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The Chemistry Behind Plant DNA Isolation Protocols

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Abstract

Various plant species are biochemically heterogeneous in nature, a single deoxy-ribose nucleic acid (DNA) isolation protocol may not be suitable. There have been continuous modification and standardization in DNA isolation protocols. Most of the plant DNA isolation protocols used today are modified versions of hexadecyltrimethyl-ammonium bromide (CTAB) extraction procedure. Modification is usually performed in the concentration of chemicals used during the extraction procedure according to the plant species and plant part used. Thus, understanding the role of each chemical (*viz.* CTAB, NaCl, PVP, ethanol, and isopropanol) used during the DNA extraction procedure will benefit to set or modify protocols for more precisions. A review of the chemicals used in the CTAB method of DNA extraction and their probable functions on the highly evolved yet complex to students and researchers has been summarized.

Keywords: DNA extraction, CTAB buffer, polysaccharide, organic phase, RNase A

1. Introduction

The isolation of good-quality DNA is the prerequisite for molecular research. Maintaining yield and quality of DNA during plant DNA extraction is one of the difficult tasks compared to that of animals, because of its rigid cell wall, which is made up of cellulose along with other variable levels of chemical components such as polysaccharides, polyphenols, proteins, and lipids that act as a contaminant during DNA extraction. The amount of these components varies according to plant species, plant part used, environmental condition, and growth stage and it is very problematic when isolating DNA. For example, cereals are rich in carbohydrates whereas medicinal plants are rich in the polyphenols wherein stressed plants have higher polyphenols. These contaminants can be removed during extraction by standardizing basic DNA extraction protocol [1–3].

Generally fresh leaves aged 15–20 days are preferred for plant tissues (fresh, freeze-dried, or frozen in liquid nitrogen) and usually ruptured by mechanical force in pestle and mortar or TissueLyser. If liquid nitrogen is unavailable, CTAB buffer can be used directly or prewarmed for grinding. The main objective of various DNA isolation methods is development of relatively quick, inexpensive, and consistent protocol to extract high-quality DNA with better yield. Generally, leaf samples contain large quantities of polyphenols, tannins, and polysaccharides. The basic principle of DNA isolation is disruption of the cell wall, cell membrane,

and nuclear membrane to release the highly intact DNA into solution followed by precipitation of DNA and removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols, and other secondary metabolites by enzymatic or chemical methods [4].

The plant DNA is extracted by either CTAB-based [5, 6] or sodium dodecyl sulfate (SDS)-based methods [7]. The majority of the protocols developed for DNA extraction are modified versions of hexadecyltrimethylammonium bromide (CTAB) extraction [8]. The role of various chemicals involved in CTAB extraction method has been described in the present communication.

2. CTAB buffer

The CTAB buffer mainly includes CTAB, sodium chloride (NaCl), and ethylenediaminetetraacetic acid (EDTA) Tris2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), polyvinylpyrrolidone (PVP), and β mercaptoethanol.

2.1 CTAB

The plant cells enclose themselves in complex polysaccharide cell wall, of which cellulose is a major constituent [9], which is crystalline in nature, due to chain-like structure and intermolecular hydrogen bonding. This can be weakened to open the cell wall, by applying mechanical force exerted during grinding along with CTAB buffer or liquid nitrogen.

Cell membrane lies next to the cell wall and cellulose and is composed of a diverse set of phospholipid molecules and proteins. It dissolves in surfactant, detergents, which are amphipathic (hydrophobic tail and hydrophilic head) in nature, very much similar to phospholipid membranes. Surfactants are characterized based on their hydrophilic group, that is, ionic, nonionic, and zwitterionic. Ionic surfactant has been always better in denaturing protein molecules, and thus in dissolving the membranes [10].

CTAB, a cationic detergent, constitutes a long hydrophobic hydrocarbon chain and a hydrophilic head. It forms micelle in water because of the amphipathic nature. During DNA extraction, under aqueous condition, CTAB comes in contact with the biological membrane, captures the lipids (**Figure 1**), and results in the release of nucleus, which is devoid of membrane [11]. Plant tissue, which is rich in complex polysaccharides and secondary metabolites, interfere and co-precipitate with DNA; CTAB along with some other chemicals like PVP is used to minimize the effect of these metabolites.

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 [12]. 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 [13].

