Chapter 3

Isolation and Purification of DNA from Complicated Biological Samples

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Abstract

The isolation of nucleic acids from a biological sample is an important step for many molecular biology applications and medical diagnostic assays. This chapter describes an efficient protocol using established acidic CTAB (with a pH value of 5.0 to 6.8) based extraction method for isolation and/or purification of high molecular weight genomic DNA from a range of fresh and difficult sources from plant, animal, fungi, and soil material. This protocol is suitable for many sequencing and genotyping applications, including large-scale sample screening.

Key words High-quality DNA, DNA extraction, Plant tissues, Woody plants, Animal tissues, Herbarium specimens, Food, Soil, CTAB

1 Introduction

Nucleic acid sequences have a variety of applications in the field of molecular biology. They are a valuable tool in many analytical and application techniques used in the field of molecular biology, health, medicine (gene therapy, diagnostics, and recombinant protein expression), forensics, and food science. Some examples of these techniques include next-generation sequencing applications, genotyping with DNA fingerprinting, detection of pathogens, and forensic identification of biological samples and environmental samples contaminated with different biological entities [1–8].

To be used as a diagnostic tool, the target nucleic acid sequence should be free of contaminants that inhibit PCR and other downstream applications. Such contaminants chemically or mechanically block or inhibit chemical and enzymatic reactions, including denaturation and hybridization of nucleic acids, and other applications used in molecular biology methods. Contaminants can also degrade or modify the nucleic acid. These include high-molecular substances, such as polysaccharides and polyphenols, as well as substances of lower molecular weight, such as pigments, secondary

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metabolites, lipids, humic substances, low-molecular enzyme inhibitors, and oligonucleotides. Therefore, in order to be able to use nucleic acids from biological materials for further analysis, it is important that these substances are eliminated entirely from the sample.

Isolating DNA or RNA that is sufficiently purified from contaminants is complicated by the diversity and complex composition of biological material from which DNA and RNA are isolated. Biological material consists of cells and tissues. Cells in liquid media, such as blood, lymph, milk, urine, and feces, and cells in culture, on an agarose or polyacrylamide gel, in soil, or in solution, usually include significant amounts of contaminants that must be removed from the DNA or RNA before molecular biology experiments. The presence of chemical or mechanical crosslinks between DNA chains and with contaminants interweaving with DNA leads to partial or complete inhibition of DNA denaturation and the appearance of artifacts. The quality of nucleic acids directly influences problems and artifacts produced by molecular biology procedures downstream. Thus, for efficient DNA amplification, for example, using the PCR method or isothermal DNA amplification, complete separation of nucleic acid strands at all lengths is required.

A variety of DNA extraction and purification methods have been developed [9–23], and are known for different characteristics. Ionic ion exchange resins were used to purify a nucleic acid already in 1953 [24]. Nucleic acids, proteins, and other contaminants are bound on a solid support by anion exchange. Nucleic acids are then eluted in a high salt concentration (7 M urea or 1.2 M NaCl) [18] and further purified by ethanol precipitation. Ultracentrifugation in a gradient of sucrose or cesium salts has also been used to purify DNA. Nucleic acids are separated from other macromolecules in accordance with their sedimentation coefficient, before extraction with phenol or phenol/chloroform and precipitation with ethanol or isopropanol. Conventional protocols for the extraction of DNA or RNA from cells are well known in the field, and described in *Molecular Cloning*, Sambrook et al. [25]. For DNA, these protocols typically include a cell lysis step, solubilization of DNA, enzymatic or chemical extraction, and separation of DNA from impurities such as proteins, RNA, and other substances [26].

A wide spectrum of methods has been developed for the purification of nucleic acids by filtration on a microporous carrier [27]. The microporous membrane as a matrix for binding and support for DNA purification has many advantages, such as compactness and ease of development. It allows differential control of the elution of desired molecules and the removal of undesirable components in the liquid phase, in parallel for a larger number of samples in a shorter period of time compared to other approaches. There are several methods based on the binding of nucleic acids on a sorbent and then washing unwanted impurities, followed by

elution of nucleic acids. Silicon dioxide particles (SiO_2, silica, sand with particle size of 10–50 μm), fiberglass (glass microfiber filters Grade GF/A), microballs, hydroxyapatite, anion exchange resins, and diatomite are used as sorbents. Nucleic acids bind reversibly on particles of synthetic silica gel in buffers containing high concentrations of chaotropic salts (sodium iodide, sodium perchlorate, or guanidine thiocyanate) or urea. Unbound cell components are then washed out, after which the pure nucleic acid is eluted from the sorbent with an aqueous solution with low ionic strength [20, 28–30]. Several companies offer DNA and RNA purification kits based on this approach. The kits contain columns with membranes of sorbents based on silicon dioxide and microporous glass. Centrifugation or vacuum filtration is used to bind nucleic acids with the sorbent, followed by washing and elution. The use of glass microfiber filters as a sorbent for purifying nucleic acids does not always result in sufficiently pure DNA for subsequent use in molecular biology protocols [12, 13, 20, 23, 24, 31, 32].

