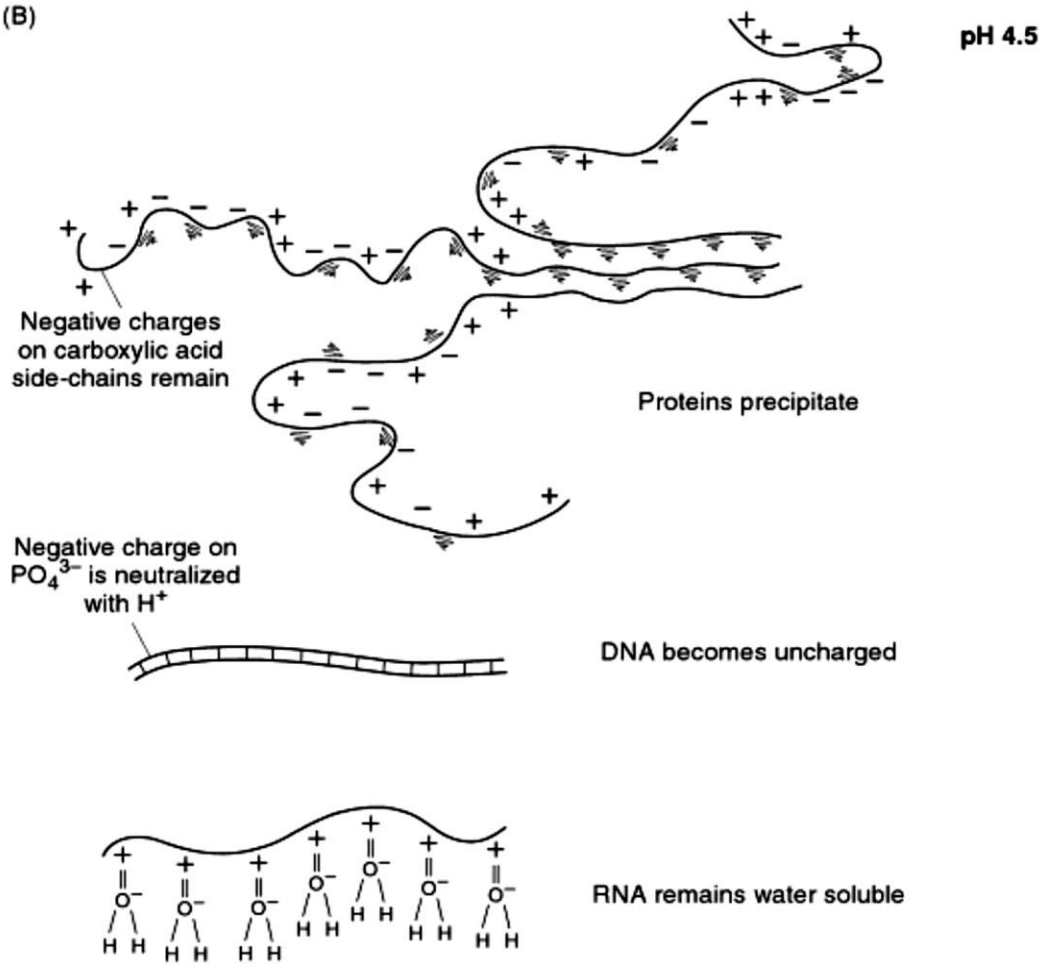
several proteins associated with DNA as well as present in solution in the presence of SDS at 55^0C incubation temperature. RNase is added to remove unwanted RNA from the solution. RNase is relatively heated stable.

3. Phenol Extraction

Phenol is an organic solvent that is used to separate from the nucleic acid. Since phenol and water are immiscible hence two phases i.e. upper aqueous and lower phenol (organic) phase develop after mixing. The density of phenol (1.03 gm/cc) is the more then water hence phenol layer forms at the bottom. Tris saturated phenol at pH 8 is used for DNA isolation as alkaline pH leaves DNA in aqueous phase whereas acidic pH leaves RNA in aqueous phase but moves DNA into phenol as DNA become uncharged at acidic pH. Phenol is hydrophobic and interacts with hydrophobic portion of protein results in denaturation and causing its precipitation in organic phase along with other hydrophobic molecule.

A mixture of phenol, chloroform and isoamyl alcohol (25:24:1) is found to be most effective in phase separation and DNA retention in the aqueous phase. As the density difference between phenol (1.03g/cc) and water (1.00g/cc) is very less. Chloroform ensures better phase separation of the two phases because chloroform is miscible with phenol and it has a higher density (1.47 g/cc) and it forces a sharper phase separation of the organic and aqueous phase. Protein is visible as flocculent material at the top of the organic phase.





4. Ethanol precipitation

It is a more commonly used method of DNA precipitation. Due to the structural difference between water and ethanol, ethanol has a much lower dielectric constant than water (24 vs 80 respectively). As solution contain negatively charged DNA and monovalent cations, the Columb force of attraction increases between the cations and anions under the influence of lower dielectric constant. So DNA is co-precipitated with the cations.

Isopropanol is used some time instead of ethanol, which is more effective in co-precipitation as compared to ethanol due to its lower dielectric constant (20) and it shows the same efficiency as ethanol with the only half volume of ethanol. But it requires more time to evaporate hence drying become tedious.

Storage of sample for DNA isolation

The quality of starting material (sample) is most important for better yield of quality DNA. Fresh tissue always yields good quality DNA. If the sample cannot be processed immediately after harvesting then it is better to store at an ultra-low temperature in ethanol. Ethanol should be replaced by fresh ethanol after the initial 7 days.

Blood sample

An anticoagulant should be added to blood samples before storage. For example, blood samples treated with heparin or EDTA can be stored at 2-8°C for a few days or at -20°C for a few weeks.

Tissue

Freshly collected tissue can be immediately frozen and stored at -20°C, -80°C or in liquid nitrogen. Lysed tissue samples can be stored in suitable lysis buffer for several months at ambient temperature. Ethanol (95%) should be used to preserve animal tissue. The sample should not be preserved in formalin as it is difficult to extract DNA from that sample.

Protocol for extraction of genomic DNA from animal tissue

1. Cell lysis

As explained in principle this step facilitates complete disruption of **the** membrane of cell and organelle to allow the release of DNA from the nucleus as well as from mitochondria. This step is critical as poor membrane disruption lead to incomplete lysis and poor DNA yield.

- Take 100-200 mg of tissue in a 2 ml sterilized microfuge tube and cut into small pieces using sterile forceps and scissors. Homogenize with the help of automated homogenizer or mortar and pestle.
- Add 500 µl of TEN buffer, 50 µl of 10% SDS and 5 µl of proteinase K, mix properly by inverting the tubes several times and incubate at 55°C for 6 hr.(overnight is optional).
- For alcohol preserved tissues, the alcohol should be removed prior to lysis.

2. Phenol Extraction

- After lysis, add an equal volume of Tris-saturated phenol to the lysate and mix well till emulsion forms.

- Centrifuge the tube at 10,000 rpm for 10 minutes and collect the aqueous phase in a fresh tube
- If the organic and aqueous phases are not well separated, then centrifuge again for a longer period.
- To each tube, add an equal volume of Phenol: chloroform: isoamyl alcohol (25:24:1) and mix by inverting the tube several times till emulsion forms.
- Centrifuge the tube at 10,000 rpm for 10 minutes at 4°C.
- Transfer the aqueous phase into a separate tube and add an equal volume of chloroform: isoamyl alcohol (24:1), mix well and centrifuge the tube at 10,000 rpm for 10 minutes at 4°C.
- Transfer the clear aqueous phase to a separate 1.5 ml microfuge tube.

3. Isopropanol precipitation

- Adjust the salt concentration, if necessary, with sodium acetate (0.3 M final concentration; pH 5.2) or ammonium acetate (2- 2.5 M final concentration).
- Add 0.6 to 0.7 volumes of isopropanol to the DNA solution and mix well.
- Centrifuge the tube at 10,000 rpm for 10 minutes.
- Remove the supernatant and wash the precipitate twice with 500 µl of chilled 70% ethanol.
- Air dry the DNA pellet for 15-20 minutes.
- Resuspend the DNA in an appropriate volume of TE buffer and store at 4°C.
- Quantify the DNA using a spectrophotometer and check the quality by running it on 0.8% agarose gel.

DNA isolation using CTAB Method

Introduction

DNA isolation from the plant material and soil sample is a challenging task due to the presence of rigid cellulosic cell wall along with another component such as polysaccharides, polyphenols, lipids etc. Most of the methods are not good enough to extract DNA from such a sample. CTAB based method is considered as one of the best methods for such a sample which uses cationic detergent CTAB along with another chemical to facilitate good quality and quantity DNA from such complex samples. This unit will elaborate different aspect of CTAB based DNA extraction method.

Chemistry of different reagents (Source-) -

CTAB buffer

The CTAB buffer which is mostly used for DNA isolation from plant material mainly includes CTAB, Sodium chloride (NaCl), Tris2-amino-2- hydroxymethyl-1, 3-propanediol (TRIS), Polyvinylpyrrolidone (PVP) and β - mercaptoethanol.

CTAB

Cellulosic fiber by means of a chainlike structure and intermolecular hydrogen bonding forms the outermost strong cell wall of a plant cell. This is disrupted by means of mechanical grinding in CTAB buffer or liquid nitrogen. The biological membrane which forms the second layer from outside is consist of a diverse group of phospholipid and protein molecules and proteins and require amphipathic (hydrophobic tail and hydrophilic head) surfactants (detergent) to dissolve. CTAB is a cationic detergent consisting of the long hydrophobic hydrocarbon chain and a hydrophilic head, forming micelles in aqueous condition and more often used for DNA extraction from plant tissue. In the process of DNA extraction, under aqueous condition, CTAB interacts with the biological membrane, capture the lipids and results in the disruption of membrane. CTAB works differently based on the ionic strength of the solution. At a low ionic strength, it precipitates nucleic acid and acidic polysaccharides (pectin, Xylan and carrageenan), while protein and neutral polysaccharides (dextran, gum locust bean, starch, and inulin) remain in the solution. However, at high ionic concentration, it gets bound

to the polysaccharides and forms complexes, that are removed during subsequent chloroform extraction. It also denatures or inhibits the activity of proteins and/or enzymes.

NaCl

Salt concentration plays a significant role in cell lysis. The hypotonic solution facilitates the movement of water inside the cell leads to swelling, rising internal pressure and finally bursting. Whereas hypertonic solution facilitates the loss of water from the cell which turns cell flaccid and leads to plasmolysis. A 0.5 M or more salt concentration build ionic strength that is needed for CTAB to precipitate polysaccharides. In most of the protocols, 1.5 M concentration of NaCl has been suggested; however, a higher concentration of NaCl/or CTAB is recommended in case sample consist of a higher amount of polysaccharides.

Tris

Tris Amino (2-amino-2-hydroxymethyl -1, 3-propanediol) has three primary alcohols and an amine group. When the pH is adjusted to 8, with HCl, it contains a mixture of a weak base and its conjugate weak acid which can act as a buffer. When the cell wall and membranes are broken during tissue grinding, compartmentalization ends, cytoplasmic material is released, because of which the pH gets altered, and consequently the stability of biomolecules like nucleic acid is disturbed. The buffer plays an important role under such a situation, and the Tris buffer maintains the pH.

