Preface

The era of molecular pathology has arrived. From its promising beginnings in research laboratories, the field has grown, and continues to grow, to become a vital part of the care of an ever-increasing number of patients.

Because of its recent emergence from the research laboratory, many molecular pathology protocols are still to be found in the primary literature, and have not appeared in a text. *Molecular Pathology Protocols* contains laboratory protocols that have been developed by many of the authors for use in clinical molecular pathology laboratories and describe in detail how to perform these assays. This book is therefore intended for clinical laboratory use by medical technologists and pathologists. It will also be of interest to research workers who are performing these assays.

In its broadest meaning, pathology is the study of disease, and therefore it follows that any disease for which the molecular basis is understood would be suitable as a topic for inclusion in this work. When selecting protocols, it was necessary to place limits on the number of chapters that could be feasibly presented in a single work. Those protocols that were selected are performed more frequently, or have achieved recognition as having important diagnostic utility in contemporary practice. A decision was made to exclude inherited genetic diseases with certain exceptions, such as those diseases that are associated with thrombotic states and are part of the traditional domain of pathology. Undoubtedly their etiology would have been of much interest to the great pathologists of the past. A small number of chapters describe methods that are available as commercial assays. These chapters, particularly in certain methodological details described in the notes, offer much fuller description of the methods than is often available.

As will be apparent, many of the protocols described have been developed as in-house methods by the authors. This point highlights a characteristic of the field of molecular pathology: the lack of standardization of most assays, even those in reasonably wide clinical practice. Unlike some other areas of clinical laboratory analysis, there are no definitive methods available for molecular pathology. It would be of advantage to the field, if only to provide consistency among laboratories, if there were to be agreement on

analytical issues such as probes and primer sets for various assays. These protocols offer methods that the authors have found to work in their laboratories. As with all clinical laboratory testing, the results should be interpreted in conjunction with other laboratory data and clinical findings.

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DNA Extraction from Fresh or Frozen Tissues

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1. Introduction

The first step in molecular analysis of patient tissues is preparation of purified, high molecular weight DNA. A number of methods and commercial kits are available for DNA isolation. Traditional organic extraction protocols ***(1,2)*** are based on the fact that DNA is soluble in water whereas lipids are soluble in phenol. In these protocols, tissues are disaggregated and then treated with detergent to lyse cell membranes followed by proteinase to digest proteins. Phenol, an organic solvent, is added to help separate the lipids and protein remnants from the DNA. Chloroform is then used to facilitate the removal of phenol. DNA is subsequently concentrated and further purified by precipitation in a cold mixture of salt and ethanol. Finally, DNA is resolubilized in Tris-EDTA buffer.

The traditional organic extraction procedure presented herein is used by many laboratories to obtain abundant high molecular weight DNA. However, in recent years, there has been a trend toward adoption of commercial nonorganic protocols that are faster and avoid the toxicity inherent with phenol exposure. A popular nonorganic extraction kit that works particularly well on blood and marrow samples is the Puregene DNA Extraction Kit (Gentra Systems, Minneapolis, MN). This kit can also be adapted for use on solid tissue samples for subsequent polymerase chain reaction (PCR) analysis.

2. Materials

2.1. Reagents

1. Lymphocyte Separation Media (ICN Biomedicals Inc., Aurora, OH).
2. 1X phosphate buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.

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3. Liquid nitrogen.
4. DNA extraction buffer: 10 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
5. 10% sodium dodecyl sulfate (SDS).
6. Proteinase K solution: 10 mg/mL of proteinase K in 50 m*M* Tris-HCl, pH 7.5; store at 4°C.
7. Phenol equilibrated with 0.1 *M* Tris-HCl, pH 8.0.
8. Chloroform: isoamyl alcohol (24:1).
9. 3 *M* Sodium acetate, pH 5.2.
10. 100% Ethanol.
11. 70% Ethanol.
12. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA.
13. Agarose.
14. 1X TAE buffer: 40 m*M* Tris-acetate, 1 m*M* EDTA.
15. Ethidium bromide (10 mg/mL).
16. 10X Gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll (type 400) in 10X TAE buffer.
17. DNA molecular weight marker.

2.2. Equipment

1. Mortar and pestle.
2. Water baths at 37, 50, and 55°C.
3. Centrifuge.
4. Spectrophotometer.
5. Horizontal gel electrophoresis apparatus.
6. DC power supply.

