A guide to DNA extraction protocols for ecologists, conservation managers, and environmental DNA researchers

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Abstract

Ecologists, conservation biologists, and environmental managers are increasingly dependent on environmental DNA (eDNA) data for research and decision making. The inherent complexity of eDNA samples, coupled with different choices involved during the DNA extraction process, can introduce biases into the final eDNA dataset. Therefore, optimization and troubleshooting of DNA extraction protocols are pivotal for the successful execution of eDNA projects. Knowledge of the basic steps and principles of DNA extraction is essential for eDNA analysis. However, traditional education in ecology, conservation, and environmental management typically does not include in-depth training in molecular methods. While DNA extraction box kits are typically user-friendly, they may fail to deliver the desired results with eDNA samples, necessitating protocol adaptations or educated selection of alternative approaches.

The primary objective of this paper is to enable scientists with an ecological background who employ DNA extraction protocols to understand the four key steps of DNA extraction, and to use this expertise to their advantage. Furthermore, we describe the purpose of commonly used reagents and chemicals, point out alternatives for each key step, explain the impact of certain choices on DNA integrity and purity, and advocate for an adaptable "mix and match" protocol when applicable.

We anticipate that the paper will enable field ecologists to develop a deeper understanding of the mechanisms and chemistry underlying DNA extraction, thus allowing them to make informed decisions regarding the best DNA extraction method for their research. Our intention is not to provide comprehensive, step-by-step protocols, but to offer guiding principles while highlighting alternative solutions. Finally, we hope that this paper will act as a useful resource to support knowledge transfer and teaching.

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Contributions
JR conceptualized the manuscript. All authors contributed to the overall structure, visualization work, writing, and editing of the manuscript.

Keywords
Environmental DNA (eDNA), DNA extraction, DNA isolation, molecular methods, conservation ecologists and biologists

Acknowledgements
We are grateful to Dragan Stajic for insightful comments on an earlier version of this manuscript. This work was funded by Innosuisse funding application no. 55760.1 IP-LS and by SNSF project 315230_204838/1.

Introduction

Environmental DNA (eDNA) has become a central analyte for ecological research, biodiversity assessment strategies, and conservation practices. eDNA is used to determine the presence/absence of individual species (Nguyen et al., 2018; Lutz et al., 2020) and to describe the composition of entire communities, either in natural settings such as estuaries (Nagarajan et al., 2022) or artificial settings such as recirculating aquaculture systems (Rieder et al., 2023). Species-specific analyses of eDNA samples are commonly used for pathogens (Bastos Gomes et al., 2017; Howell et al., 2019) and invasive species (Nevers et al., 2018; Lutz et al., 2020; Rusch et al., 2020), whereas barcoding, metabarcoding, and metagenomic approaches are used to assess biodiversity (Creer et al., 2016; Deiner et al., 2017; Taberlet et al., 2018) and taxonomic richness (Deiner et al., 2017; Rieder et al., 2023). Currently, eDNA is widely accepted as a powerful alternative to conventional capture- or culture-based methods for species and ecosystem monitoring.

Consequently, a substantial number of originally field-trained biologists and taxonomists are embracing approaches that rely on the isolation of eDNA. As pointed out by numerous studies (Adamowicz et al., 2014; Albertsen et al., 2015; Felczykowska et al., 2015; Corcoll et al., 2017), the choices made during the isolation step can have serious impacts on the detection probabilities and experimental outcomes. For community composition studies, inefficient DNA extraction may lead to over- or under-representation of species and bias (Rieder et al., 2023). Inadequate extraction may result in poor DNA quality and quantity (Sellers et al., 2018), lead to false results and conclusions, or cause a project to fail. However, choosing or developing a DNA isolation protocol tailored to the specifics of the sample and the requirements of the downstream steps requires a certain understanding of the DNA extraction process and the purpose of the chemicals used (Table 1). Many field-trained biologists may find this task daunting.

This paper is tailored to readers who are not lab-trained molecular biologists, but nonetheless need to decide on a sample- and purpose-appropriate DNA extraction approach for precious samples or need to improve, simplify, or scale up a DNA extraction protocol. We would like to enable researchers to see through the jungle of protocol mystery that either magically produces DNA or fails inexplicably by explaining the different approaches and chemistries of common protocols. Furthermore, we want to highlight the fact that DNA extraction protocols are surprisingly robust to change and adaptation and can often be tweaked to greatly improve efficiency or facilitate handling. Our objective is to enable researchers to understand what a particular section of their protocol does, to modify existing protocols, confidently mix and match kits with non-kit protocols, and to make educated choices when selecting a DNA extraction method according to their experimental needs. We do not aim to provide step-by-step instructions, which are better obtained elsewhere, but to synthesize overarching principles and the purposes of certain protocol steps, while pointing out aspects that are particularly important or particularly malleable. To this end, we list various approaches to achieving the same outcome, describe the role of key reagents, explain the impact of various approaches on DNA integrity and purity, and address common troubleshooting issues and solutions.

