**Review of DNA Extraction Methodologies and Guidelines for Protocol Development**

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DNA extraction is required for many molecular biology applications, and many commercial kits are available to isolate DNA from a variety of source materials. The quality and purity of the extracted DNA should be suitable for the intended downstream analyses. These days, most labs use commercial DNA extraction kits (rather than the traditional phenol-chloroform method), which employ spin columns, for DNA isolation. Spin columns contain a silica resin that selectively binds DNA, depending on the salt conditions and other factors influenced by the extraction method. These kits are generally much easier and faster to use than traditional methods, and do not require significant expertise. However, regardless of the kit used, and/or if the lab must optimize a protocol based on available supplies and reagents, it is important to understand the basic criteria that any method of DNA isolation must meet to determine whether a protocol is a suitable alternative, particularly for fecal DNA because of the low quantity and quality of the DNA.

For any sample type, a kit or protocol should satisfy the following criteria: 1. efficient extraction of DNA, 2. successful removal of contaminants, 3. production of sufficient amount of DNA for downstream workflow, and 4. isolation of high quality and high purity DNA. The following is a review of each basic step involved in all DNA extraction methods, and guidelines for selecting protocols and reagents to ensure the above criteria are met.

DNA extraction methods comprise three main steps:

**1. Lysis of cell walls and membranes to release DNA into solution**

The first step of DNA isolation is the disruption and lysis of cell walls and plasma membranes of cells and organelles. Lysis buffers contain a high concentration of chaotropic salts. Chaotropes have two important roles in nucleic acid extraction. First, they destabilize hydrogen bonds, van der Waals forces and hydrophobic interactions, leading to destabilization of proteins, including nucleases. Second, they disrupt the association of nucleic acids with water (which provides optimal conditions for transfer to silica if spin columns are being used). Incomplete lysis significantly reduces DNA yield. In addition to chaotropes, a detergent is often present in the lysis buffer to aid protein solubilization and cell lysis. This step also involves a protease for the digestion of cellular proteins that contaminate target DNA. Proteinase K works best under protein denaturing conditions (i.e. in denaturing lysis buffer). There are many lysis and protease solutions available:

- Buffers ATL and AL (from Qiagen kit): cell lysis solutions that break open tissue, cell, and nuclear membranes. Contains chaotropic agent guandinium chloride.

- Homemade lysis buffers commonly contain the following: 0.5% SDS, 8% PVP-10 (opt, 250 mM NaCl, 25 mM Na-DETA, 200 mM Tris-HCl; pH 7.5
- Proteinase K, 20ug/mL: a broad-spectrum serine protease. Very efficient in digesting proteins during nucleic acid preparation. Works by catalyzing the breakdown of cellular proteins by splitting them into smaller peptides and amino acids.

Description of kit-specific additions (not necessary for successful extraction if creating a homebrewed protocol):

- InhibitEX solution (Qiagen) is designed to remove inhibitors from samples which can impact down-stream processing, and may be added prior to Proteinase K. *The exact composition of this solution is proprietary information.*
- Some commercial kits that do not use columns utilize a high-salt buffer (e.g., Protein Precipitation Solution, Qiagen) during the lysis step to lower the solubility of proteins and allow protein precipitation so they can be pelleted during centrifugation.
- Some commercial kits use RNase A solution, which ensures that cellular RNA is digested during lysis.
- Phosphate-buffered saline (PBS) (Gentra Puregene) is used to isolate white blood cells from blood or other cells from fluids and prevents rupture of cells, balancing the salt concentration around surrounding cells. Contains 50 mM potassium phosphate, 150 mM NaCl; pH 7.2.

**2. Purification of DNA by precipitating proteins and polysaccharides**

Following cell lysis, proteins in the sample are precipitated out of the solution and removed following separation by centrifugation. Alcohol precipitation is used for concentrating, desalting, and recovering nucleic acids. Precipitation is facilitated by high concentrations of salt and the addition of either isopropanol or ethanol. DNA is less soluble in isopropanol and allows precipitation of lower concentrations of nucleic acids than ethanol, especially if incubated at low temperature for longer periods of time. This can be performed at room temperature, which minimizes co-precipitation of salt that interferes with downstream applications. However, because isopropanol involves the co-precipitation of salts, a second wash with ethanol is necessary. Ethanol is particularly efficient at precipitating polymeric nucleic acids and removing chaotropic salts, and ideal for precipitating very small DNA fragments. Additionally, for protocols using spin columns, the addition of ethanol further enhances the binding of nucleic acids to the silica in the spin column. Different extraction methods may use ethanol or isopropanol (or both) in washing buffers.

The percentage and volume of ethanol used are critical for optimal extraction. Too much ethanol will precipitate degraded material and small non-specific organisms, which will influence UV260 readings during DNA quantification. In contrast, too little ethanol may impede removing salts from the precipitate, which can affect down-stream reactions. It is therefore important to follow the recommendations of the kit or protocol being used, and to use fresh ethanol. If you suspect that degraded DNA is inflating your absorbance readings during quantification, consider re-optimizing ethanol concentration.

