Swayam Course - Analytical Techniques

Week: 10, Tutorial 26 - Methods for isolation, purification, quantification, and integrity check of DNA

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Objectives

- To understand the structure of DNA and its relevance to the DNA isolation.
- To state the basic process of isolation of DNA from various sources - blood, tissue, cultured cells.
- To understand that different types of DNA require different methods of isolation and the method used is dependent upon the final application.
- To explain the visualisation of DNA by gel electrophoresis
- To know the principles of quantification of DNA by spectrophotometry

Introduction

DNA (deoxyribonucleic acid) is the genetic material in all the living organisms. DNA is present in all the nucleated cells. Most of the DNA is in the cell nucleus (nuclear DNA), but a small amount of DNA can also be found in the mitochondria (mitochondrial DNA or mtDNA). In plants, chloroplast also contains DNA. Some amount of DNA is released into the blood from cells undergoing apoptosis. This DNA is known circulating free DNA (cfDNA).

DNA is the starting material for many downstream procedures like Polymerase chain reaction, sequencing and restriction digestion. If the DNA quality is not good, it leads to waste of time and resources. So, it is important to isolate a good quality DNA. In this tutorial, we will learn the methods for isolation, purification and quantification of DNA.

1. Structure of DNA and its relevance to DNA isolation, separation and quantification

1.1. Absence of 2’ OH group

DNA is a polynucleotide of 2’-deoxyribonucleotide. Absence of 2’ hydroxyl group makes DNA more stable compared to RNA. 2’ OH group is responsible for the spontaneous alkaline hydrolysis of RNA. Therefore, DNA isolation is done under slightly alkaline conditions whereas RNA isolation is done under slightly alkaline condition.

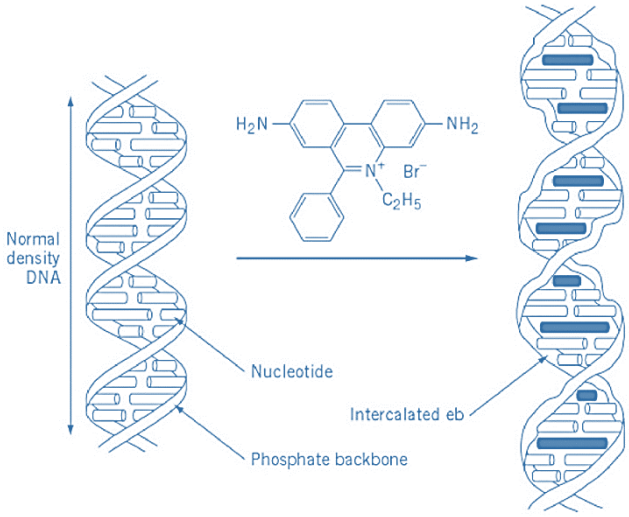
1.2. Polar and Negatively charged

DNA is a double helical molecule with bases inside the helix and the sugar phosphate backbone outside. This gives a uniform negative charge over DNA molecule. This can explain the following two things:

- DNA is water-soluble and separates into aqueous phase in organic extraction method.
- During electrophoresis, DNA moves towards the anode.

1.3. Bases in the DNA are stacked over each other

Hydrogen bonds hold the two strands of DNA together and the bases are stacked over each other. Intercalating agents like ethidium bromide can bind in between (intercalate) the bases. This property is used to visualise the DNA after electrophoresis.



1.4. Bases in DNA absorb UV light

Conjugated ring system present in nitrogenous bases of DNA absorb UV light with absorbance maxima at 260 nm. This is the basis of quantification of DNA which we will discuss later.

2. METHODS OF DNA EXTRACTION

Methods of DNA extraction is broadly classified based liquid phase and solid phase methods. Liquid phase method is of two types whether there is use of organic chemicals or not. Based on the solid phase used, there are 3 different types of solid phase methods.

Types of Liquid Phase extraction methods:

1. Organic (phenol-chloroform) extraction
2. Non-organic (proteinase K and salting out) extraction

Types of Solid Phase extraction methods:

1. Silica based method
2. Magnetic bead method
3. Chelex (ion exchange resin) extraction

3. General steps of DNA extraction

The following is the general scheme of most of the DNA extraction methods mentioned above.