EDTA

Ethylenediaminetetraacetic acid (EDTA) chelates divalent cations, such as Mg^{+2}, Ca^{+2} . Divalent cations are the cofactors for many enzymes. For example, enzyme DNase requires Mg^{+2} ions as a cofactor for its activity. Chelating Mg^{+2} ions with EDTA makes enzyme DNase nonfunctional, and thereby protect the DNA. The Mg^{+2} ions are also required for aggregation of nucleic acid with protein, whereas Ca^{+2} ions are required for cementing of cell wall's middle layer and membrane stability. Thus, harnessing them by EDTA results in destabilization of their integrity.

β-Mercaptoethanol

Globular proteins get dissolved in water. To make them insoluble, their denaturation is one of the alternatives. This can be done at tertiary and quaternary structure

level of protein by reducing intermolecular disulphide linkages. β- mercaptoetahnol's sulfhydryl reduces disulphide bonds of the protein and thus the proteins are denatured.

PVP

Polyphenol is a major component in medicinal plants, woody plants, and mature plant parts. It is present in the vacuole, while its oxidizing enzyme, Polyphenol Oxidase (PPO) is located in the plastid. During grinding of the tissue, compartmentalization breaks and PPO convert polyphenols into Quinone's, which gives brown coloration. Polyphenols bind DNA and make downstream processing difficult. Whereas, it's oxidized product, i.e. Quinone's breaks the nucleic acid by producing reactive oxygen species. Polyvinylpyrrolidone (PVP) removes polyphenolic contamination by binding it through a hydrogen bond. Thus, it prevents polyphenol oxidation, and thereby browning of DNA samples. When the extract is centrifuged with chloroform, PVP complexes get accumulated at the interphase. Cell lysate mixture with CTAB buffer should be kept in the water bath at 65 ^{0}C, which irreversibly inhibit enzyme DNase. After removing the sample from the water bath, it should be allowed to cool at room temperature, then Chloroform: Isoamyl alcohol (24:1) or Phenol: Chloroform: Isoamyl alcohol (25:24:1) shall be added.

Chloroform

Chloroform is a non-polar (hydrophobic) solvent, in which non-polar and lipids get dissolved, thus removing these contaminants. As chloroform is volatile in nature, it does not hinder the downstream process.

Isoamyl alcohol

When chloroform comes in contact with the air it forms harmful phosgene ($COCl_2$, Carbonyl chloride) gas. The gas entrapment also causes foaming or frothing, which is prevented when Chloroform is used along with Isoamyl alcohol. Isoamyl alcohol is not miscible in the aqueous solution because it is a long chain aliphatic compound, containing five carbon atoms. Thus, stabilizing the interphase between the organic and aqueous layer.

Phenol

Phenol is used along with chloroform for purification of the DNA by removing contaminating proteins and polysaccharides. When centrifugation after phenol: chloroform

steps give three layers i.e. aqueous, interphase and at bottom organic phase. At Neutral to alkaline pH, the nucleic acids are negatively charged and polar. Therefore, it is hydrophilic and remains in an aqueous phase. Proteins have polar and nonpolar amino acid stretches. In aqueous solution, hydrophobic amino acid forms a protective core. However, after denaturation, nonpolar cores (hydrophobic) get exposed, causing precipitation of protein as well as some polysaccharides at Interphase. The centrifugation after chloroform: isoamyl alcohol step should be done under room temperature, because below 15°C CTAB / nucleic acid form irreversible aggregate and that may precipitate. During this step, the DNA shall be in the supernatant phase.

Isopropanol/Ethanol

DNA remains dissolved in aqueous solution because DNA has a phosphodiester backbone, which is hydrophilic in nature. Water molecule forms hydration shell around DNA by forming hydrogen bonds. Ethanol, as well as isopropanol, is useful in precipitation of DNA, which breaks the hydration shell. Isopropanol is a good choice for precipitation of DNA. The amount of Isopropanol requirement is less (0.7 volume of supernatant) than ethanol (2.5 volume), as isopropanol has a higher capacity to reduce the dielectric constant of water than the ethanol. While Isopropanol is unique in its action, RNA (has extra 2'OH which so remains hydrogen bounded with water more strongly than DNA) tends to stay soluble in it, thus selective precipitation of DNA can be done. Isopropanol also dissolves non-polar solvent such as chloroform thus the impurities form the previous step can also be removed. Using ice-cold isopropanol is generally practiced, but many researchers say that it should be used at room temperature, otherwise it will precipitate polysaccharides also. Though the yield of DNA will be increased at low temperature, it may increase impurities.

Sodium acetate /ammonium acetate/potassium acetate

In aqueous solution, the salt dissociates into its ions because water has high dielectric constant. Monovalent cations such as Na^+ shields the negative charge of the sugar phosphate backbone thereby it reduces repulsion between DNA molecules. This is useful in aggregation and formation of tangled mass. It is also called as salting out. Nevertheless, it is not seen when salt alone is used. It requires the solution with low dielectric constant, which

allows this interaction. This is performed by either ethanol or isopropanol. Isopropanol has extra CH_3 making it less polar than ethanol, so some of the salts are not dissolved and precipitate along with the nucleic acid. NaCl has a lower solubility than the sodium acetate, so for the production of the monovalent cations, the sodium acetate is preferred. Sodium acetate with pH around 5.2 is used for precipitation of nucleic acid along with ethanol.

Ethanol

DNA precipitate is washed again with 70% ethanol to rinse excess salt**,** centrifuged and ethanol is discarded, leaving DNA in the precipitate. The precipitate can be air-dried or vacuum dried.

(Tip: Over drying should be avoided as it converts a B form of the DNA to D form, which is difficult to dissolve later.)

TRIS - EDTA (TE) buffer/sterile water

For long storage, DNA must be retained in TE buffer. TRIS AMINO constituent of TE buffer has the ability to protect DNA strands from radiation damage, in both solid state and fluid solution. As radiation produces free radicals, it may break DNA strands. Thus, in the fluid solution (ambient temperature) Tris acts by scavenging hydroxyl radicals. Sterile water can be utilized for short duration storage of DNA. (Tip: If TE buffer is used for storage of DNA, it should be diluted further with sterile water dilute EDTA concentration for making magnesium ions available for polymerase activity during PCR.)

Ribonuclease A

Genomic DNA should be treated with Ribonuclease A (RNase A) to remove the contamination of RNA. RNase A is an endoribonuclease that hydrolyzes 3', 5'-phosphodiester linkage of RNA. It makes use of the 2'OH group of the Ribose sugar for hydrolysis of phosphodiester linkage. As the DNA is devoid of the 2'OH group (Deoxy), it remains secure.(Tip: RNase may have impurities of DNase that should be eliminated by boiling water bath method during which DNase impurities are denatured, leaving heat stable RNase enzyme)

Modified CTAB method for isolation of DNA from a tissue sample

Reagent Preparation-

- Extraction Buffer- (100 mM Tris-HCL (PH 8.0), 100 mM sodium EDTA (PH 8.0), 100mM sodium phosphate (PH 8.0), 1.5 M Nacl, 1% cetyltrimrthyl ammonium bromide)
- Proteinase K -10mg/ml
- SDS-20%
- Chloroform
- Isoamylalcohol

Protocol-

Take approximately 200 mg of sample in a fresh 2 ml microfuge tube.

To this add 550µl of extraction buffer and 5µl of Proteinase K (10mg/ml) and incubate the tube in an incubator shaker at 37^0C at 225 rpm for 30 minutes.

After incubation adds 150 µl of 20% SDS and incubate for 2 hr. at 65ºC in a water bath with intermittent gentle shaking after every 15-20 minutes.

After incubation centrifuge the tube at 6000×g at room temperature for 10 min and then transfer the supernatant to another 2 ml fresh tube.

Resuspend the pellet completely in 200 µl extraction buffer and 25 µl of 20% SDS and incubate for another 10 min at 65°C.

Again centrifuge the sample at 6000×g for 10 min and transfer the supernatant to the previously collected supernatant.

Mix the combined supernatant (~700 µl) with an equal amount of chloroform: isoamyl alcohol (24:1) and centrifuge at 1000 rpm at room temperature for 10 min.

Carefully remove the upper (aqueous) layer into another fresh 1.5 ml centrifuge tube and to this add 0.6 volume of isopropanol and mix by inverting the tube 3-4 times and incubate for 1 h at room temperature following centrifugation at 16000×g for 20 min at room temperature.

Decant the supernatant and wash pellet with cold 70% ethanol and air dry in laminar flow.

Dissolve pellet in 30-40 µl of TE (1×) buffer and store at 4°C.

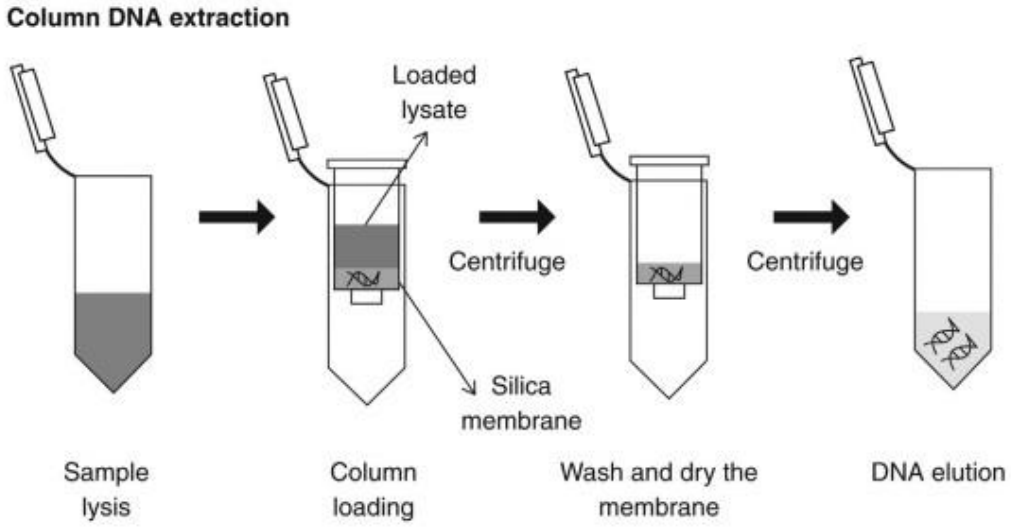
Genomic DNA isolation from a commercial kit

Introduction

Kit based genomic DNA isolation is quite easier, faster and yields a good quality of DNA in a substantial amount and most importantly do not require any expertise. Different types of commercial kits are available, mostly silica column based at different price range to facilitate DNA isolation from different types of the sample such as tissue, blood, fecal matter, soil, microbes, plant material etc. This unit is meant to throw light on the principle of column-based kit. Protocols and chemicals are provided in detail with the kit and are company specific.

Principle

The basic principle includes cell lysis, purification, washing, final purification and elution.