DNA purification generally involves two wash steps to remove residual protein and salt. The first wash is either isopropanol, or an ethanol-based solution that includes a low concentration of chaotropic salts to remove residual proteins and pigments (many names, i.e. Wash buffer 1). This is always followed with an ethanol wash to remove co-precipitated salts (e.g. Wash buffer 2), which is crucial for increasing DNA yield and purity (residual salt

contributes to high A230 readings and thus low 260/230 ratios). Residual contaminants, including salts, may also interfere with accurate quantification, and may result in reduced 260/280 ratios. Some kits may recommend two ethanol washes.
There are several versions of ethanol or isopropanol-based washing buffers to aid purification:

- Buffer AW1 and AW2/ Wash Buffer 1, 2 (Qiagen): Wash solutions that remove contaminants from the DNA attached in the column membrane.
- Homemade washing buffer I (first washing) for protocols without isopropanol should contain the following:10 mM NaCl, 10 mM Tris-HCl pH 6.5, 80% ethanol.
- For kits and protocols using isopropanol, 100% isopropanol is used for the first washing.
- Homemade washing buffer II (second washing) comprises only 70% to 95% ethanol.

Description of kit-specific additions (not necessary for successful extraction if creating a protocol):

- Glycogen solution 20mg/mL (Gentra Puregene Kit): During isopropanol precipitation, Glycogen Solution acts as a nucleic acid carrier and helps to efficiently precipitate small amounts of DNA. In addition, it facilitates visualization of the DNA pellet. *The exact composition of this solution is proprietary information. If building a protocol, note that Glycogen Solution does not work in combination with Qiagen's silica membrane column-based technologies, e.g., RNeasy, DNeasy, QIAamp, etc.*
- Some column-based kits may use a binding buffer to bind DNA to filter paper discs. Homemade binding buffer contains the following concentrations: 2M guanidine hydrochloride, 75% ethanol.

**3. Precipitation of DNA and resuspension in a buffer**

Most protocols include a drying step to remove residual ethanol, either by drying the spin column (for column-based methods) or by evaporating ethanol from the sample tubes. This step is essential for a clean eluent because the presence of ethanol will prevent full hydration, elution and interfere with downstream protocols.

DNA is resuspended from spin columns or dried sample tubes with the addition of an elution buffer. 10 mM Tris buffer will hydrate the DNA and result in higher quality elution than nuclease-free water, which has a lower pH and may not be adequate for hydrating high molecular weight DNA. For maximal DNA elution, it is recommended to allow buffer to stand in the silica membrane before centrifugation (column-based protocols), or incubation to dissolve DNA. For applications requiring intact high molecular weight DNA, elution buffer is preferred over water-based resuspension.
There are several elution buffers:
• Buffer AE and ATE (Qiagen): A solution that elutes the DNA from the membrane and allows stable storage of DNA for years in the refrigerator or freezer.

- DNA Hydration Solution (Gentra Puregene): Same purpose as above. 10 mM Tris, 1 mM EDTA; pH 7–8.
- Homemade elution buffers should contain the following concentrations: 10 mM Tris-Cl, 0.5 mM EDTA (optional); pH 9.0.

**Considerations for all reagents**

Regardless of the kit or protocol used, it is important to prepare and store reagents according to supplier recommendations. Many buffers can be stored at room temperature, but certain steps

require cooling on ice. It is also important to use the amounts of sample material indicated in the protocol, as limitations may vary based on the kit and reagents used.

For column-based extractions, avoid touching the silica membrane with the pipet tip. Extra centrifugation time may be necessary for scat samples containing debris to ensure liquid flows through the column (columns may change color, but eluate should be clear).

For some downstream applications, concentrated DNA may be required. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but this slightly reduces overall DNA yield. For column-based methods, following the first elution of 200 µl, a second elution in a new sample tube of 100 µl can increase overall DNA yield. Additionally, warming the elution buffer to promote evaporation (including by speedvac) can increase yields by further concentrating the DNA.

Aggressive vortexing can shear target DNA, as can rapid hand mixing (e.g., DNA precipitation with isopropanol and ethanol), so gentle inversion and pulse-vortexing is recommended after lysis steps to minimize mechanical degradation of target DNA.
It is the responsibility of the lab to ensure that the reagents and storage buffers used are appropriate for the desired sample processing method.

**Building an ad hoc protocol**

Silica column-based commercial extraction kits are a good choice for fecal DNA extraction because each step in these protocols are optimized to account for the low quality and quantity of fecal DNA, and troubleshooting is relatively easy when improved yields are required. When commercial kits are not available, however, the user must decide if a protocol can be built to mimic a spin column method, or if isopropanol/ethanol methods using hand mixing are more appropriate. If silica membrane spin columns are available, and the user can mix appropriate substitutes for a given commercial kit, the Qiagen scat extraction protocol is recommended. However, if a comparable commercial kit is unavailable, manual processing using modified salting-out precipitation methods are an ideal substitute due to the simplicity of protocol optimization and availability of reagents or corresponding homemade substitutes. These methods generally include isopropanol and ethanol-based washing buffers during the washing steps (reagents described in this document are used in both extraction methodologies), and do not require columns. Below is an example of a manual salting-out precipitation method that can be modified for ad hoc protocols.

