Genomic DNA Purification Instructor's Manual

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I. Purpose and Concepts Covered

The purpose of this laboratory is to isolate DNA from cells. Students will become familiar with the reagents and steps necessary for DNA purification by isolating DNA from bananas using household solutions, then applying this knowledge to the purification of genomic DNA from cultured cells using a commercially available kit. A discussion about DNA quantitation using spectrophotometry or agarose gel electrophoresis, followed by ethidium bromide staining, is provided if you choose to include these topics in this laboratory exercise (Section V).

II. Introduction

In a cell, DNA acts as the genetic repository of information, and for this reason, scientists often study DNA to learn more about cellular biology. To manipulate or amplify DNA, scientists often must remove other cellular components that might interfere with their experiment, such as proteins, RNA and lipids, without damaging the DNA. The first scientist to purify DNA was a Swiss chemist named Johann Friedrich Miescher (1844–1895).

The term "leukocyte" refers to a broad class of white blood cells, which are involved in a body's immune response to infection and disease.

Miescher was working to isolate intact leukocytes from used hospital bandages. His initial attempts were unsuccessful and yielded a viscous lysate that was impossible to handle. However, by optimizing the composition of the salt solution in which the bandages were soaked, he was able to obtain intact cells. He then went on to develop a protocol to isolate intact nuclei from these cells. From these intact nuclei, Miescher extracted an acid-insoluble, alkali-soluble substance, which he named nuclein (1,2). At the time, scientists believed that cells were made up largely of protein, but Miescher determined that nuclein was not made up of protein because it was not digested by protease. Miescher soon demonstrated that nuclein was found in many other cells.

Nuclein was later renamed nucleic acid. Today we refer to it as deoxyribose nucleic acid (DNA) to distinguish it from other types of nucleic acids such as ribonucleic acid (RNA).

Miescher's original DNA purification protocol was crude, and thus, the resulting DNA preparation was not pure, preventing an accurate chemical analysis. One of his main concerns was protein contamination. Miescher set out to modify his original protocol to obtain pure nuclein relatively free of interfering protein. He turned to pepsin, a proteolytic enzyme that he extracted from pig stomachs, to eliminate proteins in the cells' cytoplasm. With this modified protocol (Figure 1; 1,2), he obtained nuclein with sufficient purity and in sufficient quantities to perform chemical analyses and show that nuclein contained carbon, hydrogen, oxygen and nitrogen but, unlike protein, large amounts of phosphorus and no sulfur.

Since Miescher's early experiments, much work has been done to optimize and simplify DNA purification. Today, DNA purification often is considered a routine laboratory process, and most scientists use existing systems, which take advantage of protocols based on chemical and physical properties of DNA. These systems allow scientists to focus their time and energy on other experimental goals.

Wash used hospital bandages with a dilute solution of sodium sulfate [1:10 dilution of cold saturated Glauber's salt ($Na_2SO_4 \cdot 10\ H_2O$) in water]. Filter the wash solution, and examine the cells under a microscope to ensure that leukocytes are intact.

↓

Wash leukocytes three or four times with "warm alcohol".

↓

Prepare pepsin solution by washing pig stomachs with a mixture of 10 ml of fuming hydrochloric acid and 1 liter of water. Filter the solution until it is clear.

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Treat the washed leukocytes with the pepsin solution at 37–45°C for 18–24 hours.
Change the pepsin solution twice during this time.
Allow the gray sediment to settle, then remove the aqueous layer.

↓

Add a mixture of water and ether to the sediment, and shake several times to obtain intact nuclei free of cytoplasm. Collect the resulting nuclei by filtration, and wash them with water until there is no trace of protein present.

↓

Add a 1:100,000 dilution of sodium carbonate to the nuclei, then add an excess of acetic or hydrochloric acid to obtain an acid-insoluble, flocculent precipitate (nuclein).

Figure 1. Miescher's modified DNA purification protocol.

III. DNA Purification from Bananas

III.A. Preparation for the Laboratory

Dispense 15 ml of isopropyl alcohol into each test tube. Be sure test tubes are sealed to prevent evaporation. Store the tubes at –20 °C prior to the laboratory exercise and on ice or in an ice-water bath during the lab.

III.B. Protocol

Wear gloves, lab coats, closed-toe shoes and protective eyewear whenever you are working in a laboratory.

