ISOLATION OF PLANT GENOMIC DNA AND ITS PURIFICATION

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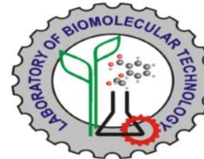

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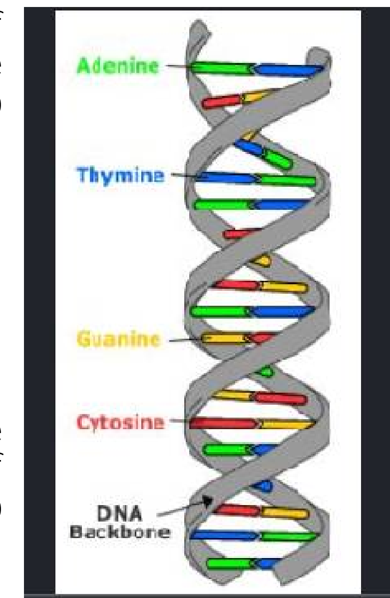
Key Topics

- DNA extraction
- DNA extraction related equipment and accessories
- Chemicals and buffers for plant DNA extraction
- CTAB plant DNA extraction method
  - Modification in CTAB DNA extraction protocol
  - Tips…..to care about

Introduction

- The DNA is identical in all living organisms, made up of Adenine, Thymine, Cytosine and Guanine. The backbone of the DNA is made up a phosphate while the hydrogen bond joins two opposite strands.
- "Extracting DNA from the cell by disrupting the cell wall/ cell membrane and nuclear membrane with the help of the chemical, physical or mechanical methods is called DNA extraction."
- In the year 1980, *Murray & Thompson* developed an inexpensive and simple protocol for the plant DNA extraction with the help of the CTAB. Later on Taylor and Powell make it possible to extract high molecular weight (> 50 kb) DNA
- Earlier Cesium density gradient methods were used.
- *Dellaporta* (1983), *Saghai Maroof* and coworkers (1984) are the pioneers in developing plant DNA extraction methods.
- Extraction of DNA from Plant cell is little difficult as compare to animal cells



Major steps in plant DNA extraction

- Lysis of cell wall with the help of the physical methods and chemicals such as grinding and using liquid nitrogen.
- Separating DNA by Extraction buffer including CTAB and SDS like chemicals.
- Precipitation of the DNA by alcohol.
- Purification of extracted DNA by 70% alcohol.
- Dissolving DNA by Tris-EDTA buffer.

DNA Extraction related equipments and accessories

Equipments

- Weighting Machine
- Autoclave
- Vortex Mixer
- Centrifuge
- Pipette and their autoclaved tips

Glassware's

- ✓ Liquid nitrogen container
- ✓ Ice box with ice
- ✓ Pre chilled Pastel Motor
- ✓ Spatula
- ✓ Different size of Measuring cylinder
- ✓ Different size flasks and beakers
- ✓ Eppendrof tubes

Chemicals and buffers

Liquid Nitrogen

- During the grinding, the heat is generated which activates enzymes such as endonuclease and exonucleases. These enzymes cleave our DNA into small pieces.
- Adding liquid nitrogen prevents the enzymatic reaction. Also, the liquid nitrogen froze the tissue and helps in making a fine powder of the tissue.
- Liquid nitrogen helps in making the homogeneous mixture of the tissue. It instantly froze the tissue to the point where it becomes ash.

Preparation of Stock and Extraction buffer or Lysis buffer

Addition of the extraction buffer to the sample prevents our sample from other damage.

S. No.	Components	Requried concentration in extraction buffer	stock solution		Requried Volume from stock solution for 30mL extractio n buffer	Requried weight (gm) for 30mL extraction buffer (C)
1	CTAB solution	2%	10%	10 gm in 100 ml	6 ml	0.60 gm
2	NaCl	1.4 M	7M	4.09 gm in 10 ml	600 µl	2.457 gm
3	Tris	100 mM	1M	12.14gm\100ml	3 ml	0.363 gm
4	EDTA	20 mM	0.5 M	14.71gm\100ml	1.2 ml	1.2 ml
5	PVP	2%	10 %	3 gm in 100 ml	6 ml	0.60 gm
6	Beta Mercaptoethanol	0.2 – 2%	-			Add just before use
7	Mili Q water	-	–		-	Up to 30 ml final volume

- 1. **CTAB (cetyltrimethylammonium bromide)**
- Also called as **Cetrimonium bromide** or hexadecyltrimethylammonium bromide.
- The molecular formula of CTAB is **$[(C_{16}H_{33})N(CH_3)_3]Br$**.
- It is **a cationic detergent** commonly used in DNA extraction.
- It is soluble in water as well as in alcohol.
- The plant cell wall contains mainly hydrophilic end groups, CTAB can dissolve both polar as well as non-polar group compound.
- **CTAB can bind with the cell wall polysaccharides by complexing with it, it co-precipitates the macromolecules along with the DNA which is removed by the centrifugation, later on.**
- **The basis for the separation of polysaccharides from nucleic acids is their differential solubilities in the presence of CTAB, which depends on the concentration of NaCl**
- Apart from its use in DNA extraction, the CTAB is the best antiseptic and commonly used against bacteria and fungus.
- Store it at room temperature or at 37°C. Storing at a low temperature will precipitate the CTAB.