1. Cell lysis: Disruption of the cell membrane and nuclear membrane.
2. Removal of cellular proteins, lipids and polysaccharides
3. RNAse digestion to remove RNA
4. Precipitating the DNA with ethanol
5. Quantitation of DNA using spectrophotometer
6. Integrity check using gel electrophoresis

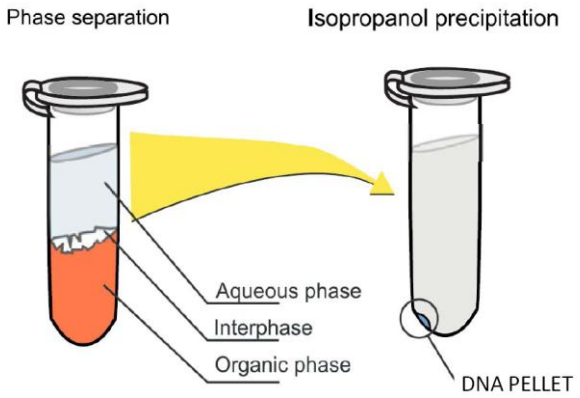
4. Principle of organic (Phenol-chloroform) DNA extraction

The term “organic” in this method refers to the use of organic solvents – phenol and chloroform.

In this method, the cells are first lysed by using lysis buffer containing proteinase K and detergent at a high temperature. This split opens the cell and nuclear membrane. Now, proteins, lipids, polysaccharides and DNA are there in an aqueous phase. We want only DNA. So, we need to remove all the other macromolecules.

So, to the above aqueous mix, we add equal volume of saturated phenol: chloroform mixture. Water cannot mix with organic solvents. So, the addition of organic solvent to aqueous mixture leads to formation of two different phases. Phenol: chloroform is denser and settles down to form the lower organic phase (as shown in the image) whereas water forms the upper aqueous phase.

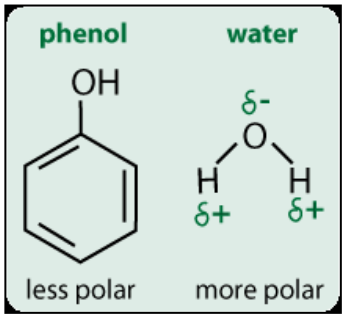
Lipids are soluble in organic phase. So, they settle down. As we have already learnt, the DNA is polar, negatively charged molecule. So, it will occupy the aqueous phase. What about proteins? Addition of phenol causes denaturation of proteins. Inside the cell, proteins exist in their correctly folded, functional state known as native state. Hydrophobic residues of the proteins are buried to the core of the protein to avoid contact with water. But, in this method, in vitro exposure of the organic solvent phenol forces the proteins to expose the hydrophobic core out. Thus, there is a “flipping out”

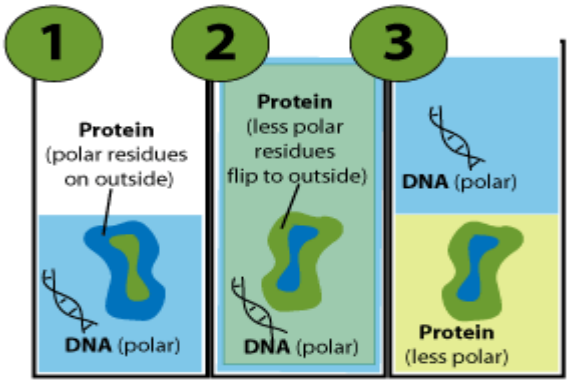


of proteins. This permanently denatures the proteins. So, they occupy the organic phase. This way of separation is known as “Phase separation” as it involves different phases.

Nearly the entire DNA is present in the aqueous phase and interphase and proteins in organic phase. The DNA in the upper aqueous phase is recovered by precipitation with ice-cold ethanol or isopropanol. After drying the DNA sample, it is reconstituted in nuclease free water or tris buffer and checked for quality and quantity.

[Note: Phenol/Chloroform/Isoamyl alcohol - 25:24:1]





DNA + Protein aqueous solution	Phenol added and vigorously mixed	P sep cen

4.1. COMPOSITION OF BUFFER

Lysis Buffer

1. NH_4Cl 8.29 g. [155 mM]

2. $KHCO_3$ 1 g [10 mM]

3. Na_2EDTA 0.034 g or 200 µl EDTA 0.5 M [0.1mM]

Fill to 1000 ml with distilled water.

Adjust to pH 7.4 with 1 M HCl or NaOH for each use.