Materials Required

- table salt
- distilled water
- clear liquid detergent containing EDTA
- 100 g piece of peeled banana
- mortar and pestle
- 60°C water bath
- ice-water bath
- beakers
- rubber band
- 100 ml graduated cylinder
- pipets or graduated cylinders capable of measuring volumes of 1–15 ml
- coffee filter
- fresh pineapple (canned pineapple cannot be substituted)
- test tubes
- test tubes containing 15 ml of ice-cold isopropyl alcohol (70–95%), kept on ice during the DNA purification
- rubber policeman or other stirring implement
- glass rod or wooden stir stick

Preparation of Detergent Solution

1. Combine 3 g of table salt and 80 ml of distilled water. Mix to dissolve the salt.
2. Add 10 ml of liquid detergent. Add water to bring the final volume to 100 ml. Mix gently to avoid foaming.

DNA Purification

Preparing the Lysate

1. Mash 100 g of banana in a mortar and pestle until you obtain a smooth pulp. Place the pulp in a 250 ml beaker, and add 100 ml of detergent solution. Mix gently but thoroughly.

   Note: Alternatively, the banana can be mashed on a clean, hard surface using a fork.
2. Place the beaker containing the homogenized banana in a 60 °C water bath for 15 minutes, mixing frequently.
3. Remove the beaker to an ice-water bath for 5 minutes to cool the pulp.
4. Insert a coffee filter into a clean beaker, fold the edge of the filter over the beaker rim and secure the coffee filter with a rubber band. The bottom of the coffee filter should be about an inch from the bottom of the beaker.
5. Carefully pour approximately 25 ml of homogenized banana into the coffee filter. After 5–10 minutes, you should have at least 5 ml of filtered solution. If the volume is less than 5 ml, add more homogenized banana into the coffee filter, being careful not to tear the coffee filter.

Removing Proteins (Optional)

6. Squeeze or crush a slice of fresh pineapple in a clean mortar and pestle to obtain at least 1 ml of juice. Pipet 1 ml of pineapple juice into a test tube.
7. Add 5 ml of filtered solution from Step 5 to the pineapple juice, and mix. Place the tube at room temperature for 2–3 minutes.

Precipitating the DNA

8. Tilt the test tube containing 15 ml of ice-cold isopropyl alcohol, and gently pipet the pineapple juice mixture down the side of the tube so that the mixture forms a layer on top of the isopropyl alcohol. Do not mix.
9. Incubate the test tube at room temperature for 4–5 minutes or until the DNA begins to precipitate. Record the appearance of the DNA in your laboratory notebook.
10. Insert the glass rod or stir stick into the tube, and slowly rotate it to spool the DNA onto the rod. Carefully remove the glass rod, and observe the purified DNA. Record the appearance of the DNA in your laboratory notebook.

III.C. Expected Results

The DNA precipitated in Step 9 will appear as milky white threads at the interface between the alcohol and filtered solution. Bubbles may form and become trapped by the DNA strands as the alcohol solution warms and gasses dissolved in the alcohol become less soluble. The DNA spooled onto the glass rod in Step 10 should be milky white or clear and stringy with a consistency of thick syrup.

III.D. Questions for Discussion

1. If no DNA was visible in Step 9 or 10, what are some possible explanations?
2. How would the appearance of the DNA change if the glass rod was removed from the alcohol solution and placed into a beaker of distilled water?
3. What was the role of each reagent in the DNA purification process? What was the purpose of each of the following steps:

   Mashing the banana?

   Adding the detergent solution?

   Heating the banana/detergent solution mixture at 60°C?

   Filtering the banana homogenate through the coffee filter?

   Adding pineapple juice to the banana homogenate?

   Adding the banana/pineapple juice mixture to the isopropyl alcohol?
4. What steps could you add to this protocol to obtain a more pure DNA preparation?

III.E. Troubleshooting

Symptom	Discussion
No visible DNA	Detergent solution was not prepared correctly. Add the specified amounts of salt and detergent, and bring the final volume to 100 ml with distilled water. Mix well before use.
	Banana was not homogenized sufficiently. Mash the banana until you obtain a smooth pulp. The detergent solution will not lyse cells in large pieces of banana efficiently, leading to poor DNA yield.
	Pulp and detergent solution were not well mixed. For efficient cell lysis, the pulp and detergent solution must be mixed well.
	Isopropyl alcohol was not ice-cold. Be sure to store the isopropyl alcohol on ice or in an ice-water bath prior to the DNA precipitation step. DNA will not precipitate as readily in room-temperature isopropyl alcohol.
	The filtered solution/pineapple juice mixture was not added to the isopropyl alcohol gently. Pipet the mixture slowly onto the isopropyl alcohol. Do not mix.
	Steps were not followed correctly, or the wrong reagents were used.