2.2 NaCl

NaCl helps to remove proteins that are bound to the DNA. It also helps to keep the proteins dissolved in the aqueous layer so they do not precipitate in the alcohol along with the DNA by neutralizing the negative charges on the DNA so that the molecules can come together.

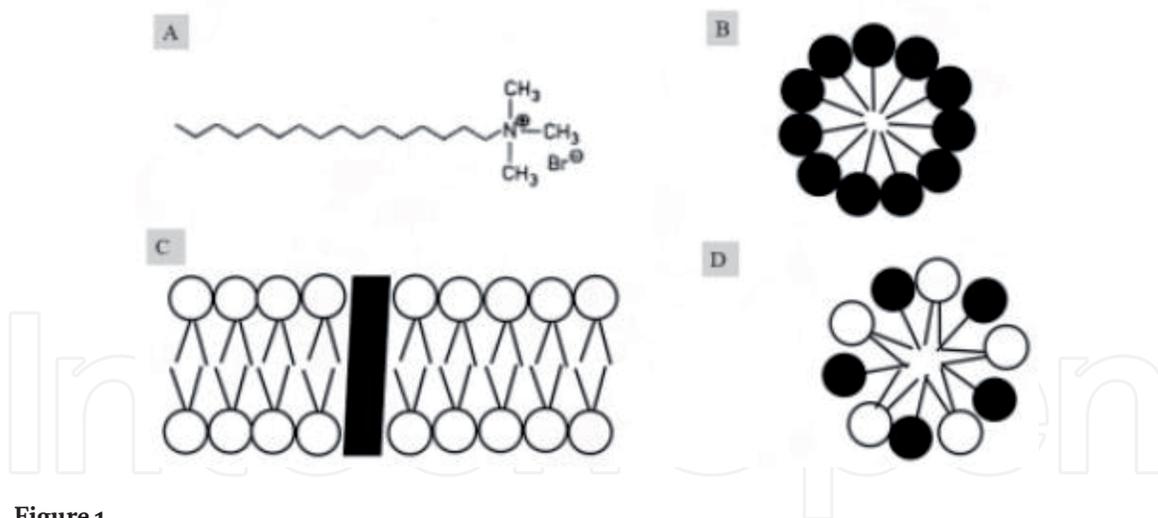


Figure 1. CTAB's role in removing membrane [25]. (A) CTAB is amphipathic in nature. It has long hydrocarbon chain (hydrophobic tail) and positively charged trimethylammonium group (hydrophilic head); (B) CTAB forms the micelle into the aqueous solution. Polar heads (hydrophilic) face outside and nonpolar (hydrophobic) hydrocarbon tail hides inside making micelle; (C) biological membrane made up of amphipathic lipids with integral protein; and (D) CTAB captures the membrane lipids and forms the hybrid micelle.

Osmosis occurs when cell is subjected to hypo or hypertonic solution. If the cells are kept in hypotonic solution, water enters inside the cell that leads to swelling, rising internal pressure and eventually bursting. On the other hand, in a hypertonic solution, water tends to ooze out from the cell and eventually plant cell shrinks and crumples, which leads to plasmolysis. Therefore, salt concentration plays a significant role in cell lysis.

The salt concentration of more than 0.5 M provides the ionic strength needed for CTAB to precipitate polysaccharides [8, 14]. In several protocols, 1.4 M concentration of NaCl has been suggested; however, in the protocols developed for getting rid of polysaccharides, higher concentration of the NaCl and/or CTAB has been recommended.

2.3 Tris

Tris is a (hydroxymethyl) aminomethane with the molecular formula $(\text{HOCH}_2)_3\text{CNH}_2$, which has three primary alcohols and an amine group with a pKa of 8.1, is an effective buffer between pH 7 and 9. When the pH is adjusted to 8, with HCl, it contains a mixture of weak base and its conjugate weak acid (**Figure 2**), which can act as a buffer and further increases the permeability of the cell wall. 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 a major role under such situations, and the Tris buffer maintains the pH of the solution.