The four steps

Four basic steps are required to move from a heterogeneous sample to pure, concentrated DNA. These include 1) disruption ("lysis") of biological structures (cells, tissues, mitochondria, microbes, spores, etc.) to liberate DNA, 2) separation of DNA from other biomolecules such as proteins and fats, 3) removal of salts, and 4) concentration and recovery of DNA (Figure 1). The order of steps three and four depends on the protocol type (with or without a DNA-binding matrix). Understanding which part of a specific protocol achieves which of these steps is the start of any protocol optimization, adaptation, and customization. For example, upstream steps from one protocol or kit can often be combined with downstream steps from other protocols. In addition, protocols can often be paused between steps, sometimes up to weeks, given the appropriate storage conditions. Of relevance, "dirty" protocols can even skip some of these steps entirely. For example, a disruption step without cleanup may suffice to perform nonquantitative PCR on an abundant target.

Step 1: Lysis

The lysis step disrupts cellular membranes and organic structures, releases DNA from the nucleus, and removes the DNA-associated proteins. Lysis can be accomplished through three approaches: 1) mechanical disruption, 2) chemical digestion, and 3) enzymatic digestion. They may be employed independently, sequentially, or in combination (Figure 1A). DNA extraction kits usually integrate physical disruption through agitation, chemical disruption using a buffer containing salt and detergents, and enzymatic digestion employing an enzyme such as Proteinase K. The lysis step is

crucial with regard to the amount and integrity of recovered DNA and is a key step in optimization and mix-and-match approaches. For example, a lysis procedure using a commercial kit can be followed by matrix-free separation via centrifugation and matrix-free isolation via DNA precipitation. Similarly, a custom-designed lysis protocol can be combined with columns from commercial kits for separation and purification.

Substance class	Role and mechanism of action	Commonly used	Abbreviation	Comments
Detergents / Surfactants	Supports the lysis step. Detergents work by inserting their long hydrophobic tails into the hydrophobic and uncharged regions of proteins and membranes, which helps to break them up. They also prevent non-specific interactions between plastics and cellular components.	Cetyl Triethyl Ammonium Bromide Sodium Dodecly Sulfate Triton X-100 Tween 20	CTAB SDS	Cationic detergent Anionic surfactant Non-ionic surfactant Non-ionic surfactant. Reduces the adsorption of DNA to plastic tubes
Chaotrophic salt*	Supports the lysis step and, for matrix dependent, also the separation step. Chaotropic salts disrupt the hydrophilic / charged interactions of proteins, thereby complementing the action of detergents.	Sodium Iodide Guanidine Thiocyanate Guanidine Hydrochloride	NaI Guanidine HCL	Helps to rupture membranes and bind DNA to silica resin; interferes with the interaction of nucleic acids with water, thereby creating optimal conditions for their transfer to silica.
Degrading enzymes	Supports the lysis step. Proteins ending in "ase" are enzymes that work on the substrate mentioned in the first part of the protein name. They are themselves sensitive to lysis reagents or conditions that remove proteins from DNA.	Proteinase K Rnase		Digests proteins Digests RNA. Very specifically used if RNA is a problem for downstream applications, not generally required for DNA isolation
Organic solvents*	Enhances the process of lysis and, in the case of matrix free, promotes the separation step. Organic solvents weaken or unwind proteins and/or attract uncharged molecules such as non-polar protein pieces, lipids, or hydrophilic molecules..	Phenol, Trizol, Phenol-Chloroform-Isoamylalcohol, Chloroform		Effective in eliminating harmful enzymes such as DNases, but the residues left in the sample can also interfere with downstream enzymatic interactions.
Salt	Enhances the DNA isolation and recovery by repelling charged water molecules, thereby increasing the insolubility of DNA in water.	Sodium Chloride Sodium Acetate	NaCl NaOAc	
Alcohol	Supports the isolation and recovery of DNA by interfering in the interaction of water molecules with DNA, and, together with the salt, reducing its solubility in water.	Isopropanol Ethanol	2-Propanol EtOH	
Chelating agent	Enhances lysis and DNA stabilization during storage by chelating ions. Chelators help to quench enzymes like DNase and RNase, which rely on calcium and magnesium ions for activity. Incorporating chelators into lysis and storage buffers can mitigate the degradation of nucleic acids.	Ethylene Diamine Tetraacetic Acid	EDTA	The utilization of chelators in DNA polymerases, which necessitates the presence of magnesium, must be carefully balanced with the needs of downstream applications when designing long-term storage buffers.
Buffering agent	DNA is chemically an acid and, as such, is susceptible to degradation in basic pH conditions. Other enzymes used during DNA extraction, such as Proteinase K, are also sensitive to pH changes and require specific conditions to function effectively. To ensure that reactions and storage are carried out in a stable and appropriate pH environment, buffering agents are used to maintain a constant and consistent pH level.	Tris(hydroxymethyl)aminomethan Tris(hydroxymethyl)aminomethan Hydrochlorid Tris-EDTA buffer	Tris Tris-HCL TE buffer	