Proteinase K (10mg/ml):

Dissolve 100 mg Proteinase K in 10 ml TE for 30 min at room temperature

Aliquot and store at –20°C

Detergent:

1. SDS
2. Triton-X 100

4.2. Functions of the buffer components

4.2.1. Ethylene Diamine Tetra Acetic acid (EDTA)

Nucleases require magnesium as cofactor. EDTA is a cation chelating agent used to chelate Mg2+ ions. It is important to note that excess magnesium inhibits the downstream processes like PCR.

4.2.2. Tris buffer

Tris(hydroxymethyl) aminomethane, commonly known as tris has the pKa of 8.08 at 25°C with buffering range of effective pH range between 7.1 and 9.1.

So, tris maintains slightly alkaline pH during DNA isolation.

4.2.3. SDS/Triton-X

Sodium dodecyl sulphate (SDS) is a strong anionic detergent. Detergents denature proteins which can be then better acted upon by Proteinase K.

4.2.4. Proteinase K

- Proteinase K is a serine protease with broad specificity.
- K in proteinase K stands for keratin because proteinase K was found to digest keratin in hair hence the name.
- It is active over a wide pH range (optimal activity between 6.5 and 9.5), under denaturing conditions (e.g., in the presence of SDS or urea) and in the presence of metal chelating agents (e.g., EDTA)
- It is active at comparatively high temperatures (optimum digestion temperature is 65°C) also.
- Proteinase K is added to digest the endonucleases and protect DNA.
- Degradation of cellular proteins for easy removal. Nucleases are broken down and rendered non-effective.

4.2.5. Role of the components (Phenol-chloroform extraction)

- Phenol – Denatures proteins.
- Chloroform - Dissolves lipids and removes excess phenol.
- Isoamyl alcohol — Antifoaming agent.
- 70% Ethanol - Removes excess salts.
- Isopropanol / Ethanol - Precipitates DNA

4.3. Advantages and Disadvantages of phenol-chloroform extraction method:

4.3.1. Advantages:

- Most effective at extracting large amounts of DNA.
- Used on a wide range of samples.

4.3.2. Disadvantages:

- Slow, labour-intensive, toxic (phenol, chloroform).
- Phenol is a corrosive and can cause chemical burns when the skin or mucosa is exposed.
- Fume hood is required.

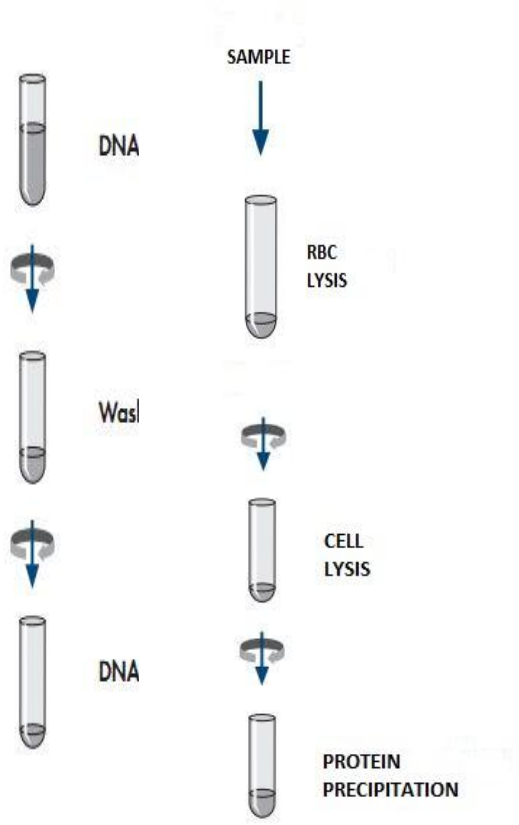
5. Liquid phase: non-organic (proteinase k and salting out) extraction:

5.1. Principle:

Cell membranes are lysed, and proteins are denatured using a detergent (such as SDS). RNA is removed with RNase. Proteins are precipitated with salt solution (Ammonium acetate, sodium chloride). DNA is precipitated with alcohol and rehydrated.