**Untested potential protocols for scat**

For labs unable to get Qiagen stool kits, a Gentra kit or the salting out method on scat may be a viable alternative. The authors have not tested this directly, so it is recommended to test these methods on samples first before using them. For salting out method protocols, see Miller, Dykes, and Polesky 1987 (provided below) as well as https://openwetware.org/wiki/DNA_extraction_-_Salting_Out .

**Purification of archive-quality DNA from fecal cells in suspension using the Gentra® Puregene® Tissue Kit**

Equipment and reagents

Phosphate-buffered saline (PBS) = 50 mM potassium phosphate, 150 mM NaCl; pH 7.2
Cell lysis solution (0.5% SDS, 8% PVP-10, 250 mM NaCl, 25 mM Na-DETA, 200 mM Tris-HCl pH7.5)

Elution buffer-10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0.
Protein precipitation solution
100% isopropanol
70% ethanol (do not use denatured alcohol)
Pipets and pipet tips
1.5 mL microcentrifuge tubes
Microentrifuge
Water bath or heat block
Crushed ice
Absorbent paper/kimwipes™
*Recommended: glycogen solution 20mg/mL*
*Optional: RNase A solution*

Procedure:

1. Dilute 1–2 g stool in 10 ml PBS, and let the debris settle.
2. After the debris has settled, transfer 100 µl of the supernatant to a clean 1.5 ml microcentrifuge tube.
3. Add 500 µl Cell Lysis Solution to 100 µl fecal cells in PBS, and mix by pipetting up and down.
   *Note: If the sample has a high protein content, 550 µl Cell Lysis Solution may be added to 50 µl sample.*
4. Add 3 µl Proteinase K (20 mg/ml) and incubate lysate at 55°C for 1 h to overnight.
   If you wish to include an optional RNase treatment, go to step 4a, otherwise proceed with step 5.
   4a. Add 3 µl RNase A Solution to the cell lysate, and mix by inverting the tube 25 times.
5. Quickly cool the sample to room temperature (15–25°C) by placing on ice for 1 min.
6. Add 200 µl Protein Precipitation Solution to the cell lysate, and vortex vigorously for 20s at high speed.
7. Incubate on ice for 5 min.
8. Centrifuge at 13,000–16,000 x g for 3 min. *The precipitated proteins should form a tight pellet. Repeat if pellet not well-formed.*
9. Pipet 600 µl isopropanol into a clean 1.5 ml microcentrifuge tube.
10. Recommended: Add 1 µl Glycogen Solution (20 mg/ml).
11. Add the supernatant from step 9 by pouring carefully. *Make sure not to dislodge the protein pellet when transferring the supernatant.*
12. Mix by inverting gently 50 times and incubate at room temperature for 5 min.
13. Centrifuge at 13,000–16,000 x g for 5 min. *The DNA may be visible as a small white pellet, depending on yield.*
14. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
15. Add 600 µl of 70% ethanol, and invert gently several times to wash the DNA pellet.
16. Centrifuge at 13,000–16,000 x g for 1 min.

17. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. The pellet might be loose and easily dislodged.
18. Allow DNA to air dry at room temperature for 10–15 min. *Make sure tube is dry before proceeding.*
19. Add 20 µl DNA Hydration Solution. *100-200 µl of elution buffer is recommended to ensure enough sample is available for downstream analyses. Low yields can be increased by concentrating the sample.*
20. Incubate at 65°C for 1 h to dissolve the DNA.

21. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

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# A simple salting out procedure for extracting DNA from human nucleated cells

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One of the obstacles encountered when extracting DNA from a large number of samples is the cumbersome method of deproteinizing cell digests with the hazardous organic solvents phenol and isochloroform. Several other non-toxic extraction procedures have been published, but require either extensive dialysis (1) or the use of filters (2). A rapid, safe and inexpensive method was developed to simplify the deproteinization procedure. This method involves salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution.

Buffy coats of nucleated cells obtained from anticoagulated blood (ACD or EDTA) were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM $Na_2EDTA$, pH 8.2). The cell lysates were digested overnight at 37°C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM $Na_2EDTA$). After digestion was complete, 1 ml of saturated NaCl (approximately 6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube. Exactly 2 volumes of room temperature absolute ethanol was added and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic spatula or pipette and transferred to a 1.5 ml microcentrifuge tube containing 100-200 µl TE buffer (10 mM Tris-HCl, 0.2 mM $Na_2EDTA$, pH 7.5). The DNA was allowed to dissolve 2 hours at 37°C before quantitating.

The DNA obtained from this simple technique yielded quantities comparable to those obtained from phenol-chloroform extractions. The 260/280 ratios were consistently 1.8-2.0, demonstrating good deproteinization. Restrictions were performed using a number of different enzymes requiring high, medium or low salt concentrations, all resulting in complete restriction. This procedure has been used in our laboratory on several thousand blood samples for parentage, population and forensic studies. This technique is used with our non-isotopic hybridization procedures (3) rendering the entire process of RFLP analysis free of toxic materials.

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