Table 1: Chemicals commonly used for DNA extraction. *DNA extraction protocols of all types (from self-prepared solutions to ready-to-use kits) use similar categories of chemicals. Detergents, chaotropic salts, or degrading enzymes help break up the materials. Chelating and buffering agents protect DNA. Salts and alcohols promote the separation of DNA from water. Importantly, compounds serving similar purposes can often be used as substitutes. *[Hazard]*

Two aspects of the lysis step have a particular impact on the recovered DNA. First, cell lysis exposes DNA to degrading enzymes, such as DNases. Therefore, lysis conditions are strategically engineered to prevent DNA degradation. This can be achieved through rapid processing at low temperatures, the employment of elevated, denaturing temperatures, and/or the introduction of enzyme-inactivating agents such as high salt concentrations, detergents, or organic solvents. Second, complex samples commonly contain both easy- and hard-to-lyse components. On the one hand, harsh lysis methods that can disrupt and liberate DNA from the latter, such as high temperatures, sonication, and mechanical beating, can simultaneously negatively impact DNA integrity, and DNA may become too fragmented for downstream sequencing (e.g., long-read sequencing). On the other hand, gentle lysis

methods, such as freeze/thaw cycling or manual grinding, may fail to recover all DNA from hard-to-lyse components but are less destructive to DNA and therefore support long-read sequencing approaches. In summary, the choice of lysis method may lead to the misrepresentation of specific hard- or easy-to-lyse taxonomic groups in the final DNA sample or may impede a desired downstream analysis method. Hence, the selection of the lysis method should be guided by the composition of the sample and specific objectives of the experiment.