2.4 EDTA

EDTA ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$) chelates divalent cations, such as Mg^{2+} and Ca^{2+} (**Figure 3**), which is present in the enzymes and reduces the enzyme activity of DNase and RNase. Divalent cations are the cofactors for many enzymes that increase the activity of the enzyme. For example, DNase enzyme requires Mg^{2+} ions as a cofactor for its activity. Chelating Mg^{2+} ions with EDTA makes enzyme DNase nonfunctional, and thereby protects 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

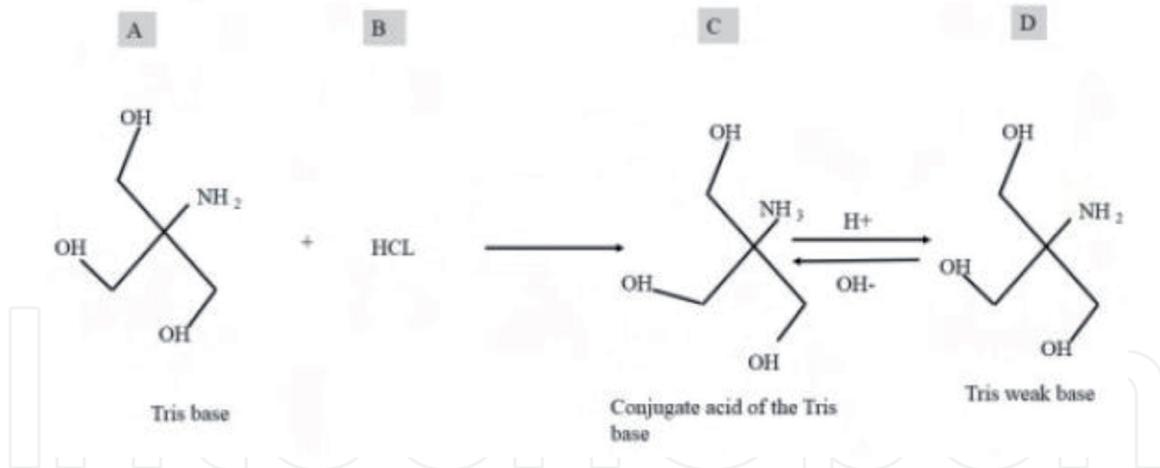


Figure 2. Tris buffer after titration of Tris base solution [25]: (A) with HCL; (B) around pH 8, it contains Tris weak base; (C) its conjugate acid; and (D) in equilibrium it acts as buffer near physiological pH range.

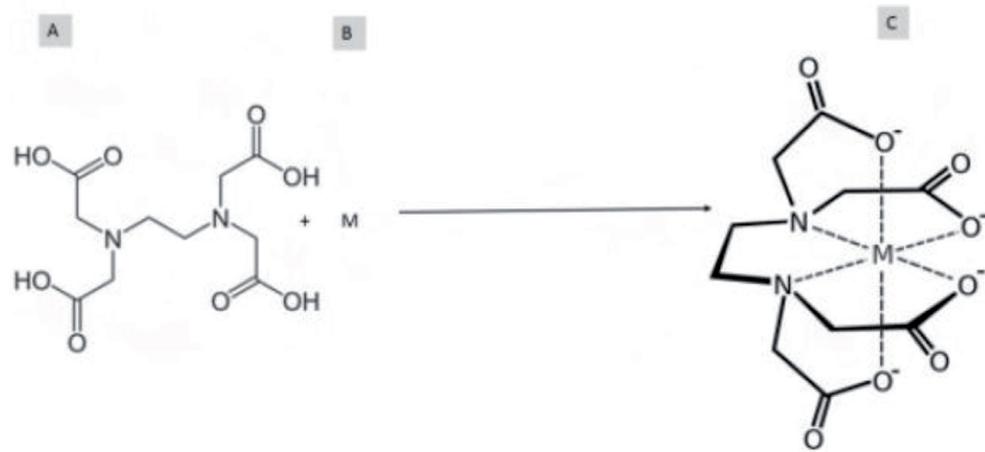


Figure 3. EDTA chelates divalent cations like magnesium and calcium [25]. (A) Structure of EDTA; (B) "M" depicts the free divalent cations like magnesium and calcium; and (C) EDTA chelates the divalent cations, thereby making unavailable to the DNase and some other activity like cell wall binding and histone-DNA complex formation.

