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SAMPLE PREPARATION FROM PARAFFIN-EMBEDDED TISSUES

Deann K. Wright and M. Michele Manos

The ability to study preserved tissues at the molecular level makes possible retrospective studies on large numbers of patients and may permit the tracking, over long periods of time, of genetic changes or infectious agents that are associated with diseases.

The most common method for preserving human tissue is fixation in formalin followed by paraffin embedding. Several research groups have performed Southern blot analyses on DNA extracted from such specimens (Goelz *et al.* 1985; Dubeau *et al.* 1986). While this approach can provide invaluable information, it is extremely laborious and therefore not suitable for the examination of large numbers of samples. DNA from old or improperly fixed samples is often degraded and cannot be analyzed by Southern blotting.

The use of PCR to examine the DNA in fixed, paraffin-embedded tissues provides a relatively simple and extremely sensitive method for examining large numbers of samples. (Impraim *et al.* 1987; Shibata *et al.* 1988a) Additionally, PCR analysis of archival tissues can be accomplished with 5- to 10-μm sections, whereas Southern blot analysis requires larger amounts of tissue. PCR does not require high-molecular-weight DNA and therefore allows the analysis of deteriorated specimens that may be inappropriate for Southern blot

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analyses. This includes tissue specimens ranging from several years old to over 40 years old. (Shibata *et al.* 1988b; Impraim *et al.* 1987).

Shibata *et al.* (1988a) have reported a simple method for the preparation of paraffin-embedded tissue sections for PCR. The method allowed amplification of 100-bp products from sections that were deparaffinized and boiled before PCR. We have developed improved methods that will have a broader spectrum of uses (Manos *et al.* 1989 and this chapter). The procedures provide increased product yield and have been used to amplify products of over 800 bp from intact samples.

Protocols

Reagents

Tissue sections (prepared as follows)
Octane or xylene
100% ethanol
HPLC-grade acetone (optional)
Proteinase K (20 mg/ml stock solution)
Digestion buffer [50 m*M* Tris (pH 8.5), 1 m*M* EDTA, plus 1% Laureth 12 (Mazer Chemicals, Gurnee, IL) or 0.5% Tween 20]

Tissue Section Preparation

Prepare sections (5 to 10 μm wide) from blocks of fixed (preferably with buffered formalin), embedded tissue. If possible, trim excess paraffin from the block before slicing. Cut the sections and remove them from the microtome dry (if the sections are hydrated, the paraffin is extracted less efficiently). Handle the sections with clean tweezers or toothpicks and place (one per tube) into 1.5-ml microfuge tubes. To avoid cross-contamination of samples, the microtome blade, tweezers, and anything else in the cutting area that the samples may have touched should be carefully cleaned with xylene between each block. Label the tubes on the caps with permanent ink.

Deparaffinizing Sections

Each section is extracted twice with octane to remove the paraffin. Alternatively, mixed xylenes can be used for this extraction. This organic extraction is followed by two washes with 100% ethanol to remove the solvent. The ethanol is removed by drying the samples under vacuum or by rinsing the sample with acetone.

1. Add approximately 1 ml of octane to each tube. Close tubes and mix at room temperature for about 30 minutes.
2. Pellet the tissue and any residual paraffin by centrifugation (3 to 5 minutes in microfuge at full speed).
3. Remove the octane from each sample with a clean (preferably plugged) Pasteur pipette. (Very old or fragile tissues often fragment when the paraffin is removed, so care must be taken to avoid losing tissue in this step.)
4. Repeat steps 1, 2, and 3.
5. Add approximately 0.5 ml of 100% ethanol to each tube. Close and mix by inverting.
6. Pellet as in step 2.
7. Remove the ethanol as done with the octane in step 3.
8. Repeat steps 5, 6, and 7. Remove as much ethanol as possible with the pipette.
9. Dry the samples under vacuum until the ethanol has evaporated completely. Before drying the samples, cover the tubes by stretching Parafilm across the top of the tube and poking several holes in it. This precaution helps to avoid cross-contamination of samples and contamination from the vacuum bottle.

OR

Add 2 to 3 drops of acetone to each tube. Keeping the tubes open, place them carefully in a heating block or water bath (37 to 50°C) to promote the evaporation of the acetone.