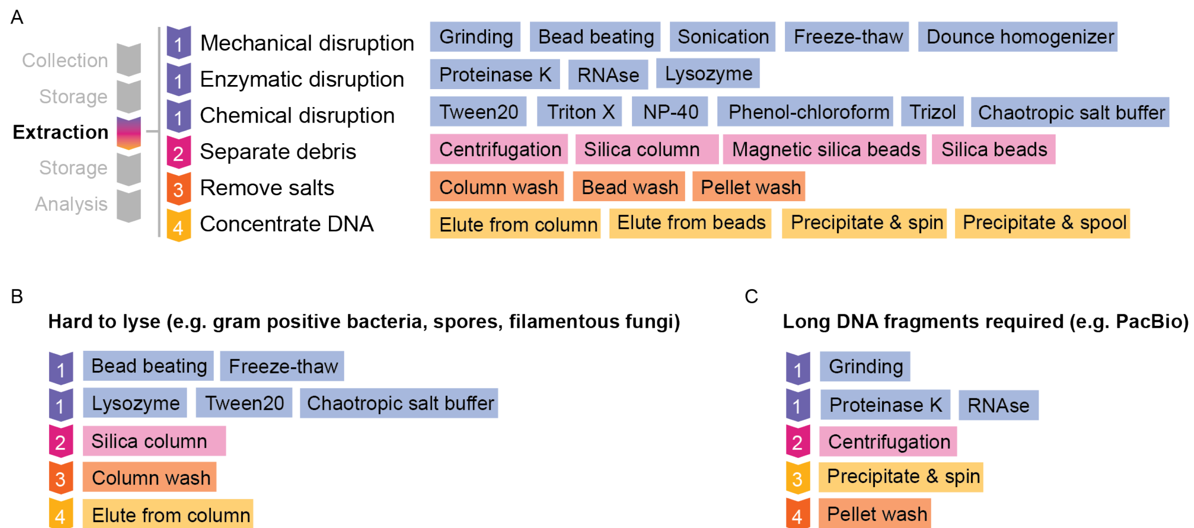

Figure 1: Mix-and-match of the four steps and various methods. *A) The four main steps in DNA extraction are (1) lysis, (2) separation of soluble DNA from other cell materials and binding of DNA, (3) removal of salts, and (4) DNA collection/concentration. Each step can be achieved using a variety of approaches with distinct properties. For example, mechanical disruption can be accomplished through grinding, bead-beating, and sonication. B) Example of an approach that can be used for hard-to-lyse organisms, such as gram-positive bacteria or spores. A variety of strong lysis methods have been combined with matrix-based separation protocols. (C) Example of an approach that can be used to recover long DNA fragments. Gentle DNA-preserving DNA extraction methods have been combined with a non-fragmenting recovery protocol to ensure that long DNA fragments are obtained for third-generation sequencing (e.g., PacBio).*

Mechanical disruption

Mechanical methods release DNA by disrupting surrounding biological materials through physical forces. Common approaches include grinding in liquid nitrogen, dounce homogenization, boiling, freeze-thaw, bead beating, and sonication (see the cell lysis review by Islam et al. (2017) for additional processes). Homogenization methods use blenders, homogenizers, or bead-beating to create a uniform mixture of samples using shearing forces. Boiling disintegrates cell membranes and proteins through entropic destabilization and protein denaturation, while freeze-thaw cycles induce the formation of ice crystals, which in turn disrupt cell walls. Mechanical disruption usually takes place in the presence of a stabilizing buffer solution, and because shearing forces create friction-induced heat, cooling is required. For instance, when using a bead mill, processing bursts can be alternated with cooling periods or by pre-cooling the adaptors beforehand.

Considerations when using mechanical disruptions are mostly related to sample composition, handling, and DNA integrity. Combining multiple mechanical methods ensures that more uniform lysis occurs, especially with respect to hard-to-lyse species (Ma et al., 2020; Anderson & Thompson, 2022) (Figure 1B). If standardization is a concern, the use of device-dependent methods such as sonication or bead beating is recommended because these options offer some degree of standardization (e.g., time and force). If fragmentation is a concern, freeze-thaw cycling or manual grinding could be a better option than harsher boiling, sonication, or mechanical beating. If cost is an issue, manual grinding or heating (both possible with kitchenware) trumps sonication or bead beating, both of which require expensive equipment. Finally, if high-throughput is required, freeze-thaw cycling or heating could be a better option than machine-based methods, which usually depend on the sample capacity of the device. Generally speaking, more time-consuming processes with individual sample handling result in less DNA fragmentation but are more challenging to standardize.

Chemical digest

+6859-+Chemical methods facilitate the release of DNA through the disintegration of cellular membranes and proteins using detergents, chaotrophic salts, or organic solvents, either individually or in combination. Detergents, at a concentration of 1% or 2 % (w/v), induce destabilization of non-charged cellular components such as lipid bilayers or lipophilic proteins. Among the different detergents, anionic variants, such as SDS, and cationic counterparts, such as CTAB, exert the most profound influence on protein structures. Non-ionic detergents, such as Triton X-100 and Tween 20, are non-denaturing and less aggressive. Zwitterionic detergents, such as CHAPS, feature characteristics between those of ionic and nonionic detergents and have a moderate impact on protein structures (Donnell et al., 2017). For a more extensive comparison of various detergents, refer to Johnson (2022).

Chaotrophic salts aid in disrupting the structure of charged molecules and destabilizing polar structures, particularly proteins, within cells. Chaotrophic salts, such as guanidine, EDTA, and urea, are strong protein denaturants. Disruption of hydrogen bonds, hydrophilic interactions, and other non-covalent forces that maintain the three-dimensional structure of proteins causes proteins to lose their original conformation and become unfolded or denatured. As a result, proteins lose water solubility and precipitate out of solution. Chaotrophic salts can also help inactivate enzymes such as DNases, which can degrade DNA during the extraction process. Importantly, EDTA can form precipitates at temperatures already at room temperature and is not recommended for use in cooled samples. Organic solvents, such as phenol or chloroform, are very effective at disrupting lipid bilayers, denaturing proteins (Saini et al., 2021), and removing organic contaminants or fat from samples.

Overall, chemical lysis methods preserve DNA integrity better than mechanical methods because they lack shearing forces and degrade DNAses. Therefore, chemical-based lysis methods are often used to isolate long DNA fragments. However, thorough cleanup, or even repeat cleanup, might be necessary after the use of organic solvents to avoid negative effects on downstream enzymatic procedures. They also pose significant concerns related to environmental and human health and may be problematic for sensitive downstream applications.