5.2. Model Protocol of DNA extraction using Non-organic (Proteinase K and Salting out) extraction

1. Dispense 450 µl of RBC Lysis Solution into a 1.5 ml microcentrifuge tube
2. Add 150 µl, whole blood and mix by inverting 10 times.
3. Incubate for 5 min, at room temperature (15–25°C). Invert at least once during the incubation.
4. Centrifuge for 2 mins at 13,000–16,000g, to pellet the white blood cells.
5. Carefully discard the supernatant by pipetting or pouring, leaving approximately 10 µl, of the residual liquid and the white blood cell pellet.
6. Add 150 µl, Cell Lysis Solution, vortex for 10sec.
7. Add 50 µl, Protein Precipitation Solution, vortex vigorously for 20 s at high speed.
8. Centrifuge for 2 min at 13,000–16,000 x g, the precipitated proteins form a dark brown pellet.
9. Pipet 300 µl isopropanol into a clean 1.5 ml tube, and add the supernatant from the previous step by pouring carefully
10. Mix by inverting gently 50 times until the DNA is visible as threads or a clump.
11. Centrifuge for 1 min at 13,000–16,000 x g
12. Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
13. Add 300 µL, of 70% ethanol and invert several times to wash the DNA pellet.
14. Centrifuge for 1 min at 13,000–16,000, carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
15. Air dry the pellet for 5 seconds and Add 50 µl, DNA Hydration Solution and vortex for 5 s at medium speed to mix.
16. Incubate at 65°C for 5 min, to dissolve the DNA.
17. Incubate at room temperature overnight with gentle shaking.

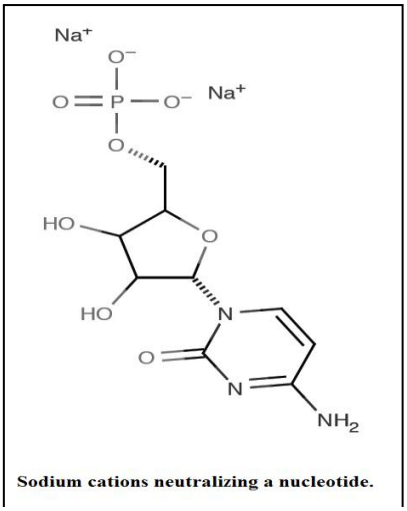


Composition of RBC Lysis solution:	Composition of Protein Precipitation solution

• Ammonium Chloride
• EDTA
• Potassium Bicarbonate
• Cell Lysis solution:
• Tris ammonium chloride
• EDTA
• SDS
• Triton-X
	• Ammonium acetate (Inorganic liquid method)
	• DNA Hydration Solution:
	  ○ Tris EDTA buffer pH 8.0
	  ○ 10 mM Tris
	  ○ 1 mM EDTA

5.3. Role of salt in DNA precipitation with ethanol:

The protease solution already contains salt. Positively charged sodium ions of sodium chloride bind to the negatively charged phosphate back bone of DNA molecules, neutralizing the electric charge of the DNA molecules. The addition of NaCl allows the DNA molecules to come together instead of repelling each other and reduces the hydrophilic nature of DNA thus making it easier for DNA to precipitate out of solution when alcohol is added.



Sodium cations neutralizing a nucleotide.

5.4. Advantages:

- Fast and easy method
- Uses nontoxic materials, no fume hood required, no hazardous materials disposal issues
- Produces high-quality DNA

5.5. Precautions to be followed during DNA extraction:

- Gentle handling to avoid shearing of the DNA
- All the plasticwares should be sterile
- Avoid using glass test tubes and glass rods as they are made of silica.
- Avoid DNA contamination from other sources

6. Gel electrophoresis of DNA

Electrophoresis is the process of movement of particles under the influence of charge and mass. As we have already discussed, due to the presence of the phosphodiester linkages, nucleic acids carry significant negative charge at physiologic pH. So, the DNA will move towards the positively charged electrode, i.e. the anode during gel electrophoresis.