2. NaCl

Use of salt is another important factor in plant DNA extraction. The DNA, as well as the polysaccharides both can be soluble in a salt solution or both, can be insoluble. It depends on the concentration of salt in the CTAB extraction buffer.
DNA is insoluble at low salt concentration and polysaccharides are soluble at low concentrations and reverse for high salt concentration.

3. Tris

- Tris (hydroxymethyl)aminomethane with the molecular formula (HOCH2)3CNH2, is a biological buffer.
- The major role of the Tris in any biological practice is to maintain the pH of the solution. During cell lysis, removal of unwanted cellular components and precipitation, Additionally, it plays a particularly important **role** in cell lysis.
- Majorly, Tris is used in biochemistry, molecular biology and chemistry. The pKa value of Tris is 8.07 (at 25°C).
- Furthermore, It can also increase the permeability of the cell wall.

4. Ethylenediamine tetra-acetic acid (EDTA)

- It is a colourless (white as in powder form), water-soluble and organic molecule. However, it can be dissolved in water at high pH (pH nearby 8.0).
- The chemical formula of EDTA is $C_{10}H_{16}N_2O_8$.
- The EDTA works as a chelating agent in the DNA extraction.
- It chelates the metal ion present into the enzymes and as we all know that the metal ions are the cofactor which increases the activity of the enzyme.
- By chelating the metal ions, it deactivates the enzyme, therefore, reduces the activity of DNase and RNase.

5. **Polyvinyl pyrrolidone (PVP)**

- The PVP binds to the phenolic ring of the tannin and prevents it's interaction with DNA and protects it.
- Hence PVP is the main ingredient along with our CTAB for the plants which contains a high level of tannin or polyphenolic compounds.

6.Beta mercaptoethanol is a strong reducing agent which can remove tannins and other polyphenols often present in the crude plant **extract**. It may also help to denature proteins by breaking disulphide bonds between cysteine residues.

Store the beta-mercaptoethanol at 4°C temperature.

7. $MgCl_2$ When membranes are busted by TRIS, there is no compartmentalization in the solution anymore. $MgCl_2$ is then used because it binds to DNA and thus protects it against DNase proteins that are now (because of lack of membranes) in direct contact with your DNA.

RNase A is an important enzyme for the removal of RNA for RNA free DNA purification

- Chilled Ethanol or 70% Ethanol
- The initial role of the ethanol and monovalent cations is to remove the solvation shell surrounding the DNA and permitting the precipitation of the DNA in pellet form.
- DNA is polar due to its highly charged phosphate backbone. If enough ethanol is added, the electrical attraction between phosphate groups and any positive ions present in solution becomes strong enough to form stable ionic bonds and DNA precipitation This usually happens when ethanol composes over 64% of the solution.
- The ethanol also serves to promote the aggregation of the DNA.
- With respect to the washing steps, typically a 70% ethanol solution is used.
- DNA is washed with 70% ethanol to remove some (or ideally all) of the salt from the pellet. Because precipitation in100% ethanol cause removal of all water molecule from DNA and Complete Dehydration, which make them not soluble, So we give 70% wash to let it retain some water molecule when make it soluble.

TE buffer or Mili-Q Water

The major role of TE buffer in DNA extraction is to dissolve DNA into liquid form. However, it is also used as a lysis buffer.

Autoclave, CTAB solution, CTAB extraction buffer and SDS solution. Further, autoclave all the plasticware and glassware before use.

Remember, never autoclave alcohol and phenol!!

Protocol

Take 500 mg of fresh plant tissue
(Mostly young leaves)

Cut it in small pieces and grind it well

Add Liquid nitrogen carefully and again grind it even harder until the tissue becomes powder

After it becomes homogeneous, take the mixture into the 2ml Eppendorf tube.

Add 500 µL of CTAB extraction buffer

Mix well with help of vortex mixer for 5 minutes

↓

incubate the sample for 30minutes at 60°C to 65°C.

↓

Centrifuge the sample at 10,000 rpm for 10-15 minutes and transfer the supernatant into another tube.

↓

Be careful, take the only supernatant, don't mess the supernatant with the debris.