Enzymatic digest

Enzymatic methods release DNA by disintegrating cellular components with enzymes, such as lysozymes, nucleases, and proteases. Many extraction kits rely on proteinase K, which nonspecifically cleaves exposed proteins. More target-specific enzymes, such as lysozymes, labiases, and achromopeptidases, disrupt the peptidoglycan layer of gram-positive bacteria (Andrews & Asenjo, 1987; Salazar & Asenjo, 2007). For gram-negative bacteria, lysozyme can be combined with detergents (e.g., EDTA) to break the cell wall and membrane (Geciova et al., 2002; Islam et al., 2017). Other enzymes also include mutanolysin and lysostaphin, which are used to extract nucleic acids from susceptible bacteria (Cho et al., 2021). The enzymes commonly used to lyse yeast cells include chitinase, zymolyase, lyticase, and glucanase (Burden, 2023). Finally, pectinases can be used to lyse plant cells.

Enzymatic lysis is a gentle method (Islam et al., 2017) that preserves the integrity of DNA, which is desirable for third-generation sequencing projects (Figure 1C). In addition, enzymatic lysis (and chemical lysis) is better suited for large sample numbers than most machine-dependent physical disruption methods. However, the DNA quality may be lower (Yang et al., 2023). Therefore, the quantity and quality requirements should be considered when deciding the use of an enzymatic digest approach.

Step 2: Separation of DNA from other cell materials

After lysis, DNA is interspersed in a complex mixture of various nucleic acids, proteins, lipids, cellular constituents, salts, enzymes, and detergents introduced during the lysis process. These components can significantly hinder the effectiveness and accuracy of subsequent analytical techniques, such as PCR, DNA sequencing, or gene expression analysis, and are usually removed, although how completely may be dependent on the project's requirements.

Protocols usually feature one of two distinct separation strategies. DNA can be isolated from other constituents using a matrix-free approach, through detergents or organic solvents that essentially attract non-DNA components from the DNA solution, or through a matrix-dependent process involving silica- or cellulose-coated surfaces that essentially pull DNA out of the solution. The most suitable approach typically depends on the nature of the sample, the specific objectives of the experiment, and the requirements pertaining to DNA purity, integrity, and quantity, in addition to budgetary considerations.

Matrix-dependent

During matrix-dependent methods, the lysis material is brought into contact with a solid phase (matrix) that binds and retains DNA, but not other cellular components. Commercial kits typically employ matrix-dependent approaches. Most commonly, the matrix consists of silica or cellulose, although other substrates have also been used. The matrix is either packed in a column or consists of a coated surface such as magnetic beads. For column-based matrices, centrifugation or vacuum suction (i.e., the use of a vacuum pump) is applied to force the sample through the matrix, whereas for coated surface matrices, the sample is separated from the solution via centrifugation or magnetic tube racks.

The binding of DNA to the matrix is facilitated by specific salt conditions, which is why a binding buffer sometimes needs to be added to the sample after lysis. Chaotropic salts, such as guanidine hydrochloride, guanidinium thiocyanate, and hydrochloride (Table 1), are common components of binding buffers that promote the binding of nucleic acids to silica (Berensmeier, 2006). Sodium iodine or sodium perchlorate may also be used but are not as common. In addition, ethanol or other organic solvents may be components of the binding buffers, further aiding the binding process. Once the DNA is safely bound to the matrix, the remaining lysis material is removed by centrifugation or suction when dealing with columns, or by pipetting away the supernatant when dealing with beads, leaving behind the matrix-bound DNA.

Matrix-dependent kits offer user-friendly features, facilitate standardization, and enable efficient processing of numerous samples. With rigorous quality control measures by manufacturers, batch-to-batch variability is minimized. Matrix-dependent kits were initially developed for use with patient samples, such as blood or tissue; however, specialized versions tailored to water, soil, microbes, or plants are now commercially accessible. The main differences between kits typically pertain to the treatment of the initial material and the lysis conditions.