(source-https://qbpatologica.files.wordpress.com/2017/06/2016-dna-extraction-finding-the-most-suitable-method.pdf)

The sample is lysed prior to the column loading, as per manufacturers' instructions. The lysate is loaded into the column in the presence of chaotropic salts that allow the DNA to bind to the silica membrane. The purification process occurs with the addition of

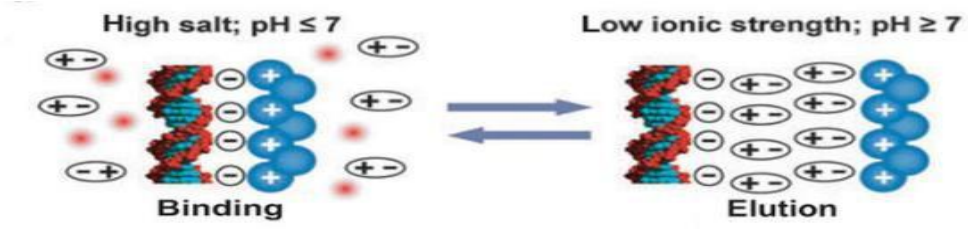
a washing reagent that goes through the column by centrifugation. Finally, DNA is eluted with an appropriate buffer.

Step 1: Cell Lysis

Most of the commercial kit uses lysis buffer for cell lysis which consists of a high concentration of chaotropic salts. Chaotropic salts destabilize proteins through destabilization of existing hydrogen bonds, van der Waals forces and hydrophobic interactions of proteins including nucleases. It also disrupts the association of nucleic acids with water, thereby providing optimal conditions for their transfer to silica. Chaotropic salts include guanidine HCL, guanidine thiocyanate, urea, and lithium perchlorate. The lysis buffer also contains detergent which also helps in the destabilization of proteins and disruption of cell membrane hence cell lysis. Some kits are also provided with broad-spectrum serine protease proteinase K, which is a highly efficient protein digesting enzymes work at a higher temperature in the presence of lysis buffer. Another popular enzyme used for the bacterial cell is lysozyme which does not work under denaturing conditions and added before the addition of denaturing salts.

Step 2: Purification – Binding of DNA to the column

Chaotropic salts are critical for lysis and binding of DNA to the column. The addition of alcohol (ethanol or isopropanol) improves the binding of nucleic acids to the silica. The alcohol prevents the dissolution of the DNA that remains bound to the membrane while the other sample components are removed. The volume and the percentage of alcohol to be added are crucial and optimal for the kit.



[The purification principle for silica matrices is based on the high affinity of the negatively charged DNA molecule for the positively charged silica particles (blue). Under high salt

conditions the DNA is tightly bound and extensive washing removes all contaminations, and purified DNA molecules can be eluted under low ionic strength.]

Step 3: Washing

Centrifugation of lysate through silica membrane allowed the release of impurities such as protein and polysaccharides along with flow-through whereas DNA remains bonded with silica membrane. However, the membrane will contain protein and salt residues. At this point, plant samples will likely contain polysaccharides and pigments, while for blood samples, the membrane may be slightly brown or yellow in color. The wash steps remove such impurities.

There are typically two wash steps, although this varies depending on sample type. The first wash will often include a low concentration of chaotropic salts to remove residual proteins and pigments. This is always followed by an ethanol wash to remove the salts. If the sample didn't contain a lot of protein starting out (e.g., plasmid preps or PCR clean ups), an ethanol wash is sufficient.

Removal of the chaotropic salts is crucial to getting high yields and purity. Some kits actually recommend two ethanol washes. Residual salt will impede the elution of nucleic acid, resulting in poor yield, high A230 readings and thus low 260/230 ratios.

Step 4: Dry Spin for ethanol-free DNA and RNA

Most protocols include a centrifugation step after washing to dry the column of residual ethanol, and this step is essential for a clean eluent. Subsequent addition of 10 mM Tris buffer or water to the membrane will hydrate the nucleic acids, thus eluting them from the membrane. Residual ethanol on the membrane at this point will prevent full hydration and elution of nucleic acids.

You will not be able to see ethanol on a spectrophotometer, but a good indicator of its presence is samples that will not sink into the wells of an agarose gel, even in the presence

of loading dye. Another indicator of ethanol contamination is samples that don't freeze at -20°C.

Step 5: Elution

The final step in the DNA extraction protocol is the release of pure DNA from the silica. For DNA extraction, 10 mM Tris at pH 8-9 is typically used. DNA is more stable at a slightly basic pH and will dissolve faster in a buffer than water. This is true even for DNA pellets. Water tends to have a lower pH of 4-5, and high molecular weight DNA may not completely rehydrate in the short time used for elution. For maximal DNA elution, allow the buffer to stand in the membrane for a few minutes before centrifugation. For applications requiring intact high molecular weight DNA, such as long-range sequencing and long read sequencing, the elution buffer is the best choice.

Agarose Gel Electrophoresis (AGE)

Introduction

A day in molecular biology laboratory without agarose gel electrophoresis is almost rare event. It is a widely used method to separates nucleic acid (DNA, RNA, plasmid) based on size (as all nucleic acid contains overall negative charge) by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration speed is determined exclusively by the molecular weight where small weight molecules migrate faster than larger ones. This movement is facilitated due to electric current which flows along with the gel mainly because of the presence of buffer and voltage or potential difference created along the electrode. The frictional force of gel act as a molecular sieve separating DNA by size. An agarose gel is a complex network of polymeric molecules whose average pore size depends upon the buffer composition and the type and concentration of agarose used. As DNA is negatively charged due to the presence of phosphate group it moves from the cathode (negative electrode) to anode (positive electrode). The lesser concentration of agarose is used for larger molecules of DNA. Larger the pore size of the gel, the greater the ball of DNA that can pass through and hence the larger the molecules that can be separated. Smaller size DNA moves faster than larger size. Overall migration of DNA along gel depends upon the following factor-

1. The strength of the voltage
2. The size and shape of the molecule
3. The concentration of agarose in the gel
4. The ionic strength and temperature of the buffe3r in the gel.
5. The relative hydrophobicity of the sample.

A lower concentration of buffer slows down the movement of DNA due to slow current flow but same time if the concentration is high it also causes much resistance to current flow results in the production of heat so the gel will melt. Hence the optimum concentration of buffer is selected for a particular concentration of agarose.

Agarose is convenient for separating DNA fragments ranging from a few hundred bases to 40 kb. Polyacrylamide gel is preferred for smaller fragments of DNA below 300 bp. whereas for DNA fragments having size more than 40 kb pulse field gel electrophoresis is used.

Requirements-

1. Agarose- Agar is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen bonding when heated in a buffer and subsequently cooling. Different concentrations of agarose are required for different size of DNA as presented in the following table-

Agarose concentration (%W/V)	DNA size (Kb)
0.3	5-60
0.5	1-30
0.7	0.8-12
1.0	0.5-10
1.2	0.4-7
1.5	0.2-3
2.0	0.05-2

So agarose shows a wide range of separation at varying level of concentration. Electrically agarose is neutral so the buffer is required to facilitate current flow through this. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "firmer" the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic.

2. Buffer-

The electrical conductivity of the gel is due to buffering only. The particular concentration of buffer is used for a particular concentration of agarose. It is also called as electrode buffer. TBE (Tris-borate EDTA) and TAE (Tris-acetate EDTA) are two commonly used buffer for gel electrophoresis of DNA. Tris base is a strong base and borate is an acid,

and both in combination maintains the pH in the nearly neutral range of 8 to 8.5. The slightly alkaline condition also protects the DNA and allows proper separation. EDTA is a chelatize Mg^{2+} ion if any present, which is required as a cofactor for DNAse hence DNA is protected from the same. Further, the buffer also helps to neutralize the charge of a water molecule. Under the electrical current, the water molecule dissociates into H^+ and OH^-. The H^+ ions can react with the PO^{3-} of DNA. So, the negative charge of buffer neutralizes the charge of the water and protects DNA. TAE buffer contains glacial acetic acid instead of borate and rest remain the same. Although TAE has lower buffering capacity as compared to TBE and it also offers some advantage over TBE such as it can interfere with enzymatic reaction and protect DNA, in contrast, whereas TBE cannot do so. Further, the borate of TBE can react with the sugar backbone of DNA, therefore, it is not always recommended. Borate of TBE also reacts with glycerol of loading dye and decreases the activity of loading dye. Hence if glycerol is the primary component in the DNA gel loading dye, TAE buffer is the best choice for getting a good result.

TAE buffer	**TBE buffer**
It has a lower buffering capacity so it can be exhausted by repeated use.	TBE has a higher buffering capacity due to the borate hence can be used repeatedly
The conductivity of TAE buffer is better so dsDNA can migrate faster as compared to TBE buffer	Because of the lower conductivity, the migration of dsDNA is lower as compared with TAE buffer. The resolution is very good for longer DNA fragments.
DNA can be easily recovered in TAE buffer so the recovery rate of TAE buffer is higher.	Borate inhibits many DNA enzymes hence the integrity of DNA is higher in TBE buffer.
It is a cost-effective and cheaper than other buffer systems, further, the working solution requirement is lower as compared with TBE buffer (only 0.5X buffer is required).	TBE buffer is a little costly and working solution requirement is higher as compared to TAE buffer (required 1X buffer).
	Borate from TBE buffer can interact with DNA so the recovery rate is lower as compared to TAE buffer.

Stock TAE buffer- 50X TAE Buffer: 242 g Tris base + 57.1 ml Glacial acetic acid + 100 ml EDTA (0.5 M; pH 8.0). Make volume to 1000 ml.

Working TAE buffer of 0.5X is used for agarose gel electrophoresis.

3. Ethidium bromide

It is a fluorescent dye which intercalates between nitrogenous bases of DNA. It binds by inserting itself between the stacked bases in double stranded DNA.

Concentration- 1 mg/ml

4. Loading buffer

It contains loading dye and TAE buffer. Loading dye is a mixture of bromophenol blue, xylene cyanol and glycerol in distilled water. Following are the role of loading buffer-

-It increases the density of the sample, mainly due to the presence of glycerol and so sample settles properly in well.

-Bromophenol blue add color to the sample thereby simplifying the loading process and also helps in tracking the path of DNA movement and its progress.

-Charge present on dye is similar to DNA so in any electric field it moves with a predictable rate along with DNA.

Loading dye (6X): 0.25% bromophenol blue + 0.25% xylene cyanol + 30% glycerol in distilled water.

Equipments-

1. Horizontal gel electrophoresis equipment.
2. UV trans-illuminator or gel documentation system.

Procedure

Pouring a Standard 1% Agarose Gel:

1. Measure 1 g of agarose.

Tip: Agarose gels are commonly used in concentrations of 0.7% to 2% depending on the size of bands needed to be separated. Simply adjust the mass of agarose in a given

volume to make gels of other agarose concentrations (e.g., 2 g of agarose in 100 mL of TAE will make a 2% gel).

2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.

Tip: TBE can be used instead of TAE, labs usually use one or the other, but there is very little difference between the two.

Note: Make sure to use the same buffer like the one in the gel box (do not mix different buffers and do not use water).

3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffers will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).

CAUTION: HOT! Be careful stirring, eruptive boiling can occur.

Tip: It is a good idea to microwave for 30-45 sec, stops and swirl, and then continue towards a boil. Keep an eye on it the solution has a tendency to boil over. Placing saran wrap over the top of the flask can help with this, but is not necessary if you pay close attention.

4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.

5. Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

CAUTION: EtBr is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical.

6. Pour the agarose into a gel tray with the comb in place.

Tip: Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.

7. Place newly poured gel at 4°C for 10-15 mins or let sit at room temperature for 20-30 mins, until it has completely solidified.

Tip: If you are in a hurry, the gel will set more quickly if you place the gel tray at 4 °C earlier so that it is already cold when the gel is poured into it.