3. Methods

3.1. Sample Preparation

3.1.1. Tissue Specimen

1. Mince fresh, solid tissue up to 3 mm^3 into small pieces (1–2 mm) with a sterile scalpel blade. Process the tissue within 2 h of collection, or freeze at –20°C or colder until the time of DNA extraction (*see* **Note 1**).
2. Place the tissue in a clean mortar filled with liquid nitrogen. (*See* **Note 2** for cleaning instructions.)
3. Using a clean pestle, grind the frozen tissue to a powder while it is submerged in liquid nitrogen. While grinding, cover the mortar with a paper towel to keep tissue fragments inside the mortar, and work under a hood to protect yourself from aerosolized powder.
4. Allow the liquid nitrogen to evaporate, leaving a dry frozen tissue powder in the mortar.

3.1.2. Ficoll Separation of Mononuclear Cells from Blood and Marrow Aspirates

Prior to DNA extraction, mononuclear cells are isolated from anticoagulated blood or bone marrow aspirates by Ficoll centrifugation. (*See* **Note 3** for information about sample stability.) About 10^7 nucleated cells yield 40 μg of DNA for Southern blot analysis, and 3×10^6 cells yield sufficient DNA for amplification testing.

1. To a 15-mL conical tube, add 4.5 mL of blood and an equal volume of PBS. For bone marrow aspirates, use 1 mL of marrow and 8 mL of PBS. If less sample volume is available, use all of it. If greater sample volume is desired, split the sample evenly among two or more tubes so that all of it is processed, and then recombine the samples on collection of the mononuclear cell layer.
2. Using a Pasteur pipet, underlay the diluted blood or marrow with 3 mL of Ficoll solution (Lymphocyte Separation Media).
3. Cap the tube and centrifuge at 400*g* for 30 min at room temperature in a swinging bucket rotor.
4. Use a plastic Pasteur pipet to aspirate the mononuclear cell layer, which is the fuzzy white layer located between the plasma and the separation medium, into a clean 15-mL conical tube. Avoid the red cell layer at the bottom of the tube. (If no mononuclear cell layer is visible, *see* **Note 4**.)
5. Resuspend the mononuclear cells in PBS to 12 mL total volume.
6. Centrifuge for 10 min at 1700*g* at room temperature. Remove and discard the supernatant by pouring it off.
7. Store the cell pellet at –20°C temporarily or at –70°C long term, or proceed directly to DNA or RNA extraction.

3.2. DNA Extraction

3.2.1. Cell Lysis and Digestion

The procedure for solid tissue differs from that of blood or marrow mononuclear cells only in the first step.

1. For solid tissue, add 920 μL of DNA extraction buffer to the tissue powder in the mortar, and gently mix with the pestle. If the buffer freezes, wait until it thaws before proceeding. Then transfer the fluid to a 15-mL conical tube or microfuge tube by gentle pipeting. For a mononuclear cell pellet (about 10^7 cells), resuspend the cells in 920 μL of DNA extraction buffer and mix well by gentle pipeting.
2. Add 50 μL of 10% SDS to the mixture and mix well; the solution should become viscous.
3. Add 30 μL of proteinase K solution to the viscous mixture. Close the cap tightly and mix vigorously by repeated forceful inversion or vortex.
4. Incubate in a 37°C water bath for at least 6 h or as long as 2 d, or at 55°C for 3 h; gently invert the tube a few times during incubation.

5. The lysed sample should be viscous and relatively clear. This sample may be stored at 4°C for up to 1 wk before subjecting it to phenol/chloroform extraction as described in **Subheading 3.2.2.**, or before proceeding with nonorganic extraction as described in **Note 5**.

3.2.2. Phenol/Chloroform Extraction of DNA

1. Add an equal volume of equilibrated phenol, close the cap tightly, and mix gently by inversion for 1 min.
2. Spin the tube at 1700*g* in a swinging bucket rotor at room temperature for 10 min.
3. With a plastic pipet, aspirate the upper clear aqueous layer and transfer it to another clean labeled tube. This should be done carefully to avoid carrying over phenol or white proteinaceous material from the interface.
4. Repeat the phenol extraction (**steps 1–3**) one more time.
5. Next, extract with an equal volume of chloroform instead of phenol, and save the supernatant to another clean tube after centrifugation.
6. Repeat the chloroform extraction; this helps eliminate all of the phenol from the DNA sample.

3.2.3. Purification and Precipitation of DNA

1. To the aqueous DNA solution add 0.1 vol of 3 *M* sodium acetate (pH 5.2), and mix well by vortexing.
2. Add 2 vol of ice-cold 100% ethanol to the tube. Close the cap tightly and mix by inversion. A white cotton-like precipitate should form.
3. Use a sterile plastic rod to spool the precipitated DNA. (If no precipitate is visible, then microfuge at full speed for 10 min, rinse the pellet with 70% ethanol, air-dry for about 10–15 min, and proceed with **step 6**.)
4. Rinse the spooled DNA thoroughly in 1 mL of cold 70% ethanol by dipping.
5. Remove the DNA-coated plastic rod and allow the precipitate to air-dry until the white precipitate becomes clear, usually about 5–10 min.
6. Dissolve the precipitate in an appropriate volume of TE buffer (typically about 100–500 µL; targeting an optimal DNA concentration 1 µg/µL), scraping the rod along the wall of the microfuge tube to help detach the viscous DNA.
7. Allow the DNA to dissolve in the TE buffer for at least 4 h at 50°C, gently shaking periodically during incubation. Failure to adequately resolubilize the DNA will result in uneven distribution of DNA within the solution.
8. The purified DNA sample may be stored for 4 wk at 4°C prior to analysis, or indefinitely at –20°C.