However, these kits have several drawbacks. First, matrix-dependent kits often rely on centrifugation or vacuum machines for processing, thus creating a batch limit (e.g., 24 samples per batch). However, some magnetic bead-based methodologies offer the advantage of being automated and applied in a multiplexed manner using systems, such as the Thermo ScientificTM KingFisherTM platform. Second, the DNA yield may be lower (Abdel-Latif & Osman, 2017) because DNA loss during binding, washing, or incomplete recovery can be substantial (Menchhoff et al., 2020). Third, most kits produce low-molecular-weight DNA (i.e., short fragments), which may or may not be an issue, depending on the aim of the project. Furthermore, the chaotropic salts used to support the binding of DNA to the matrix may inhibit downstream enzymatic reactions, such as DNA polymerase amplification (Vandeventer et al., 2012), if not removed properly. Finally, commercially available kits are costly

Matrix-free

Matrix-free DNA isolation protocols leverage the distinctive physical and chemical attributes of DNA, such as its hydrophilic and polar/charged properties, to separate it from cellular components, which are more lipophilic and less polar/charged.

During phenol-chloroform-isoamyl alcohol extraction, a commercially available mixture of these organic solvents is vigorously mixed with the lysis material through vortexing or intense shaking. This results in the denaturation of proteins and the extraction of lipid compounds into the organic solvent. Subsequently, the organic solvents and aqueous lysis buffer are separated by centrifugation into distinct layers according to their respective densities Lipids and hydrophobic cellular components accumulate in the lower solvent layer, whereas hydrophilic components such as salts and DNA accumulate in the upper aqueous layer. Protein fragments, which possess a partly polar and partly lipophilic nature, congregate at the interphase between these layers and often appear as a visible white interphase. The upper aqueous phase, containing DNA devoid of other compounds, can then be transferred to a new tube. Because phenol can significantly interfere with downstream processes, the process is then repeated with chloroform once or more to remove traces of phenol from the sample.

In the context of eDNA projects, phenol-chloroform extraction can alleviate issues related to PCR inhibitors such as humic acids (Sidstedt et al., 2015), which are effectively removed by organic solvents. Additionally, when combined with ethanol precipitation (described below), this extraction method can be used to recover a high amount of long-stranded DNA compared to fragmentation- and DNA-loss-prone matrix-dependent methods.

An alternative expedient method of separation involves the removal of cellular debris from the lysis material through high-speed centrifugation without the use of organic solvents. Subsequently, the DNA-containing supernatant is transferred to a new tube, and separation is followed up with with either column-based or precipitation-based DNA recovery procedures. It is important to note that this approach

causes some degree of DNA loss, because long fragments entangled with other cellular components are lost together with cellular debris. However, this avoids the use of organic solvents and recovers long DNA fragments.

Matrix-free protocols offer solutions for low-budget projects and/or situations in which personnel/time costs are not a major factor. The quality, quantity, purity, and integrity of the DNA obtained through phenol-chloroform extraction often surpasses those achieved by kit-based methods (Deiner et al., 2015), which is especially critical for applications such as long-read sequencing with platforms such as PacBio or MinION. However, matrix-free methods require adept pipetting skills to avoid the risk of carry-over of organic solvents, particularly phenol, which can be detrimental to downstream enzymatic reactions. It is essential to note that the chemicals employed in matrix-free procedures, such as phenol, pose risks to both users and the environment, necessitating the use of protective equipment, such as chemical hoods, and adherence to proper disposal procedures.

Steps 3 and 4: Removal of salts and DNA collection/concentration

Following the separation step, DNA is devoid of other cellular components but is not yet suitable for downstream applications. This is because it is either diluted in a substantial volume of a salty aqueous solution (e.g., following phenol-chloroform extraction) or locked in place on a solid phase in the presence of salts (when employing a matrix-based approach). Steps for concentraction/collection of DNA and removal of salts are required before DNA is used in downstream applications. DNA recovery and salt removal sequence may vary depending on the separation step. In matrix-free approaches, in which DNA is in a dissolved aqueous state, DNA recovery precedes salt removal. For DNA bound to a matrix, salts are removed before DNA recovery.

In both cases, "washing steps" rely on low-salt buffers and ethanol, and adjustments to washing steps are recommended when salt concentrations are too high for downstream applications. Repeating the washing step can solve this issue. However, it is crucial to bear in mind that each round of washing is associated with the degree of DNA loss. In both cases, the appropriate ethanol concentration is crucial. If ethanol concentrations are below 70%, which can occur through evaporation over time, serious DNA loss can occur during the salt removal process.

Matrix dependent: salt removal first, recovery second

Salt removal from matrix-bound DNA is accomplished by exposing the column or beads to wash buffer (low salt, 70% ethanol). Residual ethanol can be removed with a dry spin step; for example, columum-based extraction often suggest an additional centrifugation step with a clean collection tube. It is imperative to remove the residual ethanol to ensure the proper functionality of downstream applications. Instructions pertaining to this specific step should be adhered to.

