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Tips & Tools

Bacterial DNA Extraction Using Individual Enzymes and Phenol/Chloroform Separation †

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INTRODUCTION

The investigation of environmentally and clinically significant microorganisms is often a key component in student microbiology laboratory projects. Many of these organisms, particularly bacteria, can be pathogenic and are often the causative agent of disease in both humans and other animals (1). Others play a pivotal role in the cycling of both organic and inorganic material within the environment, which in turn plays a significant role in the bioavailability of nutrients to higher-order organisms (2). Furthermore, bacterial communities play an essential role in human digestion through the breakdown of sugars and complex carbohydrates, with microbes outnumbering human cells by a factor of ten to one (3). Determining the identity of bacteria using molecular techniques provides information that can ultimately be used to positively manipulate pre-existing microbial communities or in the classification and treatment of disease.

The purification of genomic DNA from bacterial cultures provides the basis for downstream molecular analysis, and this process is often achieved using commercially available kits. Kits are packaged (and often combined) reagent sets that are designed to reduce the tedium behind multifaceted, complex procedures. They are user-friendly and efficient, albeit often at an increased cost, and are ideal for researchers who are already familiar with the methods. However, the abundance of commercial procedural kits in research and teaching laboratory environments has resulted in a lack of general understanding of the processes involved beyond the simple explanations given in the kits' instructions. Students can achieve the desired outcomes by following a simple protocol with no understanding of the fundamental biological and chemical processes involved. The lack of this essential knowledge can hamper scientists' ability to modify a procedure as required and troubleshoot if necessary. With bacterial genomic DNA extraction kits, such as the WIZARD Genomic DNA Purification Kit (Promega), the multiple active reagents are added as a sequence of proprietary solutions with vague names such as "Nuclei Lysis Solution" or "Protein Precipitation Solution." Though somewhat informative, these titles offer no information surrounding the enzymes/chemicals responsible for degradation of various cellular components. Therefore, in a teaching environment, it is more beneficial for the participants to use traditional methods that help engage the students to learn and understand rather than kits that have been optimized for simplicity.

The protocol developed by Marmur (4) was one of the first detailed methods developed for extraction of purified DNA from bacteria. Originally written as a proof of concept biochemistry paper, it was an invaluable contribution to the field of microbiology as a standard resource and has since been adapted by researchers to produce individualized DNA extraction procedures for their specific needs (5–7). The original text is written for a scientific audience and can be difficult to follow, especially for individuals with limited experience in molecular biology. Furthermore, as a result of decades of scientific advancement, particularly with commercial enzyme availability, the paper is outdated and contains several redundant steps and superfluous information. Here, we present a modernized method for DNA extraction and discuss how this can be incorporated into a learning environment. We also provide the following educational tools to assist in student development and understanding: Pre-Laboratory Questions (Appendix 1); Pre-Laboratory Answer Key (Appendix 2); DNA Extraction Flowchart (Appendix 3); and Agarose Gel Preparation for Electrophoresis (Appendix 4).

PROCEDURE

The following is a step-by-step procedure adapted from Marmur (4). Calculating the relative concentrations of each individual enzyme as well as deciphering the

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original protocol can be a problematic and tedious process. Through providing a detailed, modernized protocol, we aim to alleviate confusion that may have otherwise occurred. Furthermore, we have included a brief description of each enzyme/reagent used for teaching purposes and how it contributes to the isolation of bacterial genomic DNA for preparation (see Table 1). We recommend that all stock solutions be premade for the students.

All reagents used are prepared aseptically using sterile deionized water and stored as per manufacturer's instruction. Though cell wall composition can be determined using the Gram stain technique, this protocol assumes cell wall structure is unknown and includes lytic agents for both gram-negative and gram-positive bacteria. Unknown environmental isolates and subsequent cultures should be handled under Biosafety Level 2 (BSL-2) practices. BSL-2 guidelines should also be adhered to for steps a. through d. within this protocol. For further instruction on BSL-2 standard procedure, the reader is directed to "Biosafety Guidelines for Handling Microorganisms in the Teaching Laboratory: Development and Rationale" (8). BSL-2 has been recommended on the assumption that this protocol will be used in the identification of unknown environmental isolates. For other bacteria, appropriate biosafety precautions should be researched and implemented.

DNA EXTRACTION

a. Transfer 10 mL of mid- to late-log-phase culture (0.5 – 0.7 at OD_{600}) to a falcon tube and pellet the cells through centrifugation at 7,500 rpm for 10 minutes. Discard the supernatant.
b. Resuspend pellet with 467 μL RNase A in Buffer P1 and transfer to a 1.5-mL microcentrifuge tube. Add 8 μL lysozyme and 5 μL achromopeptidase, gently mix and incubate at 37°C for 60 minutes.
c. Add 30 μL 10% SDS (sodium dodecyl sulfate) and 3 μL proteinase K, gently invert and incubate at 50°C for 60 minutes.
d. Add 525 μL PCI (Phenol:Chloroform:Isoamyl) solution and mix for 10 minutes by gentle inversion. Centrifuge at 12,000 rpm for 15 minutes.
    i. Extreme care and personal protective gear (gloves, lab coats, and safety goggles) should be used when working with phenol as it is corrosive and may cause severe burns. This step should be completed in a fume hood.
e. Transfer the upper aqueous phase to a sterile 1.5-mL microcentrifuge tube, taking care not to disturb the bilayer.
f. Add an equal volume of -20oC 100% ethanol and gently mix by inversion. Centrifuge at 12,000 rpm for 20 minutes.
g. Carefully decant the supernatant and thoroughly dry pellet at room temperature or in a 50°C incubator.
    i. Over drying will result in making the DNA pellet more difficult to dissolve back into solution. The pellet may or may not be visible to the naked eye.
h. Resuspend the pellet in 50 μL TE (Tris-EDTA) buffer and allow pellet to sit overnight at 4°C.
i. Confirm presence and concentration of bacterial DNA by running 5 μL of product on a 1.5% agarose gel (Appendix 4). Purified DNA will appear as a defined band when visualized under UV light (see Fig. 1).