IV. DNA Purification from Tissue Culture Cells

IV.A. Preparation for the Laboratory

1. Prepare at least 1×10^4 cells to a maximum of 5×10^6 cells per purification. The number of cells may need to be adjusted depending on cell type and function. Be sure to incubate the cells in a tissue culture incubator overnight or until the cells have adhered to the plate.
2. Prepare the 1X phosphate-buffered saline (PBS) by combining:
   0.2 g KCl
   8.0 g NaCl
   0.2 g KH_2PO_4
   1.15 g Na_2HPO_4
   Add distilled water to a final volume of 1 liter.

IV.B. Protocol

Materials Required

- Wizard® SV Genomic DNA Purification System
- 1.5 ml microcentrifuge tubes
- 95% ethanol
- microcentrifuge
- pipets and pipette tips
- 1X phosphate-buffered saline (PBS)
- microcentrifuge tube rack
- 65°C water bath (optional)
- tissue culture plate containing 1×10^4 to 5×10^6 tissue culture cells

Preparation of Solutions

Prepare the Wizard® SV Wash Solution prior to beginning the Wizard® SV Genomic DNA Purification System protocol:

Add 95% ethanol to the Wizard® SV Wash Solution bottle as directed on the bottle label. Label the bottle to indicate that ethanol has been added. Carefully seal the wash solution, and store at room temperature.

Preparation of Tissue Culture Cell Lysates

1. Remove the cell culture medium from the cells. Wash the cells once with 1X PBS.
2. Add 150 µl of Wizard® SV Lysis Buffer to the washed cells in the tissue culture plate. Mix the lysate by pipetting.
3. For each lysate, prepare one Wizard® SV Minicolumn assembly. Each Wizard® SV Minicolumn assembly consists of a Wizard® SV Minicolumn and a Collection Tube. Label the Collection Tube, and place the Wizard® SV Minicolumn assembly in a microcentrifuge tube rack.
4. Transfer the entire sample lysate from the tissue culture plate to a Wizard® SV Minicolumn assembly.
5. Place the Wizard® SV Minicolumn/Collection Tube assembly containing the sample lysate into a microcentrifuge, and centrifuge at 13,000 × *g* for 3 minutes. If some lysate remains on the column after the initial spin, centrifuge again for 1 minute at 13,000 × *g*.
6. Remove the Wizard® SV Minicolumn from the Minicolumn/Collection Tube assembly, and discard the liquid in the Collection Tube. Replace the Wizard® SV Minicolumn into the Collection Tube.
7. Verify that the ethanol has been added to the Wizard® SV Wash Solution as described above.
8. Add 650 µl of Wizard® SV Wash Solution to each Minicolumn/Collection Tube assembly.
9. Centrifuge at 13,000 × *g* for 1 minute.
10. Discard the liquid in the Collection Tube, and replace the Wizard® SV Minicolumn into the empty Collection Tube.
11. Repeat Steps 8–10 three times for a total of four washes of the Wizard® SV Minicolumn.

12. After the last wash, empty the Collection Tube, and reassemble the Wizard® SV Minicolumn/Collection Tube assembly. Centrifuge at 13,000 × *g* for 2 minutes to dry the binding matrix.

    Note: RNA may be co-purified with genomic DNA. To remove co-purified RNA, add 2 µl of RNase A Solution per 250 µl of Nuclease-Free Water prior to elution of genomic DNA from the column. Once eluted, incubate purified genomic DNA at room temperature for 10 minutes. Alternatively, the RNase A Solution (2 µl) may be added following elution from the Wizard® SV Minicolumn.

13. Remove the Wizard® SV Minicolumn, and place it in a new, labeled 1.5 ml microcentrifuge tube. Add 250 µl of room-temperature Nuclease-Free Water to the Wizard® SV Minicolumn. Incubate for 2 minutes at room temperature.

    Optional: To improve DNA yield, heat the water to 65°C before adding it to the column for elution.

14. Place the Wizard® SV Minicolumn/elution tube assembly into the centrifuge, and centrifuge at 13,000 × *g* for 1 minute.