For the recovery of DNA from a matrix - a process known as "elution" – the DNA bound to the matrix is exposed to a low salt buffer. This disrupts the ionic interactions between DNA and the matrix, allowing DNA to enter the solution. DNA can then be recovered from the matrix through centrifugation (columns) or pipetting (magnetic beads).

TE buffer is a commonly used elution buffer, although PCR-grade water can also be used. However, TE buffer is preferred for its capacity to maintain DNA stability during long-term storage, as discussed in the section on Storage Considerations; see also Panda et al. (2019). Notably, elution conditions can influence DNA yield, and various strategies can enhance recovery, such as reducing the elution volume to obtain more concentrated DNA, pre-warming the elution buffer, incubating the matrix with the elution buffer for an extended period (e.g., 10 min), or passing the eluted DNA sample through the filter for a second round. These techniques are frequently outlined in the troubleshooting sections of the respective DNA extraction kit protocols.

Matrix free: recovery first, salt removal second

To recover DNA from aqueous solutions, a process called precipitation is used to separate DNA from the liquid solution. DNA is then collected through centrifugation. DNA is a charged molecule that is highly soluble in water because of its interaction with polar water molecules. The addition of salts and alcohol to the sample disrupts these interactions and allows DNA to separate from water. Both ethanol (Xia et al., 2019) and isopropanol (Green & Sambrook, 2017) can be used for this purpose. Ethanol precipitation requires the addition of to 2-3 times the sample volume of alcohol, thus necessitating relatively large vessels. In contrast, isopropanol precipitation requires only 0.6-0.7 volumes of alcohol (Green & Sambrook, 2017), making it a better option for eDNA samples that tend to have low DNA content. The most commonly used salt is sodium acetate (Li et al., 2020); however, other salts that release positive ions can also be used.

Several factors influence the DNA efficiency and recovery during precipitation. These include: a) incubation time, where longer incubations, such as overnight incubation, can yield a higher quantity of DNA (Li et al., 2020); b) incubation temperature, where lower temperatures (from wet ice to -80 °C) can enhance recovery, especially for smaller DNA fragments; and c) carrier material, where small amounts of DNA are more readily recovered in the presence of substances such as glycogen or unrelated DNA. These conditions are usually not necessary for samples with high DNA concentrations, but if DNA recovery is unsatisfactory, precipitation at -20°C, overnight incubation, and the addition of a carrier material could be attempted. In addition, an extended precipitation step can serve as a convenient break in the day for lunch or meetings, or as a stopping point at the end of a workday, without adverse effects.

The precipitated DNA is then collected at the bottom of the tube ("pelleted") through high-speed centrifugation, typically in a cooled centrifuge. Here, extending the time or increasing the speed can enhance recovery, and extending the centrifugation step typically does not cause any damage to DNA. The ethanol-salt supernatant is then carefully removed by either pouring or pipetting, leaving the pellet behind.

To remove salt from the pellet, a wash buffer (usually 70% ethanol in molecular-grade water) is gently added to the pellet without disturbing it. Next, high-speed, chilled centrifugation is carried out with the tube placed in the same orientation as before to ensure the pellet remains in place. After centrifugation, which can be extended if it helps logistically, the supernatant is carefully removed without disturbing the sometimes nearly invisible pellet. The pellet is then air-dried at room temperature with the tube lid open, primarily to eliminate any residual ethanol that could interfere with subsequent steps. This process may take several hours to complete. Finally, the pellet is resuspended in a small volume of long-term storage buffer, most commonly TE or low-TE buffer (see the section on Storage Considerations).

Remedies for failed extractions

In the field of eDNA, samples are often exceptionally valuable. Repeating a failed experiment or reextracting additional samples is frequently not a viable option. Fortunately, some issues with DNA samples can be addressed post-extraction, to a certain extent.

For instance, samples with DNA concentrations too low for sequencing library preparation can be concentrated by vacuum centrifugation, re-precipitation, or re-elution in a smaller volume using a DNA cleanup column. These procedures may result in a small loss of DNA in terms of the total amount but can yield a sufficiently high DNA concentration per unit volume. Similarly, residual salt contamination can be addressed by dilution, re-precipitation, treatment with cleanup kits, desalting columns, or drop dialysis on dialysis membranes. Finally, PCR inhibitors are of particular concern for eDNA samples, as they can hinder amplification, even in well-concentrated and properly buffered samples. Remediation strategies for inhibition include sample dilution, addition of anti-inhibitory compounds such as BSA or commercially available anti-inhibitors to PCR reactions, or may be simply fixed with a vigorous centrifugation to collect inhibiting compounds at the bottom of the tube, followed by careful pipetting of the aliquot from the surface of the sample for PCR analysis.