Probably the most promising approach for the isolation and purification of nucleic acids is the use of electroelution techniques [33–36]. The electroelution procedure allows the purification of very clean DNA for use in all molecular biology applications. It effectively separates DNA from compounds, including high-molecular substances such as polysaccharides and polyphenols, as well as from pigments and humic substances that interfere with subsequent DNA quantification and amplification. For example, the SageELF electrophoresis system (Sage Science, Inc. USA) is commercially used to separate DNA or protein samples by size and then fractionate the whole sample. The system is equipped with pulsed-field electrophoresis for resolving large DNA.

We have developed a protocol for the isolation of DNA from biological samples using a lysis buffer (pH < 7, but preferably less than 6) containing acidic organic and inorganic salts of sodium or potassium (acetate, propionate, formate, citrate) or weak acids of zwitterionic buffering agents: HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), or MES (2-morpholin-4-ylethanesulfonic acid) [37].

The combination of acid lysis buffer and subsequent extraction with chloroform allows highly selective separation of polysaccharides, pigments, proteins, and other cellular components in the organic phase, which possesses the original color of the pigment, while in the aqueous phase the remaining pure DNA and RNA acquires a completely transparent color (*see* **Note 1**).

During DNA or RNA extraction using a lysing buffer with a weakly acidic pH (range from 5.0 to 6.8), oxidation processes and enzymatic and chemical reactions are almost completely blocked. As a result, covalent bonds are not formed between DNA and phenolics or polysaccharide components. During subsequent extraction with chloroform, contaminants that inhibit PCR

(polysaccharides, polyphenols, peptides, lipids, and pigments) are selectively removed to the interphase and an organic phase. The aqueous phase containing the DNA is collected and mixed with an equal volume of simple alcohol to precipitate the DNA. Finally, the DNA is purified by precipitation or filtration through a column with a glass/cellulose microfiber filter (*see* **Note 2**).

This protocol for DNA isolation is universal for most biological specimens. The method will effectively isolate DNA from whole blood, bones, plant samples, soil, herbarium, mycelium of fungi, and tissues rich in secondary metabolites, polysaccharides, and pigments. DNA samples obtained using the proposed method can be used in studies where the presence of contaminants in nucleic acids is undesirable; for example, during cloning, sequencing, and genotyping (*see* **Note 3**).

2 Materials

Prepare all solutions using ultrapure Milli-Q water and analytical grade reagents. Prepare and store all reagents at room temperature unless otherwise specified and away from direct sunlight. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 DNA Extraction

1. Glass beads 6 mm or tungsten carbide beads 3 mm.
2. TissueLyser II bead mill or similar Mixer Mill system, the adapter set 2 × 24 or set 2 × 96 (QIAGEN).
3. NanoDrop™ 2000/2000c Spectrophotometers or similar equipment for RNA (or DNA) concentration measures.
4. Chloroform:isoamyl alcohol mix (24:1).
5. 100% Isopropanol (2-propanol).
6. 70% Ethanol.
7. 10 mM Tris–HCL pH 8.0.
8. 0.5 M Na_3EDTA.
9. Ribonuclease A solution: 10 mg/mL in 50% glycerol, 10 mM Tris–HCL pH 8.0.
10. TE buffer: 1 mM Na_3EDTA, 10 mM Tris–HCl adjusted to pH 8.0.
11. CTAB DNA extraction buffer: 2% cetyltrimethylammonium bromide (CTAB), 1.5 M NaCl, 10 mM Na_3EDTA, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-acid) or 3-(N-morpholino)propanesulfonic acid (MOPS-acid). Combine 20 g CTAB, 25 g HEPES-acid (or 21 g MOPS-acid) and 20 ml of 0.5 M Na_3EDTA dissolved in 500 ml of Milli-Q water; then add 300 ml 5 M NaCl and bring final volume to 1 L.

2.2 Gel Electrophoresis

1. Electrophoresis Tris-Acetate-EDTA buffer (1× TAE): 40 mM Tris-base, 20 mM Acetic Acid, 1 mM EDTA (pH 8.0).
2. Gel loading buffer (10×): 20% (w/w) Polysucrose 400, 100 mM Tris–HCl pH 8.0, 10 mM EDTA, ~0.01% bromophenol blue. Dissolve 20 g Polysucrose 400 (Ficoll 400) in 80 mL 10× TE buffer. Add bromophenol blue according to the desired color intensity. Store at +4 °C.
3. DNA Ladder for electrophoresis: 100–10,000 base range. The DNA Ladder is diluted with 1× gel loading buffer to final concentration 25 ng/μL.
4. Agarose Basic for DNA Electrophoresis.