↓

Now add 5 µL to 10 µL of RNase solution to the supernatant and incubate at 37°C for 25 minutes.

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Now add 5 µL to 10 µL of proteinase K solution to the supernatant and incubate at 60°C for 2 hours (this step is additional, it can use if necessary)

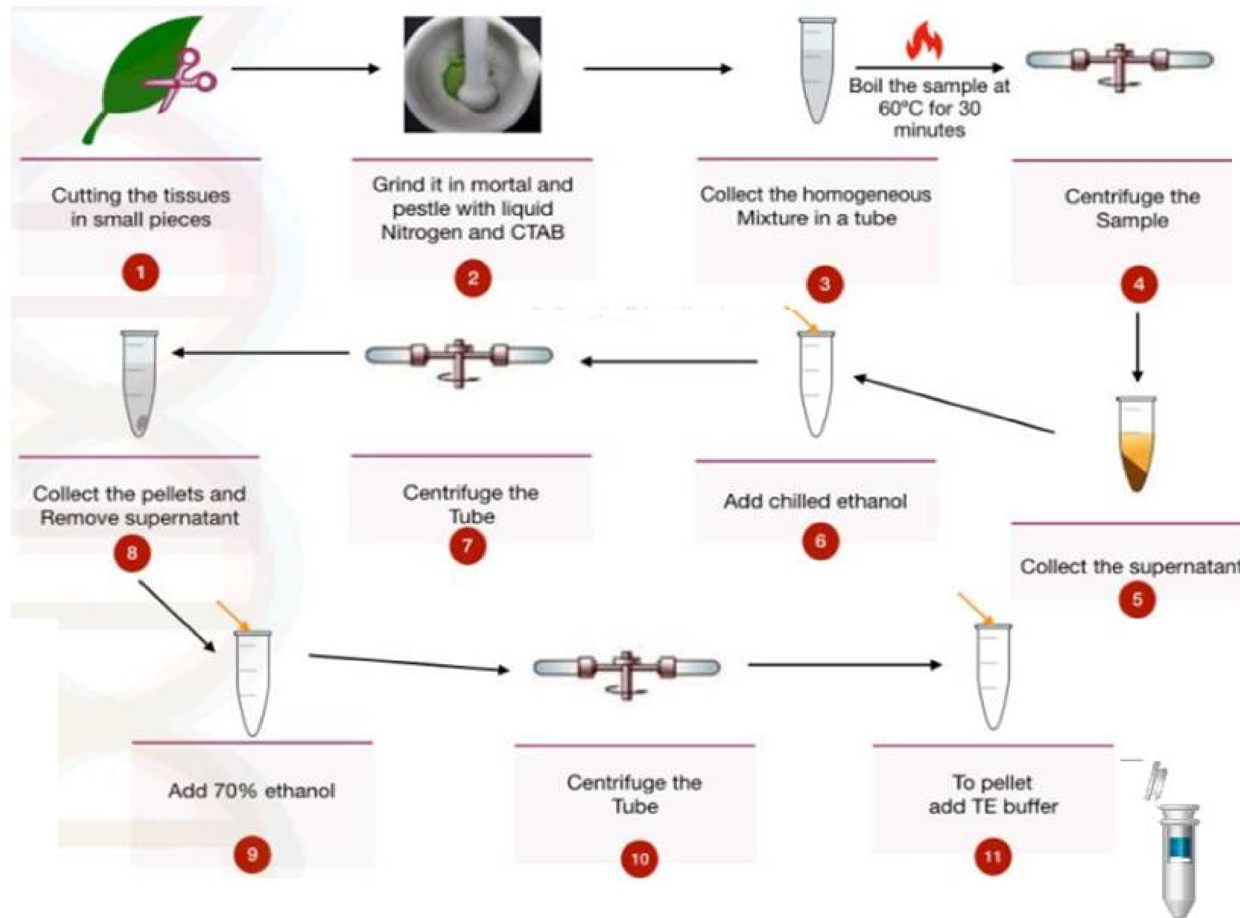
Centrifuge the sample at 10,000 rpm for 2 minutes and take supernatant to another tube.

Now add chilled isopropanol (70%),(1.5 times)

Precipitate the DNA by inverting the tube several times, after centrifugation at 10000 rpm 10 minutes throw the supernatant

Now wash the DNA pellet with prechilled 70% alcohol two times at 9000 rpm for 2 minutes.

After the clear pellet appears, add 500µL of TE buffer or elution buffer to the pellet and dissolve the DNA in it.

