| Protocol |

DNA Extraction from Formalin-Fixed, Paraffin-Embedded Tissue

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INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) tissue is one of the most widely practiced methods for clinical sample preservation and archiving. It is estimated that, worldwide, over a billion tissue samples, most of them FFPE, are being stored in numerous hospitals, tissue banks, and research laboratories. These archived samples could potentially provide a wealth of information in retrospective molecular studies of diseased tissues. While standard for histopathology and microscopic investigation (e.g., hematoxylin and eosin [H&E] staining, immunohistochemistry [IHC], and tissue microarray [TMA]), FFPE samples pose a major challenge for molecular pathologists, because nucleic acids are heavily modified and trapped by extensive protein-nucleic acid and protein-protein cross linking. Historically, FFPE samples were not considered to be a viable source for molecular analyses. Recently, however, it has been discovered that with appropriate protease digestion, it is possible to release microgram amounts of DNA and RNA from FFPE samples. The purified nucleic acids, although highly fragmented, are suitable for a variety of downstream genomic and gene expression analyses, such as polymerase chain reaction (PCR), quantitative reverse transcription PCR (qRT-PCR), microarray, array comparative genomic hybridization (CGH), microRNA, and methylation profiling. Several commercial kits are currently available for FFPE extraction. The protocol reported here is adapted from the Ambion RecoverAll Total Nucleic Acid Isolation Kit, but includes several modifications. Although our protocol focuses on DNA isolation, the RecoverAll Kit can also be utilized to recover RNA, including microRNA.

RELATED INFORMATION

Methods for quantifying DNA and RNA are described in Quantitation of DNA and RNA (Barbas et al. 2007).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

Reagents

- Ethanol (ACS grade)
- FFPE tissue sections or cores

**METHOD**

**Deparaffinization**

The time required for this portion of the method is 20-30 min.

1. Transfer FFPE sections or cores to a 2-mL microcentrifuge tube.  
   Up to 80-µm sections or 35-mg unsectioned cores (needle punch) can be processed in one tube as per RecoverAll instructions.

2. Add 950 µL of xylene. Vortex briefly. Make sure all tissue and paraffin is immersed under the xylene.  
   The standard protocol uses 1 mL of xylene. However, 950 µL is usually sufficient for deparaffinization. A smaller volume streamlines waste removal (Steps 5, 8, and 9) if using a pipette set at 1 mL.

3. Place the tube in the 50°C heat block for 3 min to melt the paraffin.

4. Centrifuge the sample at maximum speed for 2 min at room temperature to collect the tissue.  
   This step may be omitted to increase sample prep speed if needle punch cores are used, because they settle readily without centrifugation.

5. Using a 1-mL pipette, remove as much xylene as possible without disturbing the tissue pellet.

6. Add 950 µL of 100% ethanol. Vortex briefly.

7. Centrifuge the sample at maximum speed for 2 min at room temperature to pellet the tissue.  
   This step may be omitted to increase sample prep speed if needle punch cores are used, because they settle readily without centrifugation.

8. Using a 1-mL pipette, remove as much ethanol as possible without disturbing the tissue pellet.

9. Repeat the ethanol wash (Steps 6-8).

10. Briefly centrifuge the sample and remove any residual ethanol with a fine pipette.

11. Air-dry the pellet for 15 min or until no large ethanol droplets are visible.

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**Equipment**

- Heat blocks at 50°C and 95°C
- Microcentrifuge capable of 10,000 g
- Microcentrifuge tubes (2 mL)
- Microtube shaker capable of heating to 50°C (Labnet VorTemp or equivalent)

Alternatively include a heat block (Step 13), water bath (Step 13), or oven (Steps 13 and 28).

- Pipettes (1 mL and fine)
- Vacuum concentrator (optional; see Step 40)
- Vortex mixer

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**H₂O (nuclease-free PCR-grade)**  
RecoverAll Total Nucleic Acid Isolation Kit (Ambion AM1975) components for DNA isolation:  
- Collection tubes  
- Elution solution  
- Digestion buffer  
- Filter cartridges  
- Isolation additive  
- Protease  
- RNase A  
- Wash 1 concentrate  
- Wash 2/3 concentrate

=Xylene (ACS grade)
Protease Digestion

The time required for this portion of the method is 48 h.

12. Add 400 µL of digestion buffer and 4 µL of protease to each sample. Make sure that the tissue is completely covered with the digestion solution.
   To ensure consistency, prepare a master mix of digestion buffer and protease in sufficient quantity to process all of the samples.

13. Incubate the tubes for 48 h in a microtube shaking incubator set at 50°C and a moderate speed (e.g., 900 rpm).
   A shaking incubator will maximize digestion efficiency. However, a heat block, water bath, or oven may be used if a shaker is unavailable. In those cases, flick the tubes from time to time to facilitate better digestion.

14. Proceed to DNA isolation or store the digests at −20°C.
   This is a convenient stopping point if multiple batches of samples are to be processed for high-throughput FFPE DNA isolation (see Discussion).

DNA Isolation

The time required for this portion of the method is 10 min.

15. Add 480 µL of isolation additive to each sample. Vortex briefly to mix.

16. Add 1100 µL of 100% ethanol to the sample. Carefully pipette up and down to mix.

17. Assemble a filter cartridge in a collection tube.

18. Transfer 700 µL of the digest/ethanol mixture onto the filter cartridge assembly and close the lid. Centrifuge at 10,000g for 30 sec.
   Depending on the sample quantity and quality, not all tissue may be completely digested. Avoid transferring large undigested pieces to prevent filter clogging.