    Note: The total elution volume will be approximately 250 µl. Elution volumes of 250 µl are recommended for optimal DNA yield. Elution in volumes less than 250 µl will concentrate the DNA but will decrease the total DNA yield. A higher elution volume dilutes the DNA and does not improve yield.

15. Remove the Wizard® SV Minicolumn and discard. Cap the elution tube containing the purified genomic DNA, and record the appearance of the DNA in your laboratory notebook. If desired, store the DNA at –20°C to –70°C.

IV.C. Expected Results

The DNA eluted from the Wizard® SV Minicolumn will not be visible to the naked eye, and the final eluate will be identical in appearance to a tube of water because the DNA is soluble in water.

IV.D. Questions for Discussion

1. What was the role of each reagent in the DNA purification process? What was the purpose of each of the following steps:
   Washing the tissue culture cells?
   Adding Wizard® SV Lysis Buffer to the cells?
   Transferring the lysate to the Wizard® SV Minicolumn assembly?
   Washing the Wizard® SV Minicolumn with the Wizard® SV Wash Solution.
   Adding Nuclease-Free Water to the Wizard® SV Minicolumn?
2. What properties of the membrane in the Wizard® SV Minicolumn allow DNA to bind? In Step 12, there is an optional RNase A treatment of the Wizard® SV Minicolumn to remove RNA. Why would RNA also bind to the membrane?
3. Which steps of the two DNA purification protocols serve the same purpose? How do the mechanisms differ?
4. How would DNA yield be affected if you forgot to add ethanol to the Wizard® SV Wash Solution?

Supplemental Discussion Questions

1. Why did Johann Miescher get a viscous lysate when first trying to isolate intact leukocytes. What made the lysate viscous?
2. Discuss the role of each reagent in Miescher's modified DNA purification protocol.

IV.E. Troubleshooting

Symptom	Discussion
Low DNA yield	Tissue culture cells are low in genomic DNA. Genomic DNA yield may vary, depending on the number of cells used for the isolation. If yields are low, increase the amount of starting material to a maximum of 5×10^6 tissue culture cells.
	Wizard® SV Lysis Buffer was not added to washed cells. Make sure the Wizard® SV Lysis Buffer is added to all sample lysates.
	Steps were not followed correctly or wrong reagents were used. The Wizard® SV Genomic DNA Purification System is a multistep process that requires that the correct reagents are used in the correct order. This ensures that DNA remains bound to the membrane during the purification process. The Wizard® *Plus* SV DNA Purification System buffers are not compatible with this system and should not be used.
	Ethanol was not added to the Wizard® SV Wash Solution. Prepare the solution as instructed before beginning the procedure.
Clogged column	Lysate was too concentrated or viscous to pipet easily. If the lysate is too viscous, dilute with Wizard® SV Lysis Buffer until it becomes easy to pipet, then apply the entire lysate to the column.
	Too many cells were processed. A maximum of 5×10^6 cells can be processed on the Minicolumn membrane.

III.F. Additional Resources

Additional information about the Wizard® SV Genomic DNA Purification System can be found onthe Promega Web site at: **www.promega.com/tbs/tb302/tb302.html**

V. Methods for Determining DNA Yield and Purity (Optional)

Assessment of DNA yield can be carried out using many different methods, including absorbance (optical density) and agarose gel electrophoresis. Choosing which quantitation method to use is based on many factors, including access to equipment or reagents, reliability and consistency of the calculations to determine if the DNA can be used in downstream applications. Use caution when comparing yields between methods as the level of potential contaminants may cause variable determinations among the different methods. The following sections describe these optional protocols and include information on necessary accessories (e.g., equipment).

V.A. Estimation of DNA Concentration, Yield and Purity by Absorbance

The most common technique to determine DNA yield and purity is also the easiest method—absorbance. All that is needed is a spectrophotometer equipped with a UV lamp, UV-transparent cuvettes and a solution of purified DNA. Absorbance readings are performed at 260 nm (A_{260}), where DNA absorbs light most strongly, and the number generated allows one to estimate DNA concentration. Note that the spectrophotometer is most accurate when measurements are in the range of 0.1–1.0.

Spectrophotometric measurement of genomic DNA concentration is based on the Beer-Lambert equation, which defines the linear relationship between absorbance and concentration. Samples are diluted appropriately, absorbance is measured using a spectrophotometer and a quartz cuvette or 96-well plate, and the absorbance reading is used to calculate the concentration.