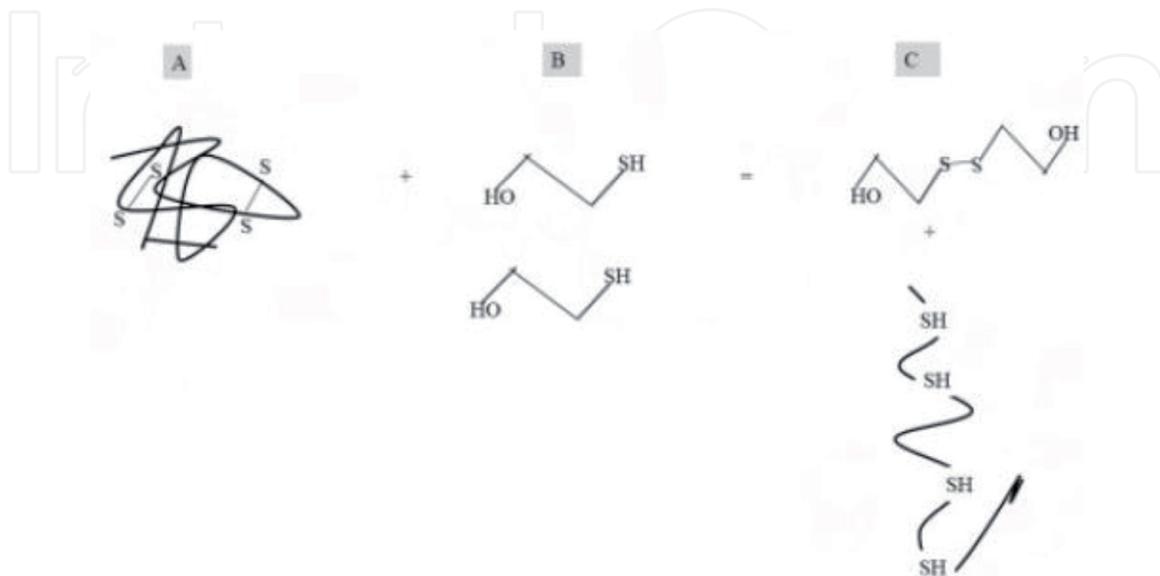


Figure 4. beta-Mercaptoethanol reduces disulfide linkage of protein, thus denaturing it [25]. (A) Protein tertiary structure with disulfide bonds; (B) beta-mercaptoethanol; and (C) oxidized beta-mercaptoethanol and protein denatured by beta-mercaptoethanol via its ability to cleave disulfide bonds.

middle layer and membrane stability. Thus, harnessing them by EDTA results in destabilization of the enzyme's integrity.

2.5 β -Mercaptoethanol

Plants are rich in phenolics compounds and to get a quality DNA these should be removed. β -Mercaptoethanol ($\text{HOCH}_2\text{CH}_2\text{SH}$) is added most of the time in extraction buffers and is a strong reducing agent to clean tannins and other polyphenols present in the crude plant extract.

Globular proteins get dissolved in water. To make them insoluble, their denaturation is one of the alternatives that can be done at tertiary and quaternary structure level of protein by reducing intermolecular disulfide linkages. β -Mercaptoethanol reduces disulfide bonds of the protein (**Figure 4**) and thus the proteins are denatured.

2.6 PVP

PVP is added to remove phenolic compounds from plant DNA extracts. 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 plastid [15]. During grinding of the tissue, compartmentalization breaks and PPO convert polyphenols into quinone, which gives brown coloration. Polyphenols bind DNA and make downstream processing difficult as they get co-precipitated with the nucleic acid. PVP removes polyphenolic contamination by binding it through hydrogen bond [16, 17]. Thus, it prevents polyphenol oxidation, and thereby browning of DNA samples [18]. 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°C , which irreversibly inhibits enzyme DNase. After removing the sample from 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:octanol (24:1) can also be used instead of chloroform:isoamyl alcohol (24:1).

3. Phenol

Phenol is an organic solvent, so it is not miscible with water and is used along with chloroform and isoamyl alcohol for purification of the DNA to remove proteins and polysaccharide contaminants. When phenol is shaken with cell extract, the nonpolar components of the cell will be fractionated in phenol, leaving polar ones in water. DNA is insoluble in phenol because phenol is a nonpolar solution. On the other side, protein has both polar and nonpolar groups present in it because of the long chain of different amino acids. Different amino acids have different groups present on their side chain. Also, the folding of the protein into the secondary, tertiary, and quaternary structure depends on the polarity of the amino acids. The bonds between amino acids are broken by the addition of phenol and protein gets denatured and ultimately the protein becomes unfolded.