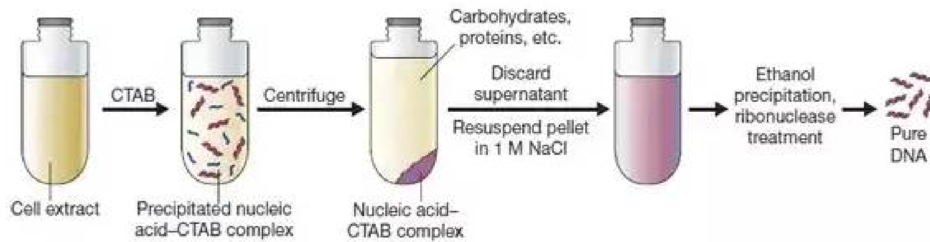


Here before step 6 we can add PCI (25:24:1), Phenol is non polar solution at the stage DNA can be separate out in the aqueous phase, phenol denature the protein and further addition of chloroform can enhance the complete denaturation of Protein and lipid also.

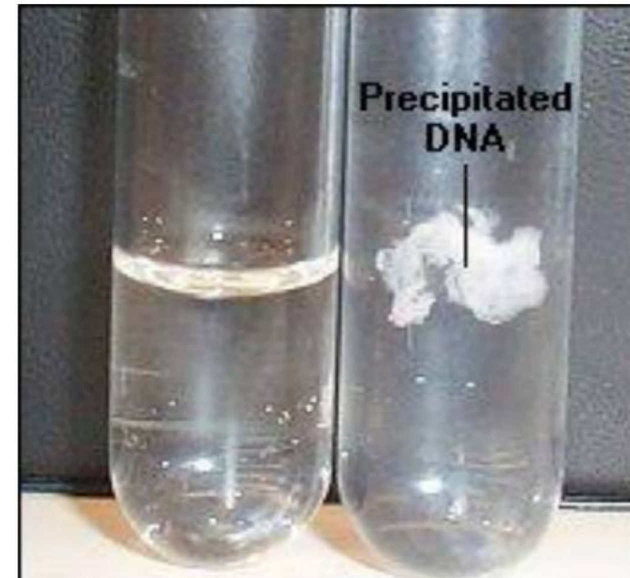
Here Isoamyl alcohol can act as antifoaming agent helping in clear separation of organic and aqueous phase.

Centrifuge 10000 rpm 10 mins.

Precipitated DNA



The CTAB method for purification of plant DNA.



Before and after adding Ethanol

Modification into the protocol

- For different types of plant species such as the plants with a high level of polyphenolic or alkaloids compound, required extra skills and modification into the protocol.
- Use of **SDS**. The sodium dodisylsulphate is the powerful anionic detergents.
- Use of the **proteinase K enzyme** . **After the RNase treatment, proceed the sample for the proteinase K digestion for overnight or for 2 hrs at 60°C.**
- We can use **PCI, phenol: chloroform: isoamyl alcohol**.
- Prepare phenol freshly PCI can be used after the proteinase K digestion step followed by the centrifugation at 2500rpm for 20 minutes.
- **Urea** is another excellent component which can be a game-changer in your plant DNA extraction protocol. We can use urea during the grinding of the tissue in the very first step.
- Urea is so powerful that it along can capable of doing DNA extraction, eliminating the need for organic solvents such as phenol or CTAB.

Tips

- Understand the physiology and anatomy of plant species you selected. Collect information on your sample, it's cell wall structure and composition. Also, note down the concentration and type of secondary metabolites and polyphenolic compound the plant secrets.
- **Tannin, the villain of plant DNA extraction**

  The tannin is the long chain of the phenolic ring which are major constituents of a polyphenolic compound. It is naturally occurring in the plant but it is secreted during the tissue damage as well. The polyphenolic compounds are the major inhibitors of PCR reaction.
- Use Mili-Q or nuclease-free water during the preparation of chemicals.
- *Remember, never autoclave alcohol and phenol!!*
- Prepare the working solution freshly every time from the stock reagents. If you have a heavy workload, prepare 10X CTAB DNA extraction buffer for the whole day or for two days.
- Store CTAB at room temperature or at 37°C. Storing at a low temperature will precipitate the CTAB.

- During phenol preparation wear gloves and eye protection, don't expose phenol in sunlight. Phenol is volatile and can burn the skin, so handle it carefully.
- Always wear gloves, goggles, cap and face mask.
- While handling phenol, always wear lab coat and goggles because phenol is volatile and can burn the skin. It can also damage our eye hence do not compromise with safety.
- The chloroform can make you faint. Also, the higher dose of the chloroform can be life-threatening. So be protective while handling chloroform.
- Along with self-precautions we have to take care of each chemical as well. Phenol can oxidize into sunlight therefore always store phenol in dark or amber bottle.
- Also, the pH can be fluctuated at a higher temperature so always protect phenol at 4°C. additionally, check the pH of the phenol periodically before proceed.
- For a short-term or single-use, dissolve DNA in water, because of the slight acidity of water the DNA dissolves fast and easily.

Thanks