PCR Amplification from Paraffin-embedded Tissues: Recommendations on Fixatives for Long-term Storage and Prospective Studies

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The development of polymerase chain reaction (PCR) DNA amplification methods has afforded molecular studies of fixed paraffin-embedded tissue samples and other archival material. Some fixation methods damage DNA, and thus deleteriously affect subsequent PCR analysis. This study addressed the effect of short- and long-term storage (2 hr to 30 days) in a variety of fixatives (10% buffered-neutral formalin [BNF], 95% ethanol, acetone, and OmniFix) before paraffin embedding. We tested the ability of prepared tissue sections to yield DNA amplification products ranging from 268 to 1327 bp. Results indicated that tissues fixed for 8 days in BNF were able to amplify 536-bp but very little 989-bp DNA fragments; after 30 days of BNF fixation only a 268-bp fragment was amplifiable. Samples fixed in OmniFix and acetone yielded products of 989 and 1327 bp, respectively, after 8 days of fixation; both fixatives yielded 989-bp amplification products after 30 days of fixation. Tissues fixed in 95% ethanol for up to 30 days efficiently produced DNA amplification fragments of up to 1327 bp in length. The results provide important information for prospective studies that involve PCR analysis from archival material. Furthermore, fixation and long-term storage in ethanol should prove par-

ticularly useful in remote areas where refrigeration or immediate sample-processing is unavailable.

The polymerase chain reaction (PCR) DNA amplification method^(1,2) is a powerful tool for the retrospective analysis of fixed, paraffin-embedded tissues (PET)⁽³⁻⁷⁾ and other archival material.⁽⁸⁾ However, not all preservation or fixation methods render DNA that is suitable for subsequent amplification.^(7,9-10) Recently, we reported the effects of commonly used fixation methods on the efficiency of subsequent PCR amplification.⁽¹¹⁾

The effect of fixation was assessed by the ability of the DNA in a treated tissue to serve as a template for the amplification of DNA fragments of various lengths. We determined that the less damaging a fixation process was to sample DNA, the longer the resulting amplification products could be. The effect of each fixation method was clearly reflected by the maximum product length that could be obtained from each fixative-treated tissue. Of the tissues tested, those most successful in subsequent PCR amplifications were those fixed in 10% buffered-neutral formalin (BNF) and acetone. A second group of fixatives including Zamboni's, Clarke's, paraformaldehyde, formalin-alcohol-acetic acid, and methacarn compromised amplification efficiency.

Tissues fixed in highly acidic solutions (Carnoy's, Zenker's, or Bouin's) were even less desirable for amplification analysis.

Most fixation procedures tested afforded the subsequent amplification of fragments 536 bp or greater in length. However, in cases where the DNA was compromised, it was recommended that a scheme involving smaller amplification products (less than 200 bp) was desirable. This approach applies also to older (greater than 5 years) paraffin-embedded tissue blocks because the maximum obtainable amplification fragment length decreases with age (C. Wheeler, pers. comm.).

The results from our first fixative study indicated that, after 24 hr of fixation, the ability to amplify large products decreased with all fixatives tested except acetone. Although many clinical laboratories routinely fix tissues for 24 hr or less, some tissues may be treated for up to several days. For example, biopsies are often placed in buffered formalin and shipped to reference laboratories for embedding and analysis. A more severe situation exists when medical studies are conducted in remote regions where samples require fixation and storage for extended periods of time before analysis. The need for specimen storage also exists in disciplines such as zoology, botany, conservation biology, and evolutionary biology, where scientists interested in biologic diversity and ecological processes require

fixation methods that will preserve precious specimens for future study.⁽¹²⁾

These challenges impelled us to seek fixation procedures that would allow at least several days to weeks of storage and maintain amplification-competent DNA and definable histologic detail. In this paper, we compared amplification of DNA fragments from tissues subjected to four different methods of fixation for fixation times ranging from 2 hr to 30 days.

METHODS

Fixatives and Tissue Processing

A fresh tonsil, without signs of autolysis, was cut into pieces approximately 6 × 6 × 8 mm. These tissue pieces were processed in each fixative for 2, 24, 72 hr, 8 days, and 30 days. Additionally, a set of four pieces was stored in a petri dish at 4°C for 24 hr, then processed for 24 hr in the respective fixatives.