Loading Samples and Running an Agarose Gel:

1. Add loading buffer to each of your DNA samples.

Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and allows you to gauge how far the DNA has migrated; 2) it contains a high percentage of glycerol that increases the density of your DNA sample causing it to settle to the bottom of the gel well, instead of diffusing in the buffer.

2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
3. Fill gel box with 1xTAE (or TBE) until the gel is covered.
4. Carefully load a molecular weight ladder into the first lane of the gel.

Note: When loading the sample in the well, maintain positive pressure on the sample to prevent bubbles or buffer from entering the tip. Place the very top of the tip of the pipette into the buffer just above the well. Very slowly and steadily, push the sample out and watch as the sample fills the well. After all of the samples is unloaded, push the pipette or to the second stop and carefully raise the pipette straight out of the buffer.

5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.

Note: Black is negative, red is positive. The DNA is negatively charged and will run towards the positive electrode. Always Run to Red.

7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 5 mins.

9. Using any device that has UV light, visualize your DNA fragments. The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

Note: When using UV light, protect your skin by wearing safety goggles or a face shield, gloves and a lab coat.

Tips and FAQ

•**How do you get better resolution of bands?**

A few simple ways to increase the resolution (crispness) of your DNA bands include: a) running the gel at a lower voltage for a longer period of time; b) using a wider/thinner gel comb; or c) loading less DNA into the well. Another method for visualizing very short DNA fragments is polyacrylamide gel electrophoresis (PAGE), which is typically used to separate 5 - 500 bp fragments.

•**How do you get better separation of bands?**

If you have similarly sized bands that are running too close together, you can adjust the agarose percentage of the gel to get better separation. A higher percentage agarose gel will help resolve smaller bands from each other, and a lower percentage gel will help separate larger bands.

•10% Rule:

For each sample you want to load on a gel, make 10% more volume than needed because several microliters can be lost in pipetting. For example, if you want to load 1.0 µg in 10 µL, make 1.1 µg in 11 µL.

Points to be taken care of during agarose gel electrophoresis

- Always use the same batch of buffer to prepare gel and for running it since small difference in ionic strength can affect migration of DNA.
- If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with distilled water.
- Mix the agarose - ethidium bromide solution well to avoid localized staining.
- Ethidium bromide is a powerful mutagen and is very toxic. Wear gloves while using it.

- Once sample are loaded, do not move the gel tray as this may cause sample to float out of the wells.
- Avoid the use of high voltages which can cause trailing and smearing of DNA bands in the gel.
- UV light can damage eyes and skin. Always wear suitable eye and face protection when working with UV light source.
- UV light damages DNA. If DNA fragments are to be extracted from the gel use lower intensity UV source if possible and minimize exposure of the DNA to the UV light.

Quantification and quality check of DNA

Introduction

Quality and quantity of nucleic acid is an important aspect of molecular work. It decides the success and efficiency of the downstream process. Reliable measurement of nucleic acid concentration and purity is important for many applications in molecular biology such as PCR, sequencing, microarray, etc. Impurities in the nucleic acid can lead to inaccurate measurement of its concentration and could potentially inhibit subsequent reactions.

Overview-

DNA concentration can be assessed using four different methods: Absorbance (optical density), Agarose gel electrophoresis, Fluorescent DNA binding dyes and a luciferase pyrophosphorylation coupled quantitation system. The two most common methods of measuring DNA purity and concentration are absorbance and agarose gel analysis.

Absorbance

Spectrophotometer based equipment such as nanodrop, microspectrophotometer etc. are used to access the quality and quantity of nucleic acid which works on a similar mechanism of absorbance. Nucleic acids absorb ultraviolet (UV) light due to the heterocyclic rings of the nucleotides; The sugar-phosphate backbone does not contribute to absorption. The wavelength of maximum absorption for both DNA and RNA is 260nm ($\lambda max = 260nm$) with a characteristic value for each base.

The absorption properties of DNA can be used for detection, quantification and assessment of purity. Although the λmax is constant, the extinction coefficient of nucleic acids depends on their environment. The absorbance of isolated nucleotides is greater than that of RNA and single-stranded DNA (ssDNA) which is in turn greater than that of double-stranded DNA (dsDNA). With the absorption of dsDNA being approximately 40% less than would be exhibited by a mixture of free nucleotides of the same concentration. This difference is due to the structural properties of the nucleic acid and is called the hypochromic effect. It results from hydrophobic and dipole-dipole interactions between the electron systems of the individual bases made possible by their stacking in the parallel array of the double helix. In ssDNA the bases have a tendency to stack on top of one another but this is maximized in

dsDNA. It is these interactions and stacking arrangement that stabilize the helical structure of DNA and also RNA rather than hydrogen bonding which determines the specificity of base pairing. Before measuring absorbance, it is important to make sure that the DNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes.

Absorption of nucleic acids also depends on the solvent used to dissolve them. Reports in the literature have compared solubilizing DNA in water with a low salt buffer (e.g., Tris or TE buffer). It was found that a low salt buffer gave very reproducible readings whereas readings in water could give up to 14% variation. This is most likely due to differences in the pH of the water caused by the solvation of CO_2 from air. The amount of CO_2 that is dissolved in the DNA/water solution is dependent on and varies with the amount of agitation used to re-suspend the DNA. As DNA can be difficult to solubilize in water thorough mixing may be needed. A260/A280 ratios measured in water were also shown to be variable and ratios obtained were typically less than 1.8. As the protein content in these examples was less than 1% these ratios were misleading. The A260/A280 ratios measured in low salt buffer were completely reproducible.

A260 Unit	Nucleic acid concentration
dsDNA	50 µg/ml
ssDNA	33 µg/ml
RNA	40 µg/ml
Oligonucleotides	20-30 µg/ml

Quantification of DNA

The absorbance at 260nm is used to calculate the concentration of nucleic acids. At a concentration of 1 µg/ml and a 1 cm path length* dsDNA has A260 = 50. The absorbance value is also dependent on the amount of secondary structure in the DNA due to hypochromicity.

To ensure the concentration reading is accurate, the absorbance reading should be within the linear range of the spectrophotometer. The Lambert-Beer law relates the absorption of light to the properties of the material through which the light is travelling. The

law states that there is a logarithmic dependence between the transmission of light through a substance and the product of the absorption coefficient of the substance and the distance the light travels through the material (i.e. the path length). The law tends to break down at very high concentrations, especially if the material is highly scattering. So for concentrated solutions, the absorbance value and therefore the concentration can be inaccurate. It is often useful to prepare and measure a series of dilutions to check not only the concentration but the accuracy of the dilutions as well. Inaccurate dilutions can occur if the DNA is not homogeneously re-suspended. For reliable spectrophotometric DNA quantification, A260 readings should lie between 0.1 and 1.0.

To improve the accuracy of DNA concentration determination allowance should be made for any impurities in the solution. This can be estimated by adjusting the A260 measurement for turbidity which is measured at an absorbance of A320. The equation below can be used:

Concentration (μg/ml) = (A260 reading – A320 reading) × dilution factor × 50μg/ml

The total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA Yield (μg) = DNA Concentration × Total Sample Volume (ml)

* Path length defines the distance that light (UV) travels through a sample in an analytical cell.

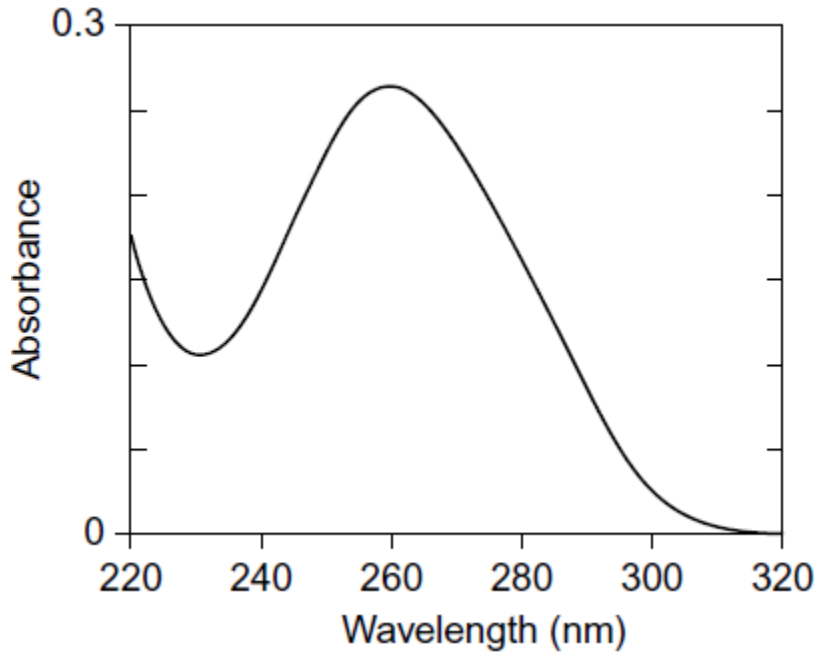


Figure - A typical spectrophotometric scan of double-stranded DNA (dsDNA). Maximum absorbance occurs at 260 nm. Absorbance at 230 nm is an indication of salt in the sample. If the sample is optically clear, it should give a very low reading at 320 nm, which is in the visible wavelength region of the spectrum.

Purity of DNA

A260/A280 ratio to measure Protein Contamination

This procedure was first described to measure protein purity in the presence of nucleic acids. However, it is now commonly used to assess protein contamination of DNA. It is important to note that the A260/A280 ratio is only an indication of purity rather than a precise answer. Pure DNA preparations have an A260/A280 ratio of greater than or equal to 1.8.

When the A260/A280 ratio is determined for a range of different DNA/protein mixtures it has been shown that the ratio is relatively insensitive to the addition of protein to the pure nucleic acid. However, the utility of the ratio becomes apparent when DNA is prepared from tissue or blood. These samples have a protein content that greatly exceeds nucleic acid on a weight basis and so in these cases the approximate purity of dsDNA preparations and

contamination by protein may be estimated by determination of the ratio of absorbance at 260nm and 280nm.

Several factors can influence the accuracy of the A260/A280 ratio. For example, readings from very dilute samples will have very little difference between the absorbance at 260 and 280nm leading to inaccurate ratios. The type(s) of the protein present will also have an effect. Absorbance in the UV range by proteins is primarily the result of aromatic ring structures. Proteins are composed of 22 different amino acids of which only three contain aromatic side chains. Thus the amino acid sequence of proteins would be expected to influence the ability of a protein to absorb light at 280 nm. For example, bovine serum albumin (BSA) has an extinction coefficient value of 0.7 for a 1 mg/ml solution at 280nm, while streptavidin has an extinction coefficient of 3.4, absorbing almost five times as much light at 280nm at the same concentration.

It is also important to note the phenol and other contaminants can also absorb at 280 nm and can affect the ratio calculation. Phenol absorbs with a peak at 270nm and has an A260/A280 ratio of 2. Nucleic acid preparations uncontaminated by phenol should have an A260/A280 ratio of around 1.8. As well as affecting the ratio calculations, contamination by phenol can significantly contribute to overestimation of DNA concentration and inhibit downstream reactions.

The pH of the solution can also affect the A260/A280 ratio, with acidic solutions having a lower ratio of up to 0.2–0.3 and alkaline solutions having an increased ratio by a similar amount.

Due to their different absorption spectra, the nucleotide composition of the bases present in DNA will have different A260/A280 ratios.

Nucleotide	A260/A280 ratio
Adenine	4.50
Cytosine	1.51
Guanine	1.15
Thymine	1.47

Table : A260/A280 ratios for nucleotides.