19. Discard the flow through. Reinsert the cartridge in the same collection tube.

20. Repeat Steps 18 and 19 two more times until all of the digest/ethanol mixture has passed through the filter.

21. Add 700 µL of wash 1 to the filter cartridge. Centrifuge at 10,000g for 30 sec.
   Be sure to add ethanol to wash 1 concentrate according to the manufacturer’s instructions.

22. Discard the flow through. Reinsert the cartridge in the same collection tube.

23. Add 500 µL of wash 2/3 to the filter cartridge. Centrifuge at 10,000g for 30 sec.
   Be sure to add ethanol to wash 2/3 concentrate according to the manufacturer’s instructions.

24. Discard the flow through. Reinsert the cartridge in the same collection tube.

25. Centrifuge at 10,000g for an additional 30 sec to remove any residual liquid from the filter.

On-Filter RNase Digestion and Final DNA Purification

The time required for this portion of the method is 45 min.

26. Prepare the RNase mix by combining 10 µL of RNase A and 50 µL of nuclease-free PCR-grade H₂O.
   To ensure consistency, prepare a master RNase mix in sufficient quantity to process all of the samples.

27. Add 60 µL of the RNase mix directly to the center of the filter cartridge.
   Make sure the entire filter is covered. Avoid “dry spots” or leaving the RNase mix on the cartridge wall.

28. Close the cap of the cartridge assembly and place in the microtube incubator or an oven. Incubate for 30 min at 37°C.
   Shaking is not necessary at this step.

29. Add 700 µL of wash 1 to the filter cartridge. Incubate for 1 min at room temperature.

30. Centrifuge at 10,000g for 30 sec.

31. Discard the flow through. Reinsert the cartridge in the same collection tube.
32. Add 500 µL of wash 2/3 to the filter cartridge. Centrifuge at 10,000g for 30 sec.
33. Discard the flow through. Reinsert the cartridge in the same collection tube.
34. Repeat Steps 32 and 33.
35. Centrifuge at 10,000g for an additional 1 min to remove any residual liquid from the filter.
36. Transfer the filter cartridge to a new collection tube.
37. Add 30 µL of elution solution or PCR-grade H₂O, preheated to 95°C, directly to the center of the filter.
   It is critical to use heated elution solution or H₂O. Using room temperature solution will significantly reduce DNA yield.
38. Close the cap and incubate for 1 min at room temperature.
39. Centrifuge for 1 min at maximum speed.
40. Repeat Steps 37-39 using the same collection tube.
   The total elution volume is ~60 µL. If more concentrated product is needed, use a vacuum concentrator.

DISCUSSION

According to a recent report, the number of archived tissue samples is estimated to be over 1 billion worldwide (Blow 2007). Thanks to advances in nucleic acid isolation technology, these archived samples, predominantly FFPE tissue, which were once considered unusable, are becoming increasingly accessible to genomic and gene expression analyses. DNA/RNA isolated from FFPE sources have been successfully used for PCR, qRT-PCR (Lehmann and Kreipe 2001), microarray (Abramovitz et al. 2008), array CGH (Little et al. 2006), microRNA (Li et al. 2007), and methylation profiling (W. Tang, B.G. Barwick, and M.M. Bouzyk, unpubl.).

Because of the nature of the FFPE process, DNA yield from such samples is expected to be relatively low. From our experience, a good yield is typically 1-3 µg from three 5-µm sections, although this will, to a large extent, depend on the quality and the usable amount of the sections. In addition, prolonged exposure to air during sample storage will further contribute to oxidation. One way to help circumvent this problem is to prepare the sections fresh, and discard the first two slices, which are the most oxidized. Typically, the DNA obtained from FFPE samples will be a population of fragments of various sizes ranging from <100 bp to >3 kb. For most PCR-based downstream genotyping applications, such as ABI TaqMan, Beckman SNPstream, or Illumina GoldenGate assays, fragmentation down to 500 bp is acceptable. However, there may be cases where a larger fragment size is needed, such as Illumina Infinium, which requires at least 2 kb. For quality control, either agarose gel electrophoresis or an Agilent microfluidics bioanalyzer could be used. We prefer the bioanalyzer, as it consumes much less DNA (nanogram amounts as opposed to typically 1 µg for gel). In addition, DNA purity should be measured by means of optical density at 230, 260, and 280 nm. A good quality DNA sample should have an OD₁₆₆/OD₂₈₀ value between 1.7 and 1.9, and an OD₂₆₀/OD₂₈₀ above 1.3 (Quantitation of DNA and RNA [Barbas et al. 2007]). We recommend a NanoDrop spectrophotometer (Thermo Scientific) because it requires only 1-2 µL of sample.

The protocol presented here is well suited for low-throughput manual extraction. For high-throughput needs, a RecoverAll/MagMAX custom kit (Ambion/Applied Biosystems) is ideal. After deparaffinization and protease digestion, the samples can be stored at -20°C until a large batch is collected. The MagMAX kit utilizes magnetic beads to purify DNA from the RecoverAll digest, in a 96-well format. In addition, a liquid handling robot or a magnetic particle processor, such as the KingFisher (Thermo Scientific), can be programmed to perform the magnetic beads purification in an automated fashion, thus further increasing the throughput. We are currently optimizing an automated protocol for large-scale genomics studies.

REFERENCES


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