2.3 Equipment

1. Power supply (minimum 300 V, 400 mA) for electrophoresis.
2. Horizontal electrophoresis apparatus without special cooling. Most commercially available medium- or large-scale horizontal DNA gel electrophoresis systems are suitable. We routinely employ an apparatus with a run length of 10 cm.
3. UV transilluminator, for visualization of Ethidium Bromide-stained or SYBR Green-stained nucleic acids, with a viewing area of 20 × 20 cm.
4. Imaging system.
5. Spectrophotometer.

3 Methods

All lab procedures are performed at room temperature. This protocol was tested with different samples from herbarium specimens, seeds including plant samples containing significant amounts of contaminants and polysaccharides (*Medicago sativa, Vicia faba, Lupinus angustitolius, Colocasia esculenta*), as well as with woody plants, soil samples, and animal tissue, like blood.

3.1 DNA Extraction Protocol

3.1.1 Tissue Grinding

1. This step can be performed using either a TissueLyser II or a mortar and pestle. The TissueLyser II option is preferred because less time is required, more samples can be extracted, and cross-contamination is minimized.
2. Collect the tissue sample (the sample mass should not exceed 50–100 mg) in 2 mL Eppendorf Safe-Lock microcentrifuge tube containing a glass ball. Place the samples (plant leaves) and the TissueLyser II adapters in an ultralow freezer and store the frozen tissue at −80 °C. Cooling is not required for dry samples (herbarium specimen).
3. Powder the tissue by shaking in the presence of the steel (glass) balls at 30 Hz for 2–10 min. Proper grinding of plant samples

with a TissueLyser II is a crucial step and the plant tissue should be ground to a fine powder after the disruption. However, for some plants one disruption step may not be enough. In these cases, repeat the disruption for 5 min at 30 Hz until the sample is thoroughly and equally homogenized.

3.1.2 Extraction of DNA from Ground Tissue

1. Add 1 mL of preheated CTAB DNA extraction buffer with 1 μL Ribonuclease A solution to the tissue powder and mix in the TissueLyser II for 1 min at 30 Hz.
2. Incubate at 65 °C for 1 h (long incubation increases DNA yield).
3. Centrifuge at maximum speed in a microcentrifuge for 2 min to remove nonsoluble debris.
4. Transfer the entire clarified supernatant to a new 2 mL microcentrifuge tube containing an equal volume of chloroform.
5. Mix well for 5 min in the TissueLyser II at 30 Hz.
6. Centrifuge at maximum speed in a microcentrifuge for 2 min.
7. Transfer the entire clarified upper aqueous layer to a new 2 mL microcentrifuge tube which contains an equal or half the volume of 2-propanol, and vortex thoroughly.
8. Centrifuge at maximum speed in a microcentrifuge for 2–5 min. A whitish DNA pellet should be visible.
9. Discard supernatant and wash the pellet by adding 1.8 mL 70% ethanol; vortex thoroughly. At this stage, DNA samples can be stored at room temperature or refrigerated.
10. Centrifuge at maximum speed for 2–5 min and carefully discard the supernatant by decanting or with a micropipette. A whitish DNA pellet should be visible during discarding of supernatant.
11. Ensure the DNA pellet does not dry and dissolve immediately in 300 μL TE buffer, pH 8.0 at 55 °C for 10–20 min.

3.2 DNA Analysis

The spectrophotometric method of DNA quantitation is commonly used to determine both concentration and relative purity of nucleic acids in a solution. A spectrophotometer is used to measure the absorbance and purity of DNA samples. Pure DNA exhibited an A_{260}/A_{230} ratio in the range of 1.8–2.0 and is acceptable down to ratios of about 1.5. Smaller values around or even below 1.0 indicate significant amounts of impurities, contamination with polysaccharides.

The rapid agarose gel electrophoresis method provides a much more accurate quantitation of the genomics DNA. The integrity of the genomic DNA samples extracted is analyzed by electrophoresis on an agarose gel. Atleast 4 samples loaded onto a gel, at least one lane should contain a series of DNA fragments of known sizes so

that a standard curve can be constructed to allow the calculation of the size of unknown DNA fragments. The most commonly used molecular weight markers are calf thymus DNA or DNA Ladder. DNA Ladder usually cover a wide range of DNA sizes.