Therefore the ratio will be approximately equal to the weighted average of the A260/A280 ratios estimated for each nucleotide if measured independently, which explains why the accepted ratio of 1.8 for pure DNA is an approximation.

The ratio can be calculated after subtracting the non-nucleic acid absorbance at A320.

DNA Purity (A260/A280) = (A260 reading – A320 reading) ÷ (A280 reading – A320 reading)

RNA Contamination

Pure RNA has an A260/A280 ratio of 2.0, therefore if a DNA sample has an A260/A280 ratio of greater than 1.8 this could suggest RNA contamination. However, due to the similarity in absorption profiles of RNA and DNA, probably the most accurate way to determine RNA contamination is to run the sample on an agarose gel where the RNA will clearly be seen migrating ahead of the DNA. RNA can easily be removed by adding RNase A during purification.

A260/A230 ratio

This ratio is used as a secondary measure of nucleic acid purity. The A260/A230 ratio values for pure samples are often higher than the respective A260/A280 ratio values. Strong absorbance around 230nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. A ratio of 260nm to 230nm can help evaluate the level

of salt carryover in the purified DNA. The lower the ratio, the greater the amount of salt present. As a guideline, the A260/A230 ratio should be greater than 1.5, ideally close to 1.8. Urea, EDTA, carbohydrates and phenolate ions all have absorbance near 230nm. The TRIzol® reagent which can be used for DNA extraction, although more commonly used for RNA preparation is a phenolic solution which absorbs in the UV spectrum at 230 nm and around 270nm. Guanidine HCL which is used in the extraction of DNA will absorb at 230nm while guanidine isothiocyanate, used for RNA isolations will absorb at 260nm.

As discussed previously, a reading at 320nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230nm to 320nm is most informative.

Gel Electrophoresis

Agarose gel electrophoresis of the purified DNA compliments the data from absorbance readings. Concentration can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the solution concentration is 50 ng/µl (100 ng divided by 2 µl). Standards used for quantitation should be labelled in the same way and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR Green is required. This method is useful for cases where concentration is too low to accurately assess with spectrophotometry and in cases where contaminants absorbing at 260nm make accurate quantitation by that method impossible.

However, the most common reason for running a gel is to access DNA quality. On a 1 to 1.5% agarose gel, intact genomic DNA should appear as a compact, high-molecular-weight band with no low-molecular-weight smears. Degraded DNA results in biased labelling.

Polymerase Chain Reaction (PCR)

Introduction:

The Polymerase Chain Reaction (PCR) was invented by Kary Mullis in the year 1985 and used extensively in molecular biology research. It has revolutionized the world of biological science. In principle, PCR results in the targeted amplification of a selected region of DNA molecule in vitro. Any region of DNA molecule can be chosen, so long as the sequences at the flanking region are known as primers are designed out of the flanking region. It is the primer which decides the target.

Previously Klenow fragment of *E.coli* DNA polymerase I was used to extend the annealed primers. But this enzyme was inactivated during the denaturation step where the higher temperature was used to separate the two DNA strands at the beginning of each PCR cycle. Therefore fresh enzyme has to be added during every cycle. The discovery of thermostable DNA polymerase has transformed the PCR into a simple, automated thermal cycling device. Thermostable DNA polymerase I enzyme isolated from *Thermus aquaticus,* called as Taq polymerase is used for amplification.

PCR can be used to amplify a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kb although some techniques allow for amplification of fragments up to 40 kb in size. The purpose of a PCR is to make a huge number of copies of a gene/DNA sequence. The principle in Polymerase chain reaction is similar to the replication of DNA in living organisms.

Basic components for a PCR:

1. **DNA template**

   It is used to copy the target region. A good quality DNA is a prerequisite for successful PCR. Presence of contamination such as phenolic compound, salt etc inhibits PCR. In general 1-2 ng of DNA template is required per µl PCR reaction volume (final concentration of DNA is 1-2ng/ µl)

2. **Primers**

   Amplification target is decided by primer pair called as forward and reverse primer. Sequence information must be available to design primer. Different freely available softwares are available to design primer. Details on primer designing will be

discussed in the primer designing chapter. Company supply primer in a lyophilized form which is diluted using 1X TE buffer. The final concentration of primer is 0.1-0.5 µM.

3. DNA polymerase

Taq polymerase is most commonly used DNA polymerase in PCR which is isolated from a bacterium found in hot spring known as *Thermus aquaticus*. It works optimally at 72ºC and over pH range 7.0-7.5, adding 100 nucleotides per second to the primer. It is a heat stable enzyme withstanding repeated Denaturation cycles. There are other types of polymerase commercially available as presented in the following table

Enzyme	Organism	Optimum temperature	Exonuclease activity	Fidelity	Stability
Taq polymerase	*Thermus aquaticus*	72-78°C	5'-3'	Low	9 minutes at 97.5°C
DeepVent polymerase	*Pyrococcus* strain GB-D	70 -80°C	3'-5'	High	480 minutes at 100°C
Pfu polymerase	*Pyrococcus furiosus*	72-78°C	3'-5'	High	240 minutes at 95°C

Most commonly used concentration of taq polymerase is 0.02 -0.03 unit/µl.

4. Deoxyribonucleotide triphosphates (dNTPs)

It is available as a mixture of deoxyadenosine triphosphates (dATP), deoxycytidine triphosphates (dCTP), deoxyguanosine triphosphates (dGTP) and deoxythymidine triphosphates (dTTP). These dNTPs attach to the free 3' hydroxyl group of the primer and form a complementary to the template strand.

All four dNTPs must present in equimolar concentrations for successful PCR if not, the fidelity of PCR can be affected. The optimum concentration range of dNTPs is around 50–200 µM. A higher concentration of DNTP adversely affects the accuracy of amplification by driving a higher rate of misincorporation by Taq polymerase whereas the lower concentration may affect the efficiency of PCR. Most of the

protocols often suggest using 200 μM of each dNTP. This amount would be sufficient to synthesize about 10 μg of PCR product.

5. **Buffer**

Buffer provides a suitable chemical environment for the DNA polymerase. Company supplying polymerase enzyme also supply buffer along with. Chemically it consists of Tris-HCl (pH 8.3), KCl, MgCl2, gelatin, Tween-20 and NP-40. Each chemical components play a significant role in PCR.

Tris HCl is a dipolar ionic buffer and its pH varies between about 6.8 and 8.3 with temperature so during PCR the pH will vary. In fact, Taq DNA polymerase has a higher fidelity at the lower pH values that occur at the higher temperatures of PCR.

KCl helps in annealing of primer with a template by reducing the repulsive force between negative charge of primer and template. Higher concentration facilitates nonspecific annealing of primer.

Magnesium is considered as one of the most critical components in the PCR as its concentration can affect the specificity and efficiency of the reaction. Taq DNA polymerase is dependent upon the presence of Mg^{2+} and shows its highest activity at around 1.2–1.3 mM free $Mg^{2+.}$ Standard PCR buffers contain 1.5 mM MgCl22. DNTP concentration affects free Mg^{2+} concentration due to equimolar binding between dNTPs and Mg^{2+}.