Centrifugation after phenol:chloroform:isoamyl alcohol in 25:24:1 ratio steps gives three layers, that is 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. 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 phenol-chloroform combination reduces the partitioning of poly (A) and mRNA into the organic phase and reduces the formation of insoluble RNA protein complexes at the interphase. Phenol retains about 10–15% of the aqueous phase, which results in a similar loss of RNA; chloroform prevents this retention of water and thus improves yields.

Only neutral phenol should be used, as acidic phenol dissolves DNA within, or phenol turns into quinones by oxidation and it forms free radical, degrading nucleic acid. Simple observation of phenol's pink color will state its acidic nature. The centrifugation after chloroform:isoamyl alcohol step should be done under room temperature, because below 15°C, CTAB/nucleic acid forms irreversible aggregates and may precipitate. During this step, the DNA shall be in aqueous phase [19].

4. Chloroform

Chloroform (CHCl_3) or trichloromethane is a nonpolar (hydrophobic) solvent, in which nonpolar proteins and lipids get dissolved to promote the partitioning of lipids and cellular debris into the organic phase, leaving isolated DNA protected in the aqueous phase. Chloroform ensures phase separation of the two liquids because it has a higher density (1.47 g/cm^3) and forces a sharper separation of the organic and aqueous phases, thereby assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase. As chloroform is volatile in nature, it does not hinder the downstream process.

5. Isoamyl alcohol

Chloroform comes in contact with the air and forms gas phosgene (COCl_2 , carbonyl chloride), which is harmful. If we simply use chloroform only, the gas entrapment causes foaming or frothing, it foams up between interphase during extraction process and makes it difficult to properly purify the DNA, which is prevented when chloroform is used along with isoamyl alcohol or isopentanol $\{(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2\text{OH}\}$ or octanol $\{\text{CH}_3(\text{CH}_2)_7\text{OH}\}$ by preventing the emulsification of a solution. Isoamyl alcohol or isopentanol is not miscible in the aqueous solution because it is a long-chain aliphatic compound, containing five carbon atoms and stabilizes the interphase between organic and aqueous layer. The aqueous phase contains DNA and the organic phase contains lipid, proteins, and other impurities. Isoamyl alcohol helps to inhibit RNase activity and to help prevent the solubilization in the phenol phase of long RNA molecules with long poly (A) portions. This will increase the purity of DNA.

6. Ribonuclease A

Genomic DNA should be treated with Ribonuclease A (RNase A) to remove the contamination of RNA for DNA purification. RNase A is an endoribonuclease that catalyzes the hydrolysis of the 3',5'-phosphodiester linkage of RNA at the 5'-ester bond in a two-step reaction. The first step is a transphosphorylation to give an oligonucleotide terminating in a pyrimidine 2',3'-cyclic phosphate. The second is the hydrolysis of the cyclic phosphate to give a terminal 3'-phosphate. Numerous chemical studies have suggested that histidine 12, histidine 119, and lysine 41 are involved in the active site of the enzyme and the DNA is devoid of 2'OH group (deoxy), it remains secure (**Figures 5 and 6**) [20].