1. Mix 10 μL of the DNA solution prepared in the previous section with 2 μL of gel loading buffer (10×) in tube or plate, a quick spin with centrifugation at 14,000 RPM (16,873 × *g*) for a few seconds.
2. Prepare 1% agarose gel in 1× TAE electrophoresis buffer containing ethidium bromide. The agarose gel must be completely melted in the microwave and then allowed to slowly cool until its temperature drops to about 50–60 °C. At that point, if desired, add the ethidium bromide solution at a rate of 20 μL per 100 mL, to bring the final concentration to 0.5 μg per mL.
3. Load the sample into one of the wells. In the adjacent wells, load equal volumes of a series of DNA concentration standards (e.g., calf thymus in the range of 25–500 ng/μL) or DNA Ladder.
4. Run the gel at 50 V when the bromophenol blue tracking dye has migrated at least 2 cm from the wells, the run can be stopped.
5. Examine the gel on an ultraviolet light transilluminator. Intact DNA will be visible as a band near wells. A smear extending from the well to the dye front indicates that the DNA has been fragmented. The images can be saved in a Gel Documentation System (*see* **Note 4**).
6. From the gel photo, estimate the quantity of DNA in the test samples by comparison to the DNA concentration standards. The yield should be in the range 5–15 μg of DNA per 300 μL, with an average size of above 50 kb.

4 Notes

1. The original techniques using CTAB for DNA isolation was first developed by Murray and Thompson in 1980 [13]. The original protocol described by the authors contains an alkaline pH on Tris buffer. However, under alkaline conditions DNA extraction takes place with oxidative processes, causing a change in the color of the lysis solution from green to brown (for plants). Therefore, water-soluble polymer polyvinylpyrrolidone (PVP) and reducing agent β-mercaptoethanol were added to the lysis buffer. The use of CTAB, a cationic detergent, facilitates the separation of polysaccharides during purification, while additives such as PVP can help remove polyphenols. Buffers based on CTAB are also used to purify

DNA from plant tissues and their metabolites. Polyphenols are compounds that contain more than one phenolic ring (e.g., tannin), a structure that binds very effectively to DNA. They occur naturally in plants, but they also form when tissue is damaged (roasting). When plant tissues are homogenized, polyphenols are synthesized by the polyphenol oxidase released. The addition of PVP prevents polyphenols from binding to DNA and phenolic rings. The presence of chemical crosslinks between the chains and impurities from the tissue, or mechanical spatial entanglements of DNA in the presence of polysaccharides, leads to partial or complete inhibition of DNA denaturation and the appearance of artifacts. When DNA is isolated, certain groups of polysaccharides form a viscous, jelly-like, uniform mass with the DNA. Serious and damaging effects are exerted by oxidants of different biochemical nature, including phenolic compounds.

2. The implementation of our protocol of isolation and purification of total DNA from a biological sample is achieved as follows. Preliminary steps are homogenization of tissue sample to complete destruction within a few seconds to minutes. Both dry and liquid samples can be used. For blood samples, start with a red blood cell lysis step and precipitation of leukocytes. Lysis of the samples is carried out using weakly acidic (pH 5.0–6.8) DNA extraction buffers containing acidic zwitterionic agents (MOPS or HEPES) during incubation at 55–65 °C. Organic extraction with chloroform results in contaminants selectively separated into interphase and an organic phase. The aqueous phase containing the DNA is collected and mixed with an equal volume of simple alcohol to precipitate the DNA. As a result, an aqueous solution containing DNA becomes completely transparent, while the organic phase possesses the original color of the pigment (or brown for hemoglobin). In some cases, organic extraction with chloroform is not possible and DNA must be precipitated immediately or purified on a column.
3. The composition of the lysis solution contains inorganic salts (sodium chloride), within the effective concentration in the range of 1–4 M. The optimal concentration of the detergent is 1.5% CTAB. To increase the efficiency of DNA extraction, proteinase K can be added to the acidic lysing solution, which retains proteolytic activity at high ionic strength and low pH values, even in the presence of strong detergents and chaotropic agents. The subsequent extraction with chloroform increases the purity of the isolated DNA, especially from complex samples (thermally treated raw materials, blood, herbarium specimen, and soils). The effective concentration of chloroform is 1–2 volumes of the total lysate. Further, the

DNA is precipitated from the aqueous phase with a water-soluble organic solvent, such as a simple alcohol (it is preferable to use isopropanol). Depending on the biological material, DNA can be precipitated by filtration through a column with a glass microfiber filter, for example, glass microfiber filters (Grade GF/A) or through cellulose paper [26]. Finally, DNA is washed by precipitation or filtration in a solution of 80% ethanol and dissolved in low ionic buffered water.

4. The most frequent cause of bad DNA resolution is improper choice of agarose concentration.

   Low percentage agarose gels should be used to resolve high-molecular-weight DNA fragments and high percentage gels for low-molecular-weight DNAs. Trailing and smearing of DNA bands are most frequently observed with high-molecular-weight DNA fragments. This is often caused by overloading the DNA sample or running gels at high voltages.

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