DNA concentration can be estimated by adjusting the A_{260} measurement for turbidity (measured by absorbance at A_{320}), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50µg/ml pure DNA.

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50 µg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

The A_{260}/A_{280} ratio can be used as an estimate of DNA purity [with a number of important limitations (3–5)]. An A_{260}/A_{280} ratio between 1.7 and 2.0 is generally accepted as representative of a high-quality DNA sample. The ratio can be calculated after subtracting the non-nucleic acid absorbance at A_{320}.

DNA purity (A_{260}/A_{280}) = (A_{260} reading – A_{320} reading) ÷ (A_{280} reading – A_{320} reading)

However, DNA is not the only molecule that can absorb UV light at 260 nm. Since RNA also has a great absorbance at 260 nm, and the aromatic amino acids present in protein absorb at 280 nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260 nm. Additionally, the presence of guanidine will lead to higher 260 nm absorbance. This means that if the A_{260} number is used to calculate yield, the DNA quantity may be overestimated (6).

To evaluate DNA purity by spectrophotometry, measure absorbance from 230 nm to 320 nm to detect other possible contaminants present in the DNA solution. The most common purity calculation is the ratio of the absorbance at 260 nm divided by the absorbance at 280 nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. The best test of DNA quality is functionality in the application of interest.

Strong absorbance around 230 nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. The ratio of absorbances measured at 260 nm and 230 nm can help evaluate the level of salt carryover in the purified DNA. The lower the ratio, the greater the amount of guanidine is present, for example. As a guideline, the A_{260}/A_{230} is best if greater than 1.5. A reading at 320 nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230 nm to 320 nm is most useful.

1. Dilute samples before measurement (if required). Samples must be diluted to give a reading within the linear range of the instrument used.
   - Usually a 1:10 dilution is appropriate. (e.g., 10µl of sample added to 90µl solvent).
   - The volumes measured in the spectrophotometer will depend on the format and minimum volume requirements of your specific instrument.
   - Quantitation of genomic DNA can be affected by the solvent used to dilute the genomic DNA before measurement (1). We recommend use of TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA [pH 8.0]).
2. Mix diluted samples by vortexing or pipetting several times.
3. Prepare the spectrophotometer to measure absorbance at 260nm, 280nm and 320nm.
4. Rinse a clean quartz cuvette with ultrapure water, and blot on a Kimwipes® tissue to remove excess liquid.
5. Add TE buffer to the cuvette, and either blank the spectrophotometer or read absorbance for autobackground correction.
6. Rinse cuvette 2 or 3 times with ultrapure water, and blot to dry. Read all samples, rinsing the cuvette between readings. If a 96-well plate reader is used, diluted samples and appropriate blanks can be added to the plate and read directly at 260 nm, 280 nm and 320 nm.

V.B. Estimation of DNA Concentration, Yield and Purity by Agarose Gel Electrophoresis

Agarose gel electrophoresis of the purified DNA eliminates the issues associated with absorbance readings. To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are needed for quantitation. The isolated DNA is loaded into a well of the agarose gel, then exposed to an electric field. The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity (7). Any RNA, nucleotides and protein in the sample migrate at different rates compared to the DNA, so the band(s) containing the DNA will be more pure.

Concentration and yield can be determined after gel eletrophoresis is complete by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the solution concentration is 50 ng/µl (100 ng divided by 2 µl). Standards used for quantitation should be the same size as the sample DNA being analyzed. To visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal (6).

We recommend separating genomic DNA on a 0.4–0.5% agarose gel at 1.5 V/cm for 12–24 hours. Handle these gels carefully because the low percentage of agarose results in soft gels that are difficult to handle. To reduce handling problems, a thin layer of supporting 0.8% gel can be poured and allowed to solidify prior to pouring the 0.4–0.5% agarose layer). To visualize DNA, ethidium bromide can be added directly to the gel, or the gel can be stained after electrophoresis using a DNA intercalating dye such as ethidium bromide or methylene blue. Protocols for preparing, running and staining agarose gels can be found in reference 7.

VI. References

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VII. Supplier and Ordering Information

Ordering Information

Product	Size	Cat.#
Wizard® SV Genomic DNA Purification System	50 preps	A2360
	250 preps	A2361

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