Storage considerations

Working with eDNA samples often necessitates long-term storage, for example, for multi-year or seasonal comparisons, or to assess pre- and post-intervention status. An ongoing concern regarding storage is the stability and potential degradation of DNA, as suboptimal storage conditions can compromise perfectly extracted samples. In general, extracted and purified DNA is more stable than environmental samples, and storage of the DNA is preferred over storage of the samples.

Recommended conditions for long-term storage involve freezing at -20°C, or for extended periods, deep freezing at -80°C, within a suitable buffer, such as TE buffer (Panda et al., 2019). Notably, the repeated thawing and freezing of DNA samples can lead to fragmentation and degradation. Therefore, aliquoting before freezing may be practical if a sample is intended for multiple uses. Conversely, if a sample is to be used within approximately a week, storing it at 4°C after extraction and freezing only for long-term storage can be beneficial.

Furthermore, DNA exhibits greater stability at higher concentrations. To enhance stability, eluting or resuspending DNA in smaller volumes and supplementing it with unrelated DNA to elevate concentrations, such as commercially available high-molecular-weight mouse DNA, can be advantageous.

Additionally, DNA tends to adhere to plastic surfaces, which may reduce the available concentration in the sample, particularly in low-concentration samples. To mitigate adsorption, surfactants can be added to the sample (e.g., elution/resuspension in TET buffer [TE buffer (pH

7.4) and 0.05% Tween 20]. However, the use of low-binding tubes is typically sufficient.

Another storage consideration pertains to the specific requirements of downstream applications, which may require minimum DNA concentrations or may be sensitive to the composition of the storage buffer. For instance, low-EDTA TE buffers should be used if downstream methods are sensitive to chelators, such as EDTA, as is the case with some Next-Generation Sequencing (NGS) techniques.

In summary, prudent planning for storage, carried out ahead of time, and before initiating a particular DNA extraction protocol is highly recommended to ensure the integrity and usability of DNA samples in environmental research.

Conclusions

The selection, modification, or optimization of a DNA extraction protocol is fundamentally guided by the specific requirements of the project in terms of DNA quantity, bias, integrity, and purity (see Box 1). These requirements are established based on downstream applications and data-collection objectives. Once these needs are well-defined, this paper aims to provide field ecologists with the confidence to adapt and combine DNA extraction protocols and kits to meet their specific project requirements.

A primary step toward achieving this objective is the identification of the sections of a candidate protocol that correspond to Steps 1, 2, 3, or 4. Moreover, we encourage field ecologists to seek guidance from molecular biology experts and rely on resources available to the scientific community, including forums like Science Learning Hub, online protocol repositories such as Cold Spring Harbor Protocols, or printed references such as the Environmental DNA book, to enhance their knowledge.

Furthermore, it is important to recognize that even minor adjustments, such as extending a 2-hour step to an overnight incubation, introducing an extra freeze-thaw cycle, or doubling the lysis volume, can significantly enhance the handling properties of a protocol or the efficiency of the protocol. Understanding which steps are amenable to modification, omission, or substitution is essential for transitioning from morphological to molecular taxonomy and ecology, with success and without frustration.

Box 1. Defining project needs.

Amount. How much DNA is required for the downstream application? Does the aim of the project tolerate the loss of DNA, incomplete recovery, or hard-to quantify low-concentration DNA samples? For example, species-specific detection by PCR, which aims for a yes/no answer, is more tolerant to low DNA concentrations than is semi-quantitative community metagenomics.

Bias. How sensitive is the detection method regarding bias, and does bias matter at all? Does the aim tolerate unequal extraction of distinct organisms, or will this impact the result? For example, the detection of easy-to-lyse gram-negative prokaryotes is probably insensitive to bias, whereas the relative quantification of chitinous fungi with respect to the entire community may be affected by incomplete lysis.

Integrity. How intact should the DNA be? Does the detection process tolerate fragmentation well, or do downstream applications require long contiguous DNA fragments? For example, 16S metabarcoding with short amplicons is robust to fragmentation, whereas any application aimed at species-level resolution using long-fragment sequencing approaches requires more intact DNA.

Purity. How clean should the DNA be? How sensitive is the downstream detection method to contamination with salts, organic solvents, or other inhibitors? For example, next-generation sequencing approaches are less tolerant to contaminations than yes/no detections using conventional PCR.

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