3.3. DNA Quantitation by Spectrophotometry

1. Mix the DNA sample by gentle vortexing and inversion.
2. Add 5 µL of the DNA sample to 495 µL of sterile water and mix well.
3. Place the diluted sample in a quartz microcuvet and measure the absorbance at 260 and 280 nm against a water blank. (Nucleic acids absorb light maximally at 260 nm whereas proteins absorb strongly at 280 nm.)

4. Compute the DNA concentration based on the concept that an OD_{260} of 1 corresponds to 50 μg/mL of double-stranded DNA, and adjusting for the 100-fold dilution factor, according to the following formula:

$$\text{DNA concentration } (\mu g/\mu L) = OD_{260} \times 5$$

5. The $OD_{260}:OD_{280}$ ratio should be between 1.7 and 2.0. Lower values indicate protein contamination, in which case the DNA can be further purified by additional phenol/chloroform extractions followed by ethanol precipitation.

3.4. Gel Electrophoresis to Analyze DNA Quality

Agarose gel electrophoresis can be used to assess the intactness of purified DNA. High molecular weight DNA is needed for Southern blot analysis, whereas partially degraded DNA might be suitable for amplification procedures.

1. Prepare a 0.7% agarose gel in 1X TAE buffer containing 0.5 μg/mL of ethidium bromide.
2. Mix an aliquot of the extracted DNA sample with loading buffer, and load into a submerged well. Control samples representing intact and degraded DNA should be loaded into adjacent wells.
3. Electrophorese in 1X TAE buffer with 0.5 μg/mL ethidium bromide at 2 V/cm, until the dye front reaches the end of the gel.
4. View the gel under UV light. High molecular weight DNA is too large to migrate well under these conditions, whereas degraded DNA contains a spectrum of smaller fragment sizes that appear as a smear across the lane.

4. Notes

1. Solid tissue samples should be processed immediately or else frozen to minimize the activity of endogenous nucleases. If frozen tissue immunohistochemistry is planned, then slice the tissue into pieces no more than 5 mm thick and snap-freeze in liquid nitrogen or in a cryostat. If morphologic preservation is not needed, then place the tissue in a –70°C freezer indefinitely, or at –20°C for up to 3 d until DNA or RNA isolation.
2. After washing with detergent and rinsing well, soak the mortar and pestle in 50% nitric acid or 10% bleach to prevent carryover of DNA to the next case, then rinse well.
3. Peripheral blood or bone marrow aspirate anticoagulated with EDTA or acid citrate dextrose should be stored at room temperature and processed as soon as possible. EDTA beneficially chelates ions to inhibit nucleases from degrading nucleic acid, and consequently DNA and RNA are often stable for up to 48 h at room temperature. Heparin anticoagulant is not recommended because residual heparin may interfere with subsequent restriction enzyme or DNA polymerase activity.

4. If no mononuclear cell layer is visible, follow these steps to recover mononuclear cells from the red cell pellet. (This procedure may be particularly helpful if the blood has been previously refrigerated, thus causing the white cells to clump and sediment with the red cells at the bottom of the tube. For this reason, refrigeration of samples is not recommended prior to Ficoll centrifugation.) Carefully remove and discard the supernatant with a Pasteur pipet. Avoid disturbing the red cell pellet. Add 3 vol of Puregene RBC Lysis Solution (Gentra Systems) to the pellet. Invert to mix several times, and incubate for 10 min at room temperature. Invert again once during the incubation. Centrifuge at 2000*g* for 10 min, remove the supernatant with a Pasteur pipet, and discard it. The mononuclear cell pellet is not always visible at this step, and if not, you may use half-volumes of the extraction reagents in the subsequent steps of extraction.
5. To avoid the use of organic solvents, numerous nonorganic extraction protocols have been developed. We recommend the Puregene DNA Extraction Kit (Gentra Systems) for blood and marrow aspirates. To adapt this kit for PCR analysis of solid tissues, follow the protocol in this chapter through SDS and proteinase K digestion. Then apply the Puregene kit according to the manufacturer's instructions, skipping the red blood cell lysis and cell lysis steps.

References

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