Tissues fixed in 10% buffered neutral formalin (Fisher Scientific) were processed and embedded using a VIP processor (Baxter Scientific) that included two 10% alcohol formalin incubations, dehydration through three Pro-Soft (Anatech, Ltd., Battle Creek, MI) washes, and three xylene washes prior to paraffin embedding. Pro-Soft was used as an alcohol substitute to reduce sample brittleness. Fixation with OmniFix (American Histology Reagent Co., Stockton, CA) or acetone was followed by 1-hr washes with 80% and 95% ethanol and three 1-hr 100% ethanol washes, followed by three xylene incubations prior to embedding. Tissues fixed in 95% ethanol were subjected to three 100% ethanol and three xylene incubations before embedding. Tissues treated in OmniFix, 95% ethanol, or acetone were processed by hand rather than in the VIP processor.

Hematoxylin and eosin (H&E)-stained sections were made from each paraffin block. The amount of tissue per section ranged from 0.2 to 0.5 mm³.

Preparation of Samples for PCR

Dry sections, 6 µm thick, were sliced from each tissue block and placed in a 1.5-ml siliconized microfuge tube with clean forceps. The microtome and blade were carefully cleaned with

xylene between each block to prevent sample-to-sample contamination. Precautions to prevent sample-to-sample contamination⁽⁷⁾ and PCR product "carryover"^(13,14) were strictly followed.

Deparaffinization of sections was performed as previously described⁽¹¹⁾ with minor modifications. Briefly, each section was extracted once with octane, followed by one 100% ethanol rinse. Tissue pellets were dried in a 55°C heat block. The pellets were suspended in 0.15 ml of digestion buffer (200 µg/ml proteinase K in 50 mM Tris-HCl, 1 mM EDTA [pH 8.5], and 1% Laureth-12 [Mazer Chemicals, Gurnee, IL]) and incubated overnight at 37°C. The proteinase K was heat-inactivated by a 10-min incubation at 95°C.

Polymerase Chain Reaction

Four oligonucleotide primer pairs⁽¹¹⁾ located in the human β-globin gene were used to produce amplification fragments of 268, 536, 989, and 1327 bp. Primer and MgCl₂ concentrations and other amplification conditions were optimized for each primer pair.

Aliquots (1 and 10 µl) of prepared samples were amplified separately (in duplicate) with each of the four primer pairs. Each 100-µl reaction contained 1 or 10 µl of sample DNA; 100 nM of each primer; 200 µM of each dNTP (dATP, dGTP, dCTP, dTTP); 2.5 units of *Taq* DNA polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT); 50 mM KCl, 4 mM MgCl₂, and 10 mM Tris-HCl (pH 8.5). A 100-µl mineral oil overlay was added to prevent evaporation during thermal cycling. Cycling parameters were 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for 40 cycles, followed by an additional 5 min at 72°C, in a DNA Thermal Cycler (Perkin-Elmer Cetus). Three nanograms of purified DNA (approximately 200 cells) from the SiHa cell line (ATCC no. HTB35) were used as a human DNA control, and sterile, distilled water was used as a negative control. Aliquots representing 1/20 of each amplification were resolved on 7% (wt/vol) polyacrylamide gels, stained with ethidium bromide, and photographed under UV light.

RESULTS

Strategy

The fixatives chosen for evaluation in

this study were 10% BNF, acetone, 95% ethanol, and OmniFix. (Acetone and 10% BNF were the two best fixatives from our previous study.) It was anticipated that the ethanol-based solutions, OmniFix and 95% ethanol, would gently precipitate DNA⁽⁹⁾ thus preserving it for PCR. To focus on the effects of each fixative and fixation time, routine clinical variables such as the type or amount of tissue processing were eliminated. Therefore, fresh tissue was obtained, cut into approximately equal pieces, and processed in each of the fixatives for times ranging from 2 hr to 30 days. Additionally, to evaluate the effect that delayed fixation would have on sample DNA, four tissue pieces were held at 4°C for 24 hr, then processed for 24 hr in each of the four fixatives prior to embedding. A section from each fixative and time point was prepared for PCR and evaluated in amplification reactions. The primers used to generate a range of amplification products lengths (268, 536, 989, and 1327 bp) are clustered in the human β-globin gene.

Effect of Fixation on PCR

Examples of the amplification results are shown in Figure 1. DNA amplification products from 10% BNF-processed tissues are shown in gel A. Successful amplifications were seen at the 2- and 24-hr time points for all product sizes. After 72 hr of fixation, there was reduction of the 989-bp product generated and no 1327-bp product was made. After 8 days of fixation, significantly less 536-bp fragment, very little 989-bp and no 1327-bp product was generated. After 30 days, only the 268-bp fragment was produced.