6.1. Factors influencing migration of DNA through agarose gel:

- The size of the DNA
- The conformation (shape) of the DNA
- The concentration of agarose
- The applied voltage
- Ionic composition of the electrophoresis buffer

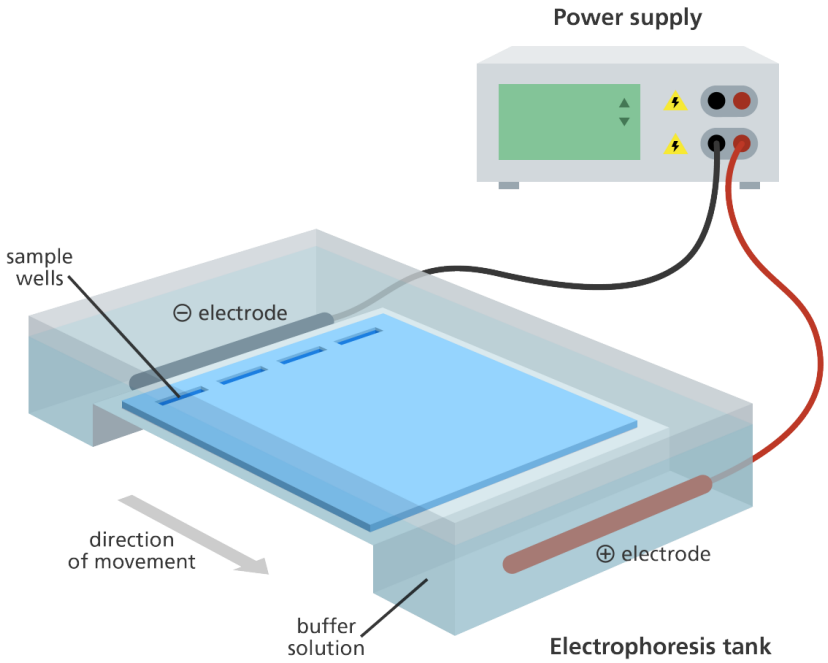
Size of the DNA: The smaller fragments move faster while the larger DNA fragments move slowly towards the anode.

Conformation of DNA: The resistance faced by the DNA in the agarose gel depends on the conformation of DNA. Supercoiled DNA contributes to less frictional resistance and so it moves fastest followed by liner DNA and nicked DNA.

Concentration of agarose: The pore size the agarose gel is inversely proportional to the concentration of the agarose. The recommended concentration of agarose based on the DNA size is given in the table below:

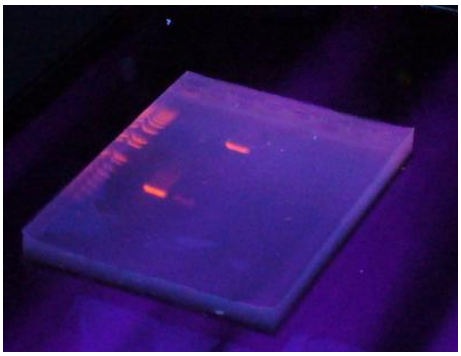
Optimum agarose concentration	Resolution (DNA size)
1.0	500–10,000 bp

1.2	400–7,000bp
1.5	200–3,000bp
2.0	50–2,000bp

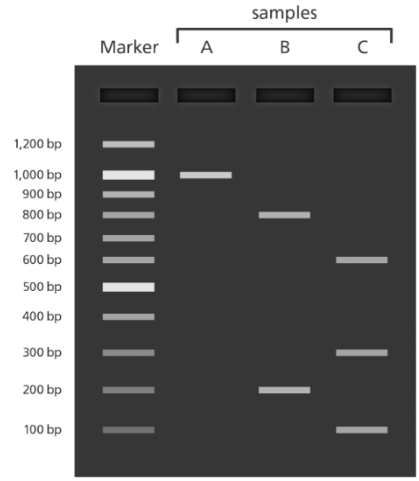


DNA samples are loaded into the wells made on an agarose or polyacrylamide gel. DNA is colourless. Then, how will we track the movement of DNA during electrophoresis and how will we visualise the DNA after electrophoresis?

1. During sample loading, we mix DNA with colored dyes like bromophenol blue. Movement of bromophenol blue helps in tracking the movement of DNA.
2. During gel preparation, we mix ethidium bromide to the agarose. Ethidium bromide intercalates with DNA. It fluoresces under UV light when it intercalates with DNA. So, after electrophoresis, we visualise the gels under UV light to look for the orange color fluorescence.



Visualisation of agarose gel under UV light. Look at the orange coloured fluorescence. By TransControl - English Wikipedia, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=2046526



Schematics of DNA electrophoresis of Marker and 3 samples. Marker is also known as DNA ladder. This is a mixture of DNA fragments of known size. This is used to find out the size of sample DNA by comparison.

6.2. Precaution:

- EtBr is carcinogenic. So, the agarose gel needs to be discarded properly after electrophoresis.