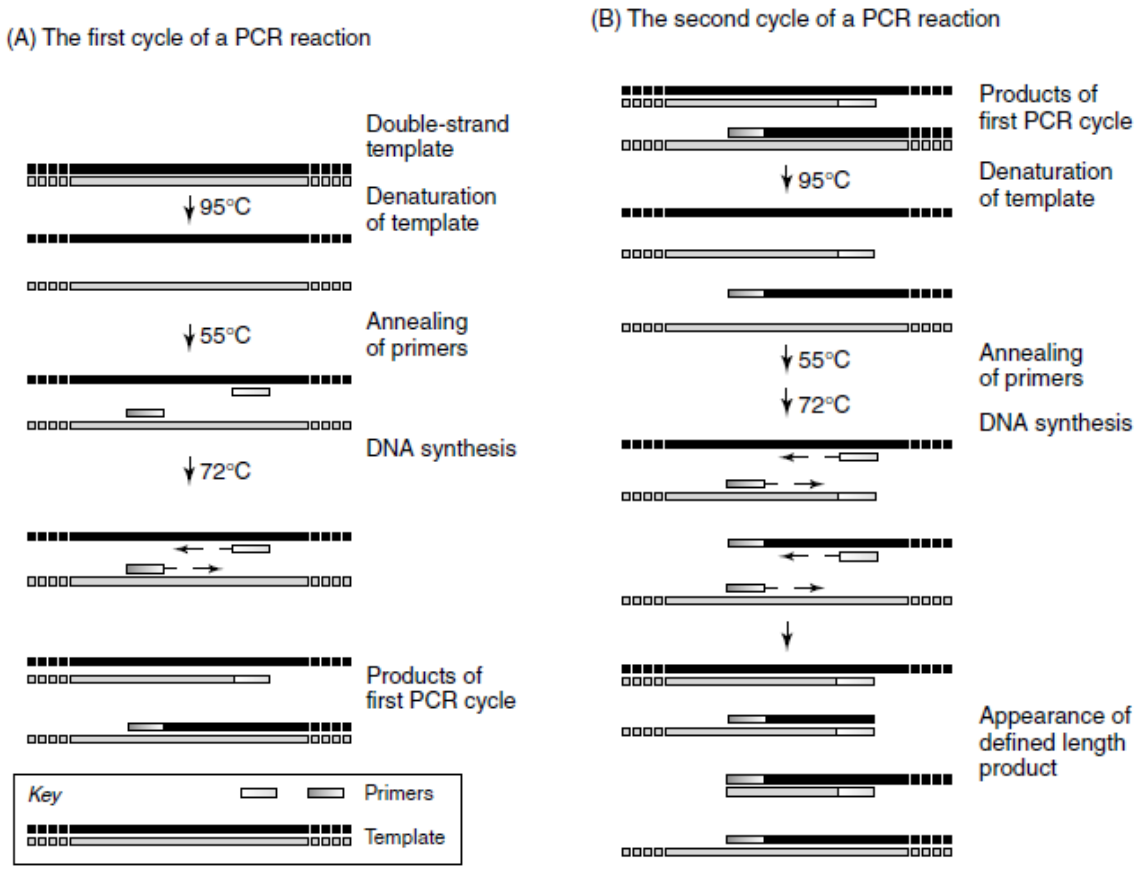
Steps in PCR

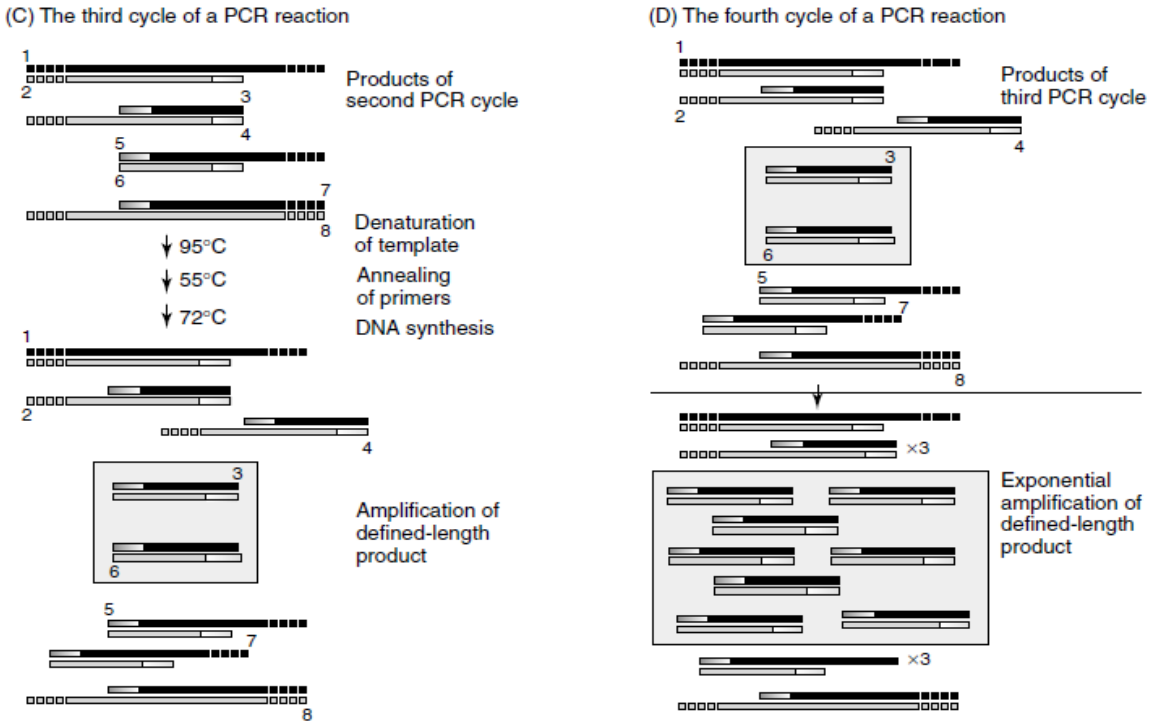
The PCR process involves three events- template denaturation by heat, primer annealing to the single stranded template and extension of the annealed primers forming daughter DNA strand by thermostable DNA polymerase. These events are repeated for several cycles to yield a large amount of product. Following diagrams shows the event happening during the initial four cycles.

A. **Denaturation:**

Higher temperature breaks the existing hydrogen bond between the complementary bases and generates single stranded DNA. Temperature is determined by the amount of G and C in the template. Higher the percentage, the higher will be the denaturation

temperature. Further more time is required to denature longer the DNA molecule and hence initial denaturation is performed for a longer duration. The temperature is also decided by the half-life of the polymerase to be used at that specific denaturation temperature. Most of the PCR involving *Taq* polymerase uses 94-95°C for 3-5 minutes in case of initial denaturation (denaturation in the 1st cycle of PCR) and 20-30 sec (increases if target size is larger) in subsequent cycles.





B. Annealing:

It includes binding of primer to the target and is critical for PCR. Higher annealing temperature leads to poor binding and hence yield will reduce. Whereas lower annealing temperature facilitates non-specific binding (binding to the non-target area) leads to non-specific amplification of unwanted segments of DNA. Annealing is usually carried out 3-5°C lower than the calculated *Tm* at which the primers dissociate from their templates. The annealing temperature can be optimized by gradient and touchdown PCR.

C. Extension or Elongation:

This step includes polymerization of nucleotide to the 3' end of primer to extend the daughter DNA strand by DNA polymerase. It is decided based on the type of DNA polymerase to be used in PCR. Most of the PCR involving Taq polymerase uses 72^0C for extension (above table). The polymerization rate of Taq polymerase is approximately 150 nucleotides per second at the optimal temperature and a thumb rule extension is carried out for 1 minute for every 1000 bp of product.

PCR involves many cycles each starting with denaturation and goes up to elongation. The number of the cycle can vary but mostly 30-35 cycles are used. After all the cycles completed at the end 5-10 minutes at 72^0C is provided for a final extension to ensure the extension of any remaining single stranded or missing places.

D. Hold:

This step at 4–15°C for an indefinite time may be employed for short-term storage of the reaction.

Post PCR agarose gel electrophoresis is performed to analyze the PCR product along with standard size marker (also called ladder)

PCR reaction composition

PCR composition is the first step in designing reaction. Amount of different component can be calculated for any reaction volume by using the following table. It is important to note the concentration of the component of stock before using them.

Sr. No.	Components	Final concentration	25	50
1	10X buffer	1X	2.5	5
2	10mM dNTP	200uM	0.5	1
3	10uM forward primer	0.2 uM (range-0.1-0.5)	0.5	1
4	10uM reverse primer	0.2 uM (range-0.1-0.5)	0.5	1
5	Template DNA	1-2ng/ul	Depends on DNA concentration in sample	Depends on DNA concentration in sample
6	*Taq* DNA polymerase	0.02-0.03 unit/ul	0.5-0.75 unit	1-1.5 unit
7	Nuclease free water	To make final volume	To make final volume 25	To make final volume 50

Reaction conditions:

Steps	Conditions		Cycles
	Temperature	Time	
Initial denaturation	94°C	3 min.	1 cycle
Denaturation	94°C	30 sec.	35cycles
Annealing	54°C * can vary (depends on the annealing temperature of primer)	20-30 sec.	
Extension	72°C	Depends on product size (generally 1sec for 150 bp.)	
Final Extension	72°C	5 min.	1 cycle
Hold	4°C	Forever	

NOTE:

Inclusion of control reactions is essential for monitoring the success of PCR reactions. Each set of PCR must always include positive and negative controls. Positive controls are required to monitor the efficiency of the PCR, whereas negative controls are required to detect contamination with DNA that contains the target sequence.

Important consideration while performing PCR

- Keep to minimum traffic in and out of the laboratory area designated for assembly of PCR.
- Wear hand gloves when working in the area and change them frequently. Use face masks and head caps to reduce contamination from facial skin and hair cells.
- Make up your own sets of reagents and disposable items (including PCR tubes, mineral oil and wax beads). Use new glassware, plasticware and pipettes that have not been exposed to DNA in the laboratory to make up and store solutions. Store the enzymes and buffers in small aliquots in a designated section of a freezer located near the flow

hood. Discards aliquots of reagents after use. Never use PCR reagents for another purpose.

- Before opening microfuge tubes containing reagents, centrifuge them briefly (10 seconds). This centrifugation deposits the fluid in the base of the tube and reduces the possibility of contamination of gloves or pipetting devices.
- Prepare dilutions of DNA used as templates in PCR at your own laboratory bench and take as much of the dilution needed into the PCR area.

PCR Primer Designing

Introduction

Oligonucleotide primers are necessary when running a PCR reaction. One needs to design primers that are complementary to the template region of DNA. They are synthesized chemically by joining nucleotides together. One must selectively block and unblock repeatedly the reactive groups on a nucleotide when adding a nucleotide one at a time. The main property of primers is that they must correspond to sequences on the template molecule (must be complementary to template strand). However, primers do not need to correspond to the template strand completely; it is essential, however, that the 3' end of the primer corresponds completely to the template DNA strand so elongation can proceed. Usually, guanine or cytosine is used at the 3' end, and the 5' end of the primer usually has stretches of several nucleotides. Also, both of the 3' ends of the hybridized primers must point toward one another.

The size of the primer is very important as well. Short primers are mainly used for amplifying a small, simple fragment of DNA. On the other hand, a long primer is used to amplify a eukaryotic genomic DNA sample. However, a primer should not be too long (> 30-mer primers) or too short. Short primers produce inaccurate, nonspecific DNA amplification product, and long primers result in a slower hybridizing rate. On average, the DNA fragment that needs to be amplified should be within 1-10 kB in size.

The structure of the primer should be relatively simple and contain no internal secondary structure to avoid internal folding. One also needs to avoid primer-primer annealing which creates primer dimers and disrupts the amplification process. When designing, if unsure about what nucleotide to put at a certain position within the primer, one can include more than one nucleotide at that position termed a mixed site. One can also use a nucleotide-based molecular insert (inosine) instead of a regular nucleotide for broader pairing capabilities.

Important Consideration During Primer Designing

The primer you design impacts the entire DNA amplification process. DNA polymerases, the enzymes that catalyse DNA replication, can only initiate the replication process by adding nucleotides to primers. In order to produce the desired DNA sequence, you must start with the right primer. Thus, proper primer design is necessary for successful DNA amplification. Here are 14 basic guidelines for constructing primers:

- Primers are always specified 5' to 3', left to right. Verify that your primers are designed and ordered in the correct orientation.
- Primers for PCR and sequencing should be between 18 to 25 nucleotides in length.
- Primers for PCR and sequencing should have a GC content between 40 and 60%, with the 3′ of a primer ending in C or G to promote binding.
- The 3' end of the primer should be an exact match to the template DNA, because extension by DNA polymerase, during PCR, depends on a good match at the 3' end.
- In the last 5 bases at the 3′ end of the primer, make sure that there are at least 2 G or C bases (GC clamp). G-C base pairs have a stronger bond than A-T base pairs (3 hydrogen bonds versus 2).
- Where a restriction site has been added onto the end of a primer, typically, 5-6 nucleotides are added 5’ of the restriction enzyme site (aka a “leader sequence”) in the primer to allow for efficient cutting.
- Try to avoid runs of 4 or more of one base or dinucleotide repeats (for example, ACCCC or ATATATAT) as this can cause primer mispriming.
- Avoid regions of secondary structure; namely intra-primer homology (more than 3 bases that complement within the primer) or inter-primer homology (forward and reverse primers having complementary sequences). These circumstances can lead to self-dimers/hairpins or primer-dimers instead of annealing to the desired DNA sequences. Thus have a balanced distribution of GC-rich and AT-rich domains. Tools like IDT Oligo Analyzer can help you to detect secondary structure.
- In general, the ΔG value for dimer analysis should be between 0 to −9 kcal/mole for optimal design. Values more negative than this may adversely affect PCR reactions.

- For hairpins, the melting temperature, (Tm) of the hairpin should be lower than the annealing temperature for the reaction; on average it should range between 55°C and 65°C. The Tm for the strongest hairpin should be at the very least 50°C and below the annealing temperature. If the Tm of your primer is very low, try to find a sequence with more GC content, or extend the length of the primer a little.
- Primer pairs should have similar Tm's with a maximum difference of 5°C and should not be complementary to each other.
- If you are using the primers for mutagenesis, try to have the mismatched bases towards the middle of the primer.
- Verify your primers' specificity so they won't bind to other genomic regions through NCBI Primer BLAST or UCSC in-silico PCR.
- In order to verify the Tm, try an annealing temperature gradient PCR reaction to find the optimal Tm according to your primer and enzyme.