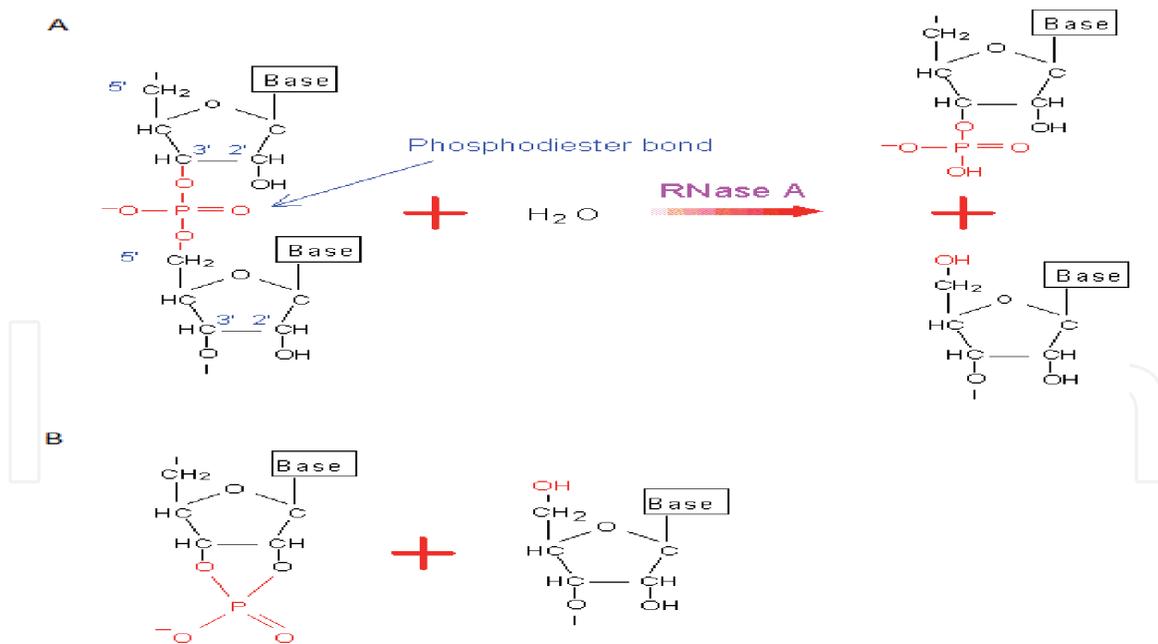


Figure 5. (A) The hydrolysis reaction catalyzed by RNase A. An RNA molecule is a chain of nucleotides linked by the phosphodiester bond, which may be cleaved by RNase [27]. (A) This figure shows only two nucleotides adjacent to the cleavage site and (B) the intermediate product (transition state) of this reaction.

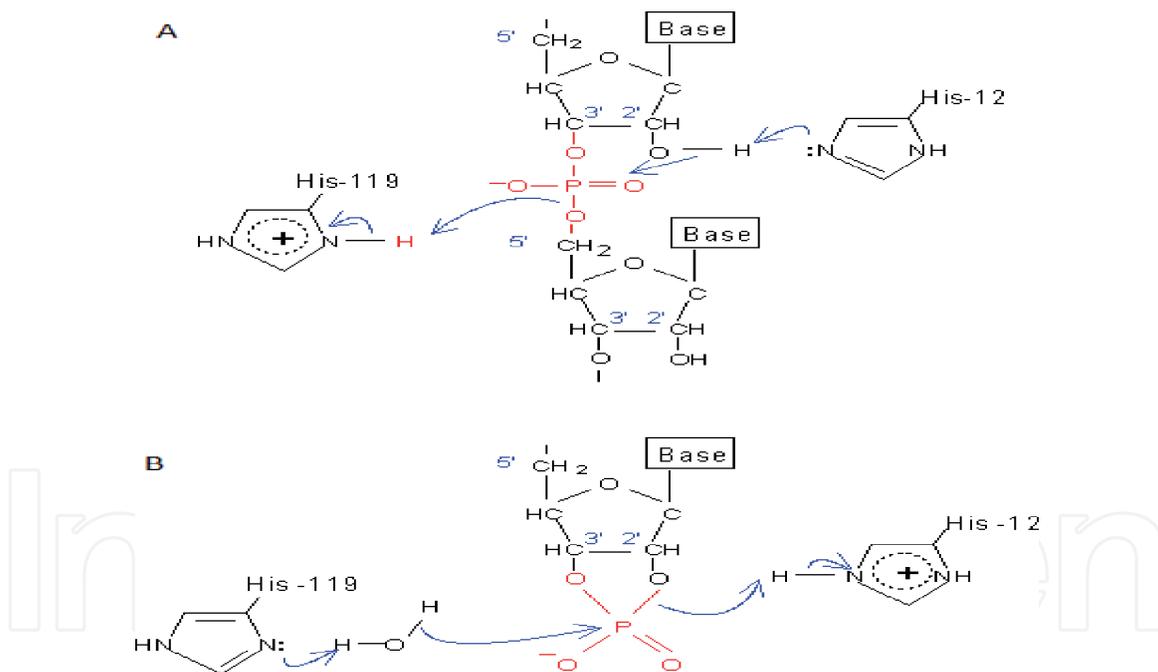


Figure 6. The catalytic mechanism of RNase A, which contains two critical residues: His-12 and His-119 [27]. (A) The transition state is formed by electron transfer from His-12 to His-119, passing through 2'-OH and (B) after the transition state is formed, the electron can move from His-119 to His-12, generating the final product. DNA lacks the critical 2'-OH and thus cannot be catalyzed by RNase A.