Tissues processed in OmniFix (gel B) allowed successful amplifications of all fragment sizes through 72 hr of fixation. After 8 days there was significant reduction in the 989-bp fragment and no 1327-bp fragment was produced. When the tissue was fixed for 30 days, the ability to produce the 989-bp fragment was severely reduced.

Acetone and 95% ethanol-treated samples (gels C and D) provided similar amplification results through the 8-day time-point. After 30 days of fixation, the acetone treatment afforded successful amplifications of fragment sizes up to the 989-bp product, but not

FIGURE 1 Examples of PCR results. Amplification products (1/20 of reaction) were electrophoresed on 7% acrylamide gels, stained with ethidium bromide, and photographed under UV light. (A) Amplification fragments from samples fixed in 10% BNF. (B) OmniFix. (C) Acetone. (D) 95% ethanol. Sizes of products are noted at center. Far left unmarked lanes contain a 123-bp ladder (BRL), and the far right lanes show ϕ X174 DNA restricted with *Hae*III (BRL) as molecular weight markers. Lanes 1, 6, 11, and 16 are amplifications from tissues fixed in for 2 hr; lanes 2, 7, 12, and 17 are from those fixed for 24 hr; lanes 3, 8, 13, and 18 are from those fixed for 72 hr; lanes 4, 9, 14, and 19 are from those fixed for 8 days; lanes 5, 10, 15, and 20 are from those fixed for 30 days.

the 1327-bp product. In contrast, after 30 days of fixation, the ethanol-treated samples yielded abundant amplification products of all lengths.

Tissues held for 24 hr at 4°C prior to a 24-hr fixation afforded equivalent amounts of amplification to those immediately fixed for 24 hr (data not shown).

Table 1 summarizes the amplification results from reactions containing 10 μ l of prepared sample. In all cases but one, the amplifications containing 10 μ l of sample were equal to or better than those containing 1 μ l of sample.

Effects on Histology

Although a comprehensive examination of the effect of the different fixatives on sample histology was not undertaken, some general observations were made. Tissues fixed in 10% BNF, OmniFix, and 95% ethanol afforded good histological detail. There did not appear to be a significant trend in loss

or improvement of detail after prolonged fixation. Histologic detail from acetone-fixed tissues was poor.

DISCUSSION

In a previous study⁽¹¹⁾ we evaluated 11 fixatives at fixation times up to 24 hr, conditions often found in clinical settings. In this study our goal was to determine the effect of longer fixation times (up to 30 days) on the ability of fixed tissue to serve as template for DNA amplification of a range of DNA fragment lengths. We tested the two most efficacious fixatives (acetone and 10% BNF) from our previous study and also evaluated 95% ethanol and OmniFix. Unquestionably, 95% ethanol and acetone-fixed tissues provided the best amplification results, followed by OmniFix and 10% buffered neutral formalin. These results are in agreement with previous data^(9,11,15) which suggested that ethanol-based neutral-pH fixatives are less damaging to DNA.

Neither this nor our previous study addressed the possible effect of fixation on the integrity of the sequence information in the amplified DNA. DNA damage resulting from fixation may cause replication errors, such as point mutations, during PCR. Furthermore, elegant work by Pääbo et al.⁽¹⁶⁾ demonstrated that DNA strand scission and apurinic sites found in ancient or highly degraded DNA can cause the extending strand to "jump" to another template during PCR. Additional studies will be necessary to determine if PETs and other archival material are appropriate for studies of subtle DNA sequence polymorphisms or mutations.

A rigorous comparison of the histologic detail afforded by the tested fixatives was not included in this study. Because the histologic requirements for different tissue types would be too varied, we chose to focus only on the effects on PCR. The suitability

of a fixation procedure for histologic analysis must be evaluated individually for each tissue type. Of course, histology may not be of great importance in all studies. Although formalin is the most commonly used fixative in clinical laboratories, other fixatives such as 95% ethanol or OmniFix may provide acceptable histology and are certainly better for ensuing PCR amplifications. Alternatively, specimens fixed in ethanol can be post-processed in a desired fixative to improve histologic detail. When possible, multiple samples may be taken and processed by different methods, or a single biopsy may be bisected and processed by different methods.

There was no apparent difference between amplification product yields from tissues held at 4°C for 24 hr prior to fixation and those from tissues fixed immediately. The apparent lack of effect of delay in sample fixation in this study was surprising. Dubeau⁽¹⁷⁾ observed that delay in tissue processing leads to significant degradation of DNA, rendering the material unusable for Southern blot analysis. Although a problem for Southern blot and possibly in situ hybridization analysis, this is not as detrimental for analysis using the exquisitely sensitive PCR method. Although autolysis was apparent histologically, the remaining DNA was a sufficient template for successful amplification. However, many factors not evaluated here, such as type, size, or the amount of necrosis or infection in a specimen, may lead to significant variability in other studies.