CONCLUSION

The extraction of DNA from microorganisms, especially bacteria, has become an essential method in nearly all modern microbiology research. Therefore, competent

TABLE 1.
Descriptions and required concentrations of enzymes/reagents used in DNA extraction.

Reagent	Concentration	Description
RNase A (ThermoFisher Scientific)	100 μg/mL	Degrades single stranded RNA. Buffer P1 is a resuspension buffer comprising 50 mM Tris-Cl (pH 8.0), 10 mM EDTA
Achromopeptidase (Sigma Aldrich)	50 kU/mL	Lysis enzyme with strong bacteriolytic activity against gram-positive bacterial cell walls
Lysozyme (Sigma Aldrich)	24,000 kU/mL	Lysis enzyme with bacteriolytic activity against gram-negative bacterial cell walls
Sodium dodecyl sulfate (SDS)	10%	Solubilization of cell membrane lipids
Proteinase K (ThermoFisher Scientific)	20 mg/mL	Digestion of proteins
Phenol:Chloroform:Isoamyl alcohol (PCI) solution		Separation of DNA from other cellular components. Comprised of Phenol:Chloroform:Isoamyl alcohol in a 25:24:1 ratio
Ethanol	100%	Precipitates DNA from solution
Tris-EDTA (TE) Buffer		Buffer solution used to store purified DNA comprised of 10 mM Tris (pH 8.0), 1 mM EDTA

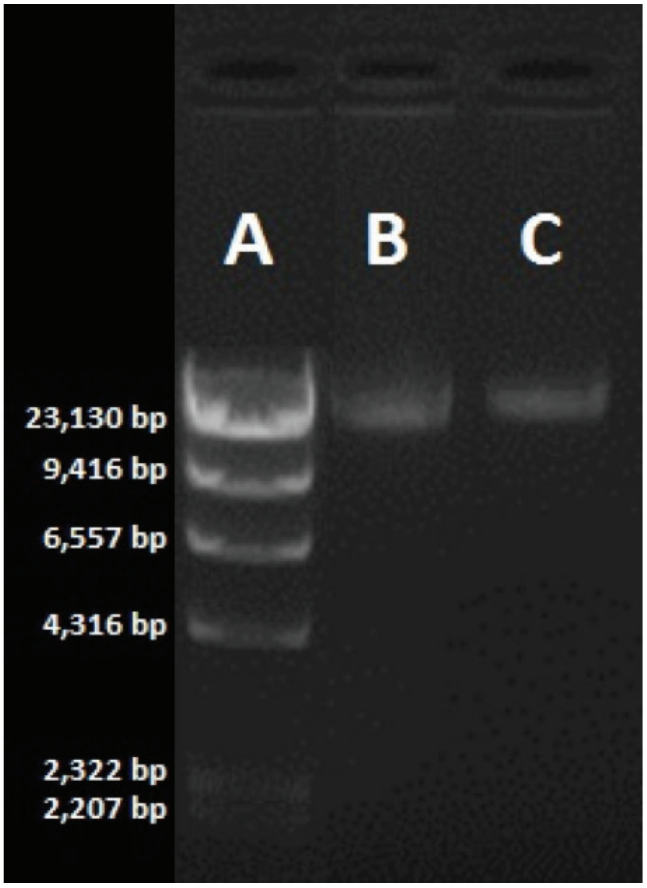

FIGURE 1. Agarose gel containing λHind III linear standard (A) and purified *Escherichia coli* genomic DNA (B, C). Visualized under UV light.

application and understanding of the procedure is fundamental to the education of any student aspiring to pursue a career as a researcher. The reported DNA protocol has been incorporated into an undergraduate molecular microbiology course for many years with consistent success. The project is designed to increase both the confidence and competence of students in the laboratory, thus enabling them to use acquired skills to complete more complex laboratory tasks either in research or as part of routine analyses. Students are given a semester-long project where they are to isolate, characterize, and identify an unknown environmental bacterium by combining both molecular and culturing techniques. After initially isolating an environmental bacterium, students are then required to purify the genomic DNA from their isolate and subsequently amplify and sequence the 16S rRNA gene as part of the molecular component of the course. Students are assessed on their understanding of technical aspects of the laboratory techniques, e.g., DNA extraction. Assessment is in the form of a seminar, with targeted questions, and a written report. Feedback suggests that students thoroughly enjoy and value this mode of teaching and assessment.

Students are taught the DNA extraction protocol as well other molecular techniques without the aid of kits as a means of increasing their knowledge and understanding of the science behind the mechanisms surrounding each individual constituent. Through using a step-by-step approach to DNA extraction, we allow students to leave the course with a much deeper understanding of this process. Furthermore, if students encounter problems along the way, they are in a position to analyze the process and troubleshoot where appropriate.

SUPPLEMENTAL MATERIALS

Appendix 1: Pre-laboratory questions
Appendix 2: Pre-laboratory answer key
Appendix 3: DNA extraction flowchart
Appendix 4: Agarose gel preparation for electrophoresis

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