Note: *If you will be including a restriction site at the 5' end of your primer, note that a 3-6 base pair "clamp" should be added upstream in order for the enzyme to cleave efficiently (e.g. GCGGCG-restriction site-your sequence).*

Primer Designing Tools

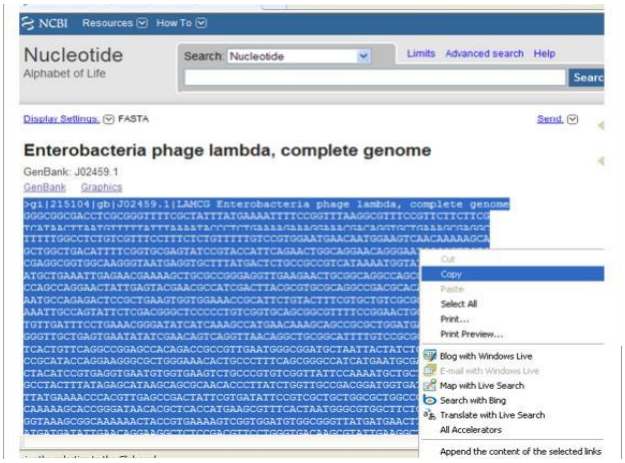
- Genome Compiler - Automatic and manual primer design, primers' inventory management, automatic detection and attachment of complementary primers to your project's sequence.
- SciTools OligoAnalyzer by IDT - a relatively complete suite of online tools for primer analysis
- PerlPrimer - Open-source, downloadable PCR primer design software
- Primer3 - Open-source PCR primer design software. Offers both downloadable and web versions
- Primer-BLAST - Web software for designing primers that combines features of both Primer3 and BLAST.
- PrimerX - Web software for designing primers for site directed mutagenesis.
- Primer3 multiple primer design Web SW for designing overlapping 600 bp pcr primers for long sequences.
- VectorNTI

Use the Right Tools

On the Genome Compiler platform, there are many useful tools that allow you to uniquely design your primers to fit your specific needs. With Primer3 algorithms embedded into the software, capabilities range from auto designing detection primers, cloning primers, and soon sequencing primers to tailoring the design of your own primers with Genome Compiler's manual primer design feature. Additionally, Genome Compiler has step by step wizards to assist with primer and oligo design for Restriction Ligation and Gibson Assembly. Throughout the design process, Genome Compiler will also compile any design errors such as too high or too low GC content, or an undesirable Tm etc., with direct connections to IDT oligo analyser to check for hetero/homo dimer formation. Taking advantage of these helpful tools will simplify your primer design process and reduce the risk of errors.

Standard Primer Design

Aim of the experiment: Design primers for cellulose gene from *Acetobacter*



1. Open NCBI
2. Download the FASTA format of cellulose gene sequence for Acetobacter sp
3. Copy the sequence by selecting the text as shown.

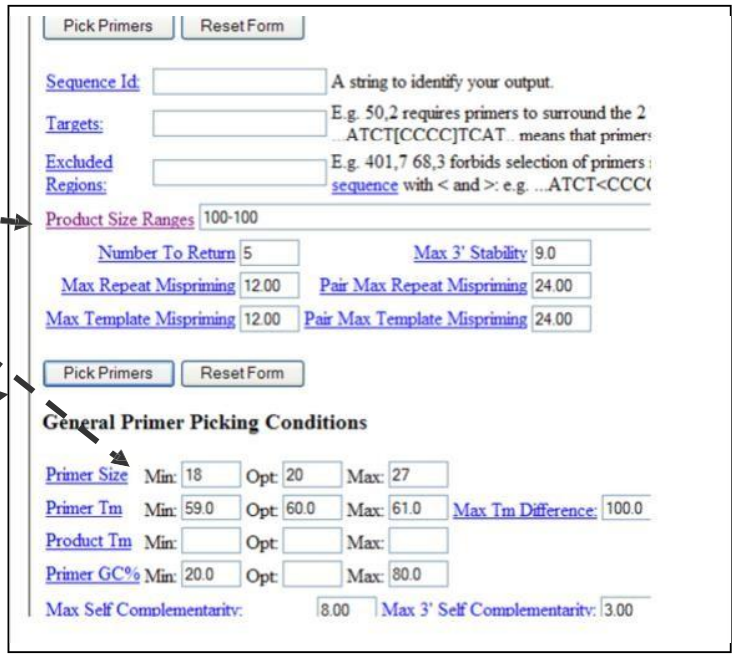
4. Go to the "Primer 3" program

http://frodo.wi.mit.edu/primer3/

Paste your sequence in the main window as shown

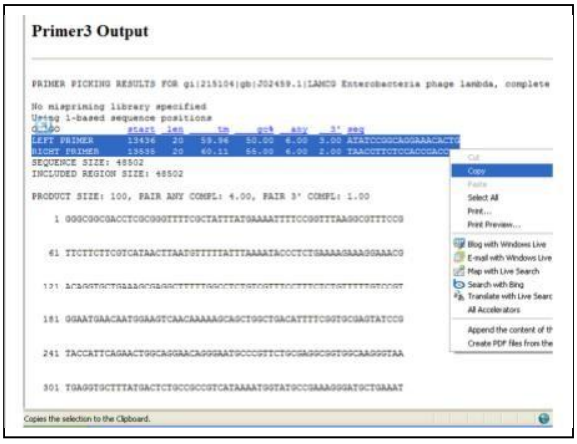
Enter YOUR needed product size as shown, then enter the Tm as a range from 59.0 60.0 and 61.0 as shown

Select “PICK PRIMERS”
The product size shown is for 100 bp; the 100-100 values indicate a minimum and maximum size. By selecting the same number, you are forcing the program to pick that exact size.



The Primer Tm is a narrow range from 59.0 to 61.0 degrees. By using such a narrow Tm, we are attempting to create a series of primers that will all anneal at the same Tm, thus improving throughput when we perform PCR.

5. If the program was able to design primers, copy the sequences below and these are your primers of interest



6. Check the specificity of the primers using Primer BLAST and ePCR

DNA Quantification Using Nanodrop

Introduction

DNA quantification is a basic requirement before PCR, sequencing, cloning or any downstream procedure. Nanodrop spectrophotometer is the fastest and simplest method to check both the **concentration** and **purity** of DNA using only **1–2 µl** of sample.

Principle

Nanodrop works on UV absorbance. DNA absorbs UV light strongly at **260 nm** due to the presence of nitrogenous bases. Purity is checked by measuring absorbance ratios:

- **A260/A280** → indicates protein contamination
  • Pure DNA ratio = **1.8**
- **A260/A230** → indicates organic/salt contamination
  • Pure DNA ratio = **> 2.0**

Requirements

- Nanodrop spectrophotometer
- 1X TE buffer or nuclease-free water
- Clean Kimwipes
- DNA sample (1–2 µl)

Procedure

1. Switch on the Nanodrop and select "Nucleic Acid" program.
2. Clean both upper and lower pedestals with lint-free tissue.
3. Pipette **1–2 µl** of TE buffer or water and blank the instrument.
4. Wipe the pedestal and load **1–2 µl** of DNA sample.
5. Close the arm and click "Measure".
6. Record the concentration (ng/µl) and purity ratios.
7. Clean the pedestal after every reading.

Interpretation

- **Concentration:**
  Shows how much DNA is present in ng/µl.
- **Purity:**
  - A260/A280 ≈ **1.8** → pure
  - < 1.7 → protein contamination
  - 2.0 → RNA contamination
  - A260/A230 < 2.0 → salts/phenol present

Common Errors & Solutions

Error	Reason	Fix
Low A260/A280	Protein / phenol	Re-precipitate DNA
Low A260/A230	Chaotropic salts	Extra ethanol wash
Very high reading	Dirty pedestal	Clean properly
DNA too low	Dilute sample / pipetting error	Repeat

Gel Documentation System (Gel Doc) Imaging

Introduction
After agarose gel electrophoresis, DNA bands are visualized using a gel documentation system. It helps capture high-quality images for records, publications, and reports.

Principle
Gel Doc uses UV or blue light and a high-resolution digital camera. DNA stained with dyes such as Ethidium Bromide or SYBR Safe emits fluorescence when exposed to light, producing visible bands.

Requirements
- Gel documentation system
- Agarose gel with DNA
- Protective goggles
- Computer software (Bio-Rad Image Lab or similar)

Procedure
1. Switch on the Gel Doc system.
2. Place the gel carefully on the UV/blue transilluminator surface.
3. Close the chamber lid to prevent UV exposure.
4. Select dye type (EtBr/SYBR) in software.
5. Adjust exposure time:
   • Low DNA → longer exposure
   • High DNA → shorter exposure
6. Capture the image.
7. Save the file in TIFF (high quality) and JPEG (report-ready) formats.
8. Generate molecular weight measurements if required.

Safety Notes
- UV light can damage skin and eyes—never open chamber during UV exposure.
- Handle gels with gloves; EtBr is a mutagen.

Troubleshooting

Problem	Cause	Solution
Weak bands	Low DNA / low dye	Increase exposure, restain gel
Smearing	Overloaded sample / degraded DNA	Reduce DNA amount, use fresh sample
Bright background	Too much dye	Reduce stain, destain gel if needed
Uneven bands	Gel not level / incorrect voltage	Place gel properly, use correct voltage

Applications
- PCR band confirmation
- Restriction digestion analysis
- Checking DNA extraction quality
- Gel-based quantification
- Teaching and training demonstrations

Biological Databases

Introduction

Biological databases are online collections of biological information such as **DNA sequences, protein sequences, gene functions, genomes, structures, pathways**, and more. These databases help researchers quickly find, compare, and analyse biological data without doing experiments again and again.

They are the **backbone of bioinformatics** and are used in almost every research activity genome analysis, gene prediction, drug discovery, evolutionary studies, and molecular biology experiments.

Types of Biological Databases

Biological databases are mainly divided into **three groups**:

1. Primary Databases (Raw data)

These store experimentally generated data such as DNA, RNA, and protein sequences.

Examples:

- **GenBank** (NCBI) – DNA sequences
- **EMBL-EBI** – European molecular database
- **DDBJ** – Japanese DNA database
- **UniProtKB** – Protein sequence database

Use: For searching sequences, downloading FASTA files, and BLAST searches.

2. Secondary Databases (Annotated / processed data)

These databases add interpretation, prediction, and extra information on top of primary data.

Examples:

- **Pfam** – Protein domains
- **InterPro** – Protein families and functional domains
- **Prosite** – Motifs and signatures
- **Swiss-Prot** – Manually curated protein information

Use: To understand protein structure, domains, motifs, and functions.

3. Specialized Databases

These focus on a specific organism, pathway, disease, or dataset.

Examples:

- **PDB** – 3D structures of proteins
- **KEGG** – Pathways, metabolism, diseases
- **ZFIN** – Zebrafish genomic database
- **Ensembl Genome Browser** – Genome-level information
- **FishBase** – Information on fish species
- **ENSEMBL Metazoa / NCBI Genome** – Fish genomes (trout, carp, salmon, etc.)

Use: Drug discovery, protein modeling, disease studies, pathways, comparative genomics.

Important Databases and Their Uses

1. NCBI (National Center for Biotechnology Information)

NCBI is the most commonly used database for:

- DNA and protein sequences
- BLAST searching
- Gene annotation
- Genome records

Popular NCBI tools:

- **GenBank** – DNA sequences
- **BLAST** – Sequence similarity search
- **PubMed** – Research papers
- **Protein** – Protein sequence information
- **GEO** – Gene expression datasets

2. UniProt

The world's most trusted protein database.

Sections:

- **Swiss-Prot** – Manually curated
- **TrEMBL** – Automatically annotated

Gives:

- Protein sequence
- Function
- Domains
- Localization

- Pathways

3. PDB (Protein Data Bank)

Used for:

- 3D structure of proteins
- Molecular modeling
- Docking studies
- Structural biology

Files stored in **.pdb format**.

4. KEGG (Kyoto Encyclopedia of Genes and Genomes)

Used for:

- Metabolic pathways
- Disease pathways
- Drug interactions
- Functional annotation
- Linking genes to biological processes

Very useful for enrichment and pathway analysis.

5. Ensembl

Ensembl provides complete **genome-level information**.