Proteinase Digestion

1. Add 100 μl of digestion buffer (above) containing 200 μg/ml of Proteinase K to the extracted, dried samples. Samples containing large amounts of tissue should be resuspended in 200 μl of digestion buffer.

2. Incubate for 3 hours at 55°C (alternatively, 37°C overnight).
3. Spin the tubes briefly to remove any liquid from the cap.
4. Incubate at 95°C for 8 to 10 minutes to inactivate the protease. Avoid heating longer than 10 minutes.
5. To use for PCR, pellet any residual paraffin or tissue by centrifuging for about 30 seconds. Use an aliquot of the supernatant for the amplification (typically 1 to 10 μl).
6. Store prepared samples at −20°C.

PCR

The amount of supernatant that is optimal for an amplification reaction is determined by numerous factors and may be specific to each sample. It is useful to test several concentrations of each sample in each amplification (e.g., 1 versus 10 μl per 100-μl PCR). Samples extracted from sections that contained large amounts of tissue may necessitate the use of a smaller fraction of the supernatant (e.g., 0.1 μl). The amount of supernatant that is "tolerated" in a PCR may be affected by residual fixation chemicals, excessive tissue debris, etc.

Amplification from prepared paraffin-embedded tissue is less efficient than amplification from other types of templates such as purified DNA or extracts from fresh clinical material. To compensate for this reduced efficiency, we modify the thermocycling parameters by increasing the number of cycles and lengthening the time at each temperature within the cycle.

A comparison of thermocycling conditions for amplification of the human papillomavirus L1 product (see Chapter 42) from purified DNA (or fresh clinical material) versus amplification from a paraffin sample follows.

	Purified DNA	Paraffin sample
95°C	30 seconds	1 minute
55°C	30 seconds	1 minute
72°C	1 minute	2 minutes
	30 cycles	40 cycles
3 to 5 minutes	72°C	(final extension)

Discussion

Preparation of paraffin-embedded tissue sections for PCR involves a great deal of manual manipulation (e.g., Eppendorf tubes are opened and closed and transferred from centrifuge to rack, relatively large volumes of solvents are added to and removed from the samples, tubes may be covered and uncovered with Parafilm). This provides ample opportunity for cross-contamination between samples or contamination from extraneous PCR products or plasmids within the work area. In the extraction/digestion procedure, common vehicles for such contamination are the experimenter's gloves (or fingers). Caps and rims of microfuge tubes are most susceptible to this. We recommend frequent glove changes. When opening and closing tubes, do not touch the rims or insides of caps and be careful to keep tubes adequately spaced in racks. If samples are dried under vacuum, cover tubes and caps with Parafilm to minimize cross-contamination. After it has been removed from the tubes, the Parafilm should be considered contaminated waste.

Amplification of extracted, digested samples that have been stored is not always as successful as is amplification of samples that have been amplified immediately after digestion. Possibly storage or freeze/thawing somehow promotes breaking or nicking of the DNA.

We have noticed a length limit on amplification products from extracted, digested tissue from formalin-fixed, embedded sections. PCR products in the range of 450 to 650 bp can be amplified from most sections of this type (unless the blocks are very old). Larger products can be amplified from some, but not all, of these samples. The integrity of the DNA (or the extent of modification or crosslinking) is likely related to the fixative used and the fixation time (Dubeau *et al.* 1986). Particular fixatives (other than buffered formalin) may modify DNA and thus reduce the amplification "competency" of the section. Alternatively, particular fixatives may contain chemicals that inhibit PCR. We are currently investigating the effects of a variety of fixatives. The inhibitory effects of certain fixatives can be overcome by further purification (phenol-chloroform extraction and ethanol precipitation after the protease digestion) of the tissue extracts. Since such further purification involves much manipulation and therefore more potential for contamination, it is not recommended routinely.

Because of the increased number of amplification cycles and the extended annealing and polymerization times used in PCR of paraffin samples, nonspecific products are often produced in addition to the specific product(s). Such nonspecific products are often visible by ethidium bromide staining, and they are most often smaller than the specific product. However, on a Southern blot, these products typically do not hybridize with a probe specific for the desired product. The presence of nonspecific products complicates purification of the desired product and may affect procedures such asymmetric PCR or direct sequencing.

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