In locations where optimal processing technology is unavailable, sample collection in a nondeleterious fixative such as ethanol would be of tremendous advantage. Such long-term storage methods will also be useful to institutions such as museums and genetic source repositories that have an interest in the long-term survival of genetic information. These procedures should also prove beneficial to epidemiological studies of disease transmission using alcohol-preserved insect vectors. Other scientists, such as conservation biologists, zoologists, and botanists, will have a simple and convenient method for storing field specimens until they can be returned to the laboratory for analysis.

TABLE 1 Summary of Amplification Results

Fixation	Fixation time	268 bp	536 bp	989 bp	1327 bp
10% BNF	2 hr	+	+	+	+
	24 hr	+	+	+	+
	72 hr	+	+	+	-
	8 days	+	+	+	-
	30 days	+	-	-	-
Acetone	2 hr	+	+	+	+
	24 hr	+	+	+	+
	72 hr	+	+	+	+
	8 days	+	+	+	+
	30 days	+	+	+	-
95% Ethanol	2 hr	+	+	+	+
	24 hr	+	+	+	+
	72 hr	+	+	+	+
	8 days	+	+	+	+
	30 days	+	+	+	+
OmniFix	2 hr	+	+	+	+
	24 hr	+	+	+	+
	72 hr	+	+	+	+
	8 days	+	+	+	-
	30 days	+	+	+	-

Successful amplification of the desired fragment size, denoted by a plus, was determined by visualization of a band of the expected migration on an ethidium-stained polyacrylamide gel (see Fig. 1). A minus denotes that no amplification product was visible.

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REFERENCES

1. Faloona, F. and K. Mullis. 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods Enzymol.* **155**: 335.
2. Saiki, R.K., S. Scharf, F. Faloona, et al. 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350.
3. Frye, R.A., C.C. Benz, and E. Liu. 1989. Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene* **4**: 1153-1157.
4. Cao, M., X. Xiao, B. Egbert, T.M. Darragh, and T.S.B. Yen. 1989. Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. *J. Invest. Dermatol.* **82**: 391-392.
5. Resnick, R.M., M.T.E. Cornelissen, D.K. Wright, et al. 1990. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J. Natl. Cancer Inst.* **82**: 1477-1484.
6. Shibata, D.K., N. Arnheim, and W.J. Martin. 1988. Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. *J. Exp. Med.* **167**: 225-230.
7. Wright, D.K. and M.M. Manos. 1990. Sample preparation from paraffin-embedded tissues. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 153-158. Academic Press, Berkeley.
8. Pääbo, S. 1989. Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. *Proc. Natl. Acad. Sci.* **86**: 1939-1943.
9. Ben-Ezra, J., D.A. Johnson, J. Rossi, N. Cook, and A. Wu. 1991. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. *J. Histochem. Cytochem.* **39**: 351-354.

10. Crisan, D., E.M. Cadoff, J.C. Mattson, and K.A. Hartle. 1990. Polymerase chain reaction: Amplification of DNA from fixed tissue. *Clin. Biochem.* **23**: 489–495.
11. Greer, C.E., S.L. Peterson, N.B. Kiviat, and M.M. Manos. 1991. PCR amplification from paraffin-embedded tissues: Effects of fixative and fixation time. *Am. J. Clin. Pathol.* **95**: 117–124.
12. Arnheim, N., T. White, and W.E. Rainey. 1990. Application of PCR: Organismal and population biology. *Bioscience* **40**: 174–182.
13. Kwok, S. 1990. Procedures to minimize PCR-product carry-over. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 142–145. Academic Press, Berkeley.
14. Kwok, S. and R. Higuchi. 1989. Avoiding false positives with PCR. *Nature* **339**: 237–238.
15. Persing, D.H., S.R. Telford, P.N. Rys, D.E. Dodge, T.J. White, S.E. Malawista, and A. Spielman. 1990. Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* ticks. *Science* **249**: 1420–1423.
16. Pääbo, S., D.M. Irwin, and A.C. Wilson. 1990. DNA damage promotes jumping between templates during enzymatic amplification. *J. Biol. Chem.* **265**: 4718–4721.
17. Dubeau, L., L.A. Chandler, J.R. Gralow, P.W. Nichols, and P.A. Jones. 1986. Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. *Cancer Res.* **46**: 2964–2969.

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References

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