7. Quantification of DNA using spectrophotometry

We get to know about the integrity of DNA by electrophoresis. Knowing the exact quantity of DNA is important for any downstream applications like polymerase chain reaction, Sequencing. As we have already learnt, absorbance of UV light at 260 nm is the basis of DNA quantification. Nanodrop (commercial name) spectrophotometer is widely used for the estimation of DNA quantity.

7.1. Protocol for estimating DNA using Nanodrop:

1. Clean the sensor of the machine using a clean Kim wipe.
2. Open the software program in the computer. Click the nucleic acids tab.
3. Select – DNA-50
4. To blank the instrument, use the buffer in which DNA was eluted, e.g. TAE buffer
5. Load 1.5 microliter of DNA sample on the senor
6. Save the result displayed in the window
7. After measurement of all the samples, clean the sample entry port.

One A260 unit is the amount of nucleic acid contained in 1 mL and producing an OD of 1.

1 A260 unit of dsDNA = 50 µg
1 A260 unit of ssDNA = 33 µg
1 A260 unit of ssRNA = 40 µg

Recently, fluorometry based nucleic acid quantification techniques have emerged to be superior to nanodrop. Fluorometry based technique involves binding of specific nucleic acid binding dyes to the sample.

8. Plasmid DNA isolation by alkaline lysis

Plasmids are extrachromosomal, autonomously replicating circular DNA found in bacteria. Plasmids are not essential for the survival of the bacteria. They encode beneficial genes for bacteria like antibiotic resistance gene.

Isolation of plasmid DNA is the first step before restriction digestion, cloning, transfection and gene therapy etc. The challenge in plasmid DNA isolation is to get rid of the chromosomal DNA. So, we can't use the genomic DNA isolation method here. Plasmid isolation steps are explained briefly below.

8.1. Culture and Harvesting

The bacteria harbouring the desirable plasmid is grown in liquid culture. After achieving sufficient growth, bacteria are pelleted down by centrifugation.

8.2. Resuspension

Bacterial culture pellet is resuspended in Tris, EDTA, Glucose and RNAse A containing solution. We have already discussed the role of Tris, EDTA, and RNAse A. Glucose maintains the osmotic balance.

8.3. Alkaline lysis

Then the alkaline lysis buffer is added. This contains SDS and Sodium hydroxide. SDS solubilizes the membrane and denatures the proteins. Role of NaOH is crucial. It disrupts the hydrogen bonding between DNA, be it genomic or plasmid. Thus, all the DNA is converted into ssDNA.

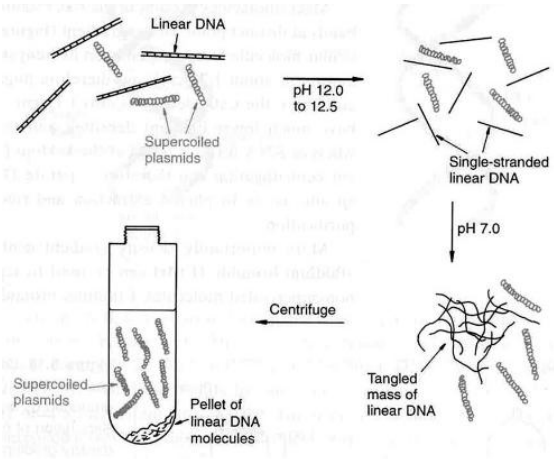
8.4. Neutralisation

Potassium acetate is added. This decreases the pH and helps to re-establish the hydrogen bonding between strands of DNA which were separated because of alkaline lysis. Genomic DNA is huge and renaturing of the whole genomic DNA is not possible, but it is easy for the small plasmid DNA to get renatured.

Double stranded plasmid DNA is soluble and come in the aqueous phase whereas single stranded genomic DNA, denatured proteins form a pellet because of hydrophobic interaction. These can be separated by centrifugation.

8.5. Purification and concentration

Plasmid DNA mixed with salt and debris is purified by phenol/chloroform extraction followed by ethanol precipitation or by affinity chromatography-based methods using a support that preferentially binds to the plasmid DNA.



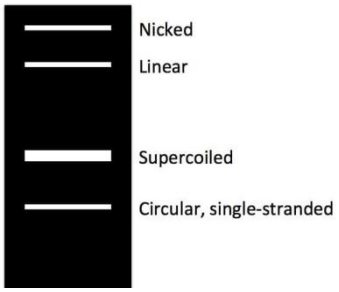
Schematic illustration of alkaline lysis

8.6. Visualisation of plasmid DNA

Plasmid DNA may appear in any one of the following five conformations.