7. Isopropanol/ethanol

Alcohol is used to precipitate the DNA out of the extraction solution, so we can wash all those salts and chemicals away and then dissolve it in our final solvent—usually water or some variant of Tris-EDTA solution. DNA remains dissolved in aqueous solution because DNA has phosphodiester backbone, which is hydrophilic

in nature. Water molecule forms hydration shell around DNA by forming hydrogen bonds. Isopropanol/ethanol is used 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 (0.6–0.7 volume of supernatant), as isopropanol has a higher capacity to reduce the dielectric constant of water than the ethanol (2–3 volume) and also requires a fair amount of salt to work. RNA which has extra 2'OH 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 nonpolar solvents such as chloroform, thus the impurities from 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 [21]. Though the yield of DNA will be increased at low temperature, it may increase impurities [22].

8. Sodium acetate/ammonium acetate/potassium acetate/sodium chloride/lithium chloride/potassium chloride

The role of the salt in the extraction protocol is to neutralize the charges on the sugar phosphate backbone of the DNA. Sodium acetate with pH 5.2 is commonly used for precipitation of nucleic acid along with ethanol [23]. In solution, sodium acetate dissociates into Na^+ and $[\text{CH}_3\text{COO}]^-$. The positively charged sodium ions neutralize the negative charge on the PO^{3-} groups on the sugar phosphate backbone of nucleic acids reducing repulsion between DNA molecules, making the DNA molecule far less hydrophilic, and therefore much less soluble in water. The electrostatic attraction between the Na^+ ions in solution and the PO^{3-} ions on the nucleic acid are dictated by Coulomb's Law, which is affected by the dielectric constant of the solution. Water has a high dielectric constant, which makes it fairly difficult for the Na^+ and PO^{3-} to come together. 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 affected by either ethanol or isopropanol, which has a much



Figure 7. Role of salt in DNA precipitation [25]. (A) DNA molecules in aqueous solution have the negative charge and repel each other; (B) sodium acetate dissociates into the water into sodium and acetate ion; and (C) sodium ion shields the negative charge on the DNA molecules by neutralizing it and helps in aggregation and precipitation.

lower dielectric constant, making it much easier for Na^+ to interact with the PO_3^{3-} , shield its charge, and make the nucleic acid less hydrophilic, causing the DNA to drop out of solution (**Figure 7**).

9. Ethanol

DNA precipitate is washed again with 70% ethanol to rinse excess salt that might come along with the extraction buffers from the pellet [24], centrifuged, and ethanol is discarded, leaving DNA in the precipitate. Precipitate is air-dried or vacuum-dried. Over drying should be avoided as DNA converts B form to D form, which is difficult to dissolve later [25].

10. Tris-EDTA (TE) buffer/sterile water

In older times in DNA isolation methods, DNA used to be stored dry and diluted when required. Nowadays, for long-term storage, it is prudent to store DNA in a buffer that maintains its pH and keeps it from getting degraded. TE buffer contains Tris (10 mM) and EDTA (1 mM), where Tris is the buffering component and EDTA the chelating component. For DNA isolation, the pH is usually set to 7.5–8.5, the slight alkalinity of TE buffer also prevents chances of acid hydrolysis that may further disrupt the stability of DNA stored in water. 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 at ambient temperature Tris acts by scavenging hydroxyl radicals [26]. The purpose of EDTA is to chelate Mg^{2+} ions in solution necessary for DNase or RNase action, thus protecting the DNA from DNases or RNase.

Sterile water can be utilized for short-duration storage of DNA. If TE buffer is used for storage of DNA, it should be diluted further with sterile water to dilute EDTA concentration for making magnesium ions available for polymerase activity during PCR because if DNA has to be sent for sequencing afterward, the buffer components in TE hinders the process. The same EDTA that chelates ions to degrade magnesium also hinders the action of DNA polymerases during PCR, which can be overcome by adding more magnesium to the master mix, or perhaps diluting the DNA sample so that the already low concentrations of EDTA do not actually disrupt PCR. In fact, in a large number of cases, they do not.

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