Useful for:

- Gene locations
- Exon–intron structure
- Variants (SNPs)
- Comparative genomics

Fish genomes like **rainbow trout, Atlantic salmon, zebrafish**, etc. are available here.

6. Fish-Specific Databases

These are very useful for fisheries and fish biotechnology.

- **FishBase** – Fish taxonomy, distribution
- **ZFIN** – Zebrafish genetics and genomics

- **FAO Fisheries Data** – Production statistics
- **SalmonBase / TroutBase** – Salmonid genomic datasets

Applications of Biological Databases

Biological databases are used for:

- Designing primers (using NCBI, Ensembl)
- Identifying genes and proteins
- Checking functional domains (SMART, InterPro, Pfam)
- Homology modeling
- Molecular docking and simulation
- Genome annotation
- Comparative genomics
- Disease gene prediction
- Evolutionary studies

Example Workflow

If you are studying a **gene in rainbow trout**:

1. Search gene in **NCBI or Ensembl**
2. Download **FASTA sequence**
3. Check domains in **Pfam/InterPro**
4. View pathways in **KEGG**
5. Find 3D structure in **PDB** or predict using AlphaFold
6. Use for docking or simulation

Summary

Biological databases store essential biological information and help in fast, accurate, and large-scale analysis. Modern research depends heavily on these databases, and understanding them is crucial for genomics, proteomics, bioinformatics, and molecular biology.

RNA Isolation

This method uses a monophasic solution of **phenol + guanidinium thiocyanate (TRIzol)** to lyse cells, inactivate RNases, and separate RNA from DNA and proteins.

Materials

Chemical / Reagent	**Purpose**
TRIzol reagent (or RNAzol)	Lyses cells, dissolves cellular components & inactivates RNases
Chloroform	Helps in phase separation to isolate RNA in aqueous layer
Isopropanol	Precipitates RNA from aqueous phase
75% Ethanol prepared in DEPC Water	Washes pellet to remove salts & impurities
Nuclease-free water (NFW)	Dissolves final RNA pellet

Procedure

Step- 1 Tissue Homogenization

- Take 50–100 mg tissue
- Add 600–1000 µL TRIzol
- Vortex 30 sec to homogenize
- Incubate 5 min at room temperature (RT)

Reason: TRIzol breaks cell membranes and denatures proteins including RNases to protect RNA.

Step- 2 Phase Separation

- Add 0.2 mL chloroform per 1 mL TRIzol
- Vortex vigorously 15–20 sec
- Incubate 5 min at RT
- Centrifuge 12,000 rpm for 15 min at 4°C

You will see three phases:

- Aqueous phase (top): RNA
- Interphase: DNA
- Organic phase (bottom): Proteins + lipids

Carefully transfer the aqueous phase to a new RNase-free tube.

Reason: Chloroform helps separate RNA (water-soluble) from DNA and proteins (remain in organic phase)

Step- 3 RNA Precipitation

- Add equal volume of Isopropanol (i.e., ~500 µL for every 1 mL TRIzol used)
- Mix gently 5–6 times
- Incubate 10–15 min at RT
- Centrifuge 12,000 rpm for 10 min at 4°C

A white RNA pellet will form.

Reason: Isopropanol reduces RNA solubility → RNA precipitates so it can be collected.

Step- 4 RNA Washing

- Remove supernatant carefully
- Add 1 mL 75% Ethanol
- Vortex gently
- Centrifuge 9,000 rpm for 5 min at 4°C
- Discard ethanol completely

Reason: Ethanol removes salts and phenol contaminants without dissolving RNA.

Step- 5 Drying and Solubilizing RNA

- Air dry 5–10 min (don't over-dry)
- Add 10–25 µL nuclease-free water
- Flick or pipette gently to dissolve RNA

Reason: NFW ensures RNA stays RNase-free for stable storage and use.

Step- 6 Storage

- Store purified RNA at –80°C

➢ **Output**

• High-quality RNA suitable for RT-PCR, qPCR, cDNA synthesis, RNA-Seq, etc.

Quick Summary

Step	Reagent	Why it's used
Cell lysis	TRIzol	Denatures proteins, inactivates RNases
Phase separation	Chloroform	Separates RNA from DNA & proteins
Precipitation	Isopropanol	Concentrates RNA into pellet
Washing	75% Ethanol	Removes salts + phenol
Dissolving	NFW	Maintains RNA stability & purity

cDNA (complementary DNA) synthesis

Principle

- cDNA (complementary DNA) is synthesized from **mRNA or total RNA** using the enzyme **reverse transcriptase**.

  This is the first step before PCR, qPCR, etc.

Materials / Reagents

- RNA template
  - Total RNA or mRNA (good quality, no degradation)
- Reverse transcriptase enzyme (e.g., M-MLV, AMV, etc.)
- Primer (anyone or combination):
  - Oligo(dT) primer → binds poly(A) tail of mRNA
  - Random hexamer → binds at many points on RNA
  - Gene-specific primer → for one particular gene
- dNTP mix (10 mM each dATP, dTTP, dCTP, dGTP)
- RT buffer (10X) – supplied with enzyme
- $MgCl_2$ (often included in buffer)
- RNase inhibitor
- Nuclease-free water
- Optional: RNase H (to remove RNA after cDNA synthesis)

Example reaction setup (20 µL total volume)

Step 1 – Mix RNA + Primer + dNTPs

In a sterile, RNase-free PCR tube:

- RNA: 1 µg total RNA (adjust volume as per concentration)
- Primer (e.g. oligo dT, 0.5–1 µg) or random hexamer (2 µL, 50 µM)
- dNTP mix (10 mM each): 1 µL
- Nuclease-free water: up to 10 µL

Procedure:

1. Mix gently.

2. Heat at **65°C for 5 min** (to denature secondary structures in RNA).
3. Immediately chill on **ice for at least 1 min**.

Step 2 – Add RT master mix

Prepare on ice:

- 5X RT buffer: 4 µL
- RNase inhibitor (20–40 U/µL): 0.5–1 µL
- Reverse transcriptase (e.g., 200 U/µL): 1 µL
- Nuclease-free water: add to bring final volume to 20 µL

Add this 10 µL RT mix to the 10 µL RNA/primer mix from Step 1 → total 20 µL.

Mix gently, spin down briefly.

Incubation conditions

Typical program (check kit instructions for exact temps):

1. **Primer annealing** (needed mainly for random primers):
    - 25°C for 5–10 min (optional, for random hexamer)
2. **cDNA synthesis (extension):**
    - 42–50°C for 30–60 min

      (AMV often at 42–50°C; some engineered RTs can go up to 55°C)
3. **Enzyme inactivation:**
    - 70°C for 10–15 min

Cool to 4°C or put on ice.

After cDNA synthesis

- Your cDNA is now ready.
- You can:
    - Use 1–2 µL as template for PCR or qPCR.
    - Store remaining cDNA at –20°C (short term) or –80°C (long term).

Important precautions

- Always use RNase-free tips, tubes, water.
- Keep RNA and enzymes on ice while setting up.

- Check RNA quality (A260/280 ~1.8–2.0; clear bands on agarose gel).
- Include a –RT control (no reverse transcriptase) to check for genomic DNA contamination.

Plasmid Isolation

Buffers Required

Buffer P1: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/mL RNase A (stored at 4°C)

Buffer P2: 0.2 N NaOH, 1% SDS

Buffer P3: 3 M Potassium acetate, pH 5.5

Procedure

1. Inoculation

A single fresh bacterial colony containing the plasmid of interest is inoculated into 5 mL LB broth supplemented with the appropriate antibiotic. The culture is incubated overnight at 37°C with shaking. The next day, the culture is centrifuged at maximum speed (≈ 10,000 rpm) for 1 minute at room temperature to pellet the bacterial cells. The supernatant is discarded completely.

2. Cell Lysis

The cell pellet is resuspended thoroughly in 350 µL of Buffer P1 ensuring no clumps remain.

Next, 350 µL of Buffer P2 is added, and the tube is gently inverted several times to mix (do not vortex). The mixture is incubated at room temperature for up to 5 minutes to lyse the cells. Over-incubation should be avoided to prevent chromosomal DNA contamination.

Then 400 µL of Buffer P3 is added, followed by gentle inversion to mix. A white precipitate forms immediately indicating neutralization. The sample is centrifuged at maximum speed (≈ 12,000–14,000 rpm) for 10 minutes at room temperature. The supernatant containing plasmid DNA is carefully transferred to a fresh tube without disturbing the pellet.

3. Isopropanol Precipitation

To the collected supernatant, an equal volume of isopropanol is added and mixed gently. The tube is incubated for 2 minutes at room temperature (incubation can be extended up to 1 hour on ice if needed for betteryield).

Samples are centrifuged for 5 minutes at maximum speed to pellet the nucleic acids. The supernatant is discarded carefully without disturbing the pellet.

The pellet is washed by adding 500 µL of chilled 70% ethanol and centrifuged again for 5 minutes.

Ethanol is completely removed, and the pellet is air dried for 5–10 minutes at room temperature.

4. **DNA Recovery**

The dried DNA pellet is dissolved in 50–100 µL of nuclease-free water or TE buffer. For better solubilization of DNA, samples may be incubated at 65°C for 10 minutes (optional). The extracted plasmid DNA can be stored at −20°C for long-term use.

(Optional) Additional DNA Precipitation Using Lithium Chloride

After the isopropanol precipitation step, the pellet may be dissolved in boiled water and 0.4 volumes of LiCl can be added. The sample is kept for 20 minutes at −20°C and then centrifuged at 11,000 rpm for 10 minutes at 4°C. The pellet is washed and dried as described above for increased DNA purity.

5. **Storage**

Store the final plasmid DNA at −20°C until use.

Transformation Protocol

Procedure

1. Competent *E. coli* cells stored at −80°C are taken out and placed on ice for 20–30 minutes to thaw slowly.
2. Select LB agar plates containing the appropriate antibiotic from storage at 4°C. Allow them to warm to room temperature and, if required, pre-incubate briefly at 37°C so that the plates are dry and ready for plating.
3. Add 1–5 µL of plasmid DNA (usually 10 pg – 100 ng) into 20–50 µL of competent cells in a pre-chilled microcentrifuge tube. Mix gently by tapping or flicking the tube; do not vortex to avoid damaging the cells.
4. Incubate the DNA-competent cell mixture on ice for 20–30 minutes to allow DNA binding to the cell surface.
5. Perform heat shock by placing the bottom two-thirds of the tube into a 42°C water bath for 30–60 seconds (45 seconds is usually ideal). The exact time may vary depending on the cell strain used.
6. Immediately transfer the tubes back onto ice for 2 minutes to stabilize the cells and allow membrane closure.
7. Add 250–1,000 µL of LB or SOC medium without antibiotic to the tube. Incubate at 37°C for 45 minutes in a shaking incubator to allow the bacteria to recover and express the antibiotic resistance gene. *Note: This outgrowth step is especially critical when using antibiotics other than ampicillin.*
8. Plate appropriate volumes (e.g., 50–100 µL) of the transformation mixture onto LB agar plates containing the required antibiotic.
    - It is recommended to plate small and large volumes on separate plates to ensure colony recovery.
    - If the culture volume is too large, pellet cells by brief centrifugation, resuspend in a small volume of LB, and then plate.
    - Ensure the agar surface is not too wet; otherwise, bacterial growth may be uneven or diffuse.
9. Incubate the plates overnight at 37°C to allow colony formation.