Nicked open circular DNA is the DNA with cut in one of the stands. Relaxed circular DNA is fully intact without any cuts but without supercoils, i.e. relaxed. Linearised DNA is the one with free ends. Supercoiled DNA (covalently closed circular DNA) is fully intact with both strands uncut and supercoiled. Supercoiled denatured DNA is the minor fraction. It is similar to supercoiled DNA with unpaired regions that make it slightly less compact.

The electrophoretic pattern of the five fractions is illustrated in the image below:



9. Applications of DNA extraction
   The isolated DNA can be used for various downstream procedure. Some of the potential application of DNA isolation is given below:
   1. Diagnostic purposes: e.g. Mutation analysis in sickle cell anemia.
   2. Forensic applications
   3. Constructing genomic libraries
   4. Detect genomic polymorphisms
   5. Genetic engineering

10. Summary
    - DNA isolation is done under slightly alkaline conditions whereas RNA isolation is done under slightly alkaline condition.
    - During electrophoresis, DNA moves towards the anode.
    - Conjugated ring system present in nitrogenous bases absorb UV light at 260 nm.
    - Intercalating agents like ethidium bromide can bind in between (intercalate) the bases. This property is used to visualise the DNA after electrophoresis.
    - K in proteinase K stands for keratin because proteinase K was found to digest keratin in hair hence the name.
    - For pure DNA, 260/280 ratio is 1.8 to 2 and $OD_{260}/OD_{230} > 2.0$
    - Plasmid DNA isolation is done by alkaline lysis method.

References & Further Reading

- Wilson and Walker's Principles and Techniques of Biochemistry and Molecular Biology - Andreas Hofmann, Samuel Clokie, Cambridge University Press; Eighth edition (2018)
- Molecular Cell Biology, Lodish, WH Freeman; 8th edition (2016)
- Gene Cloning and DNA Analysis: An Introduction, TA Brown, Wiley-Blackwell
- The Isolation of DNA by Polycharged Magnetic Particles: An Analysis of the Interaction by Zeta Potential and Particle Size, Yazan Haddad et al. nt J Mol Sci. 2016 Apr; 17(4): 550.

Multiple Choice Questions

1) All of the following can be used as samples for DNA isolation, EXCEPT
   a) Leucocytes
   b) Buccal mucosal cells
   c) Erythrocytes
   d) Fibroblasts
2) K in proteinase K stands for

a) Keratin
b) Potassium
c) Vitamin K
d) Karyon

3) In Which of the following blood collection tube will you collect the blood sample meant for DNA isolation?
   a) EDTA tube
   b) Heparin tube
   c) Plain tube
   d) Serum tube
4) Which of the following is a liquid phase DNA extraction method?
   a) Phenol-chloroform method
   b) Silica based method
   c) Magnetic bead method
   d) Ion exchange resin method
5) Which of the following is NOT a step of DNA isolation and quantification?
   a) Cell lysis using detergents
   b) Removal of cellular proteins and lipids
   c) RNAse digestion
   d) Precipitation of DNA with water

Answers:

1) C) Erythrocytes
2) A) Keratin
3) A) EDTA tube
4) A) Phenol-chloroform method
5) D) Precipitation of DNA with water

Short Answer Questions

1. Why DNA isolation is done under slightly alkaline conditions whereas RNA isolation is done under slightly alkaline condition?
2. Mention the role of following components in DNA isolation:
   a. Phenol
   b. Chloroform
   c. Isoamyl alcohol
   d. 70% ethanol
3. What is the role of salt in DNA precipitation with ethanol?
4. Write a short note on Proteinase K
5. What is the ideal 260/280 ratio for DNA sample to be used for DNA sequencing? Can nanodrop technique differentiate DNA and RNA?

Long answer questions

1. Describe the structure of DNA with relevance to the DNA isolation
2. Describe the principle, procedure, and precautions to be followed during DNA agarose gel electrophoresis
3. Briefly describe the plasmid DNA isolation by alkali denaturation method.
4. Explain the principle and protocol of DNA quantification using spectrophotometry.
5. Differentiate how plasmid DNA isolation is different from genomic DNA isolation.