Noncryogenic Preservation of Mammalian Tissues for DNA Extraction: An Assessment of Storage Methods

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Reliable field methods for the storage of tissues to be used for DNA extraction and amplification are critical to many studies employing molecular techniques. Protection from DNA degradation was compared among three commonly used methods of noncryogenic storage of tissues over a time scale of 2 years. All three methods prevented DNA degradation during storage for at least 6 months. DMSO (dimethyl sulfoxide)-salt solution provided the best protection from DNA degradation of tissues stored for up to 2 years. High molecular weight DNA was recovered from lysis buffer in which tissue was stored for 2 years, however, moderate amounts of degraded DNA was also present. High molecular weight DNA was recovered from tissues stored in ethanol for 2 years, however, the yield was relatively small compared to the other two noncryogenic storage techniques. Much of the DNA degradation in ethanol preserved tissues appeared to occur during the extraction procedure and can be reduced by soaking the tissue in lysis buffer for a few hours prior to beginning the extraction. The yield of PCR products was greatest from DNA extracted from DMSO-salt solution preserved tissues, whereas DNA from tissues stored in either lysis buffer or ethanol produced lower yields.

KEY WORDS: tissue storage; noncryogenic; DNA extraction; degradation.

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INTRODUCTION

With the increased use of DNA techniques in evolutionary and ecological studies, the methods used for preservation of tissues for DNA extraction are important to protect these potentially valuable resources. Dessauer *et al*. (1996) have summarized the appropriate methods of collecting and storing tissues and have concluded that freezing is the most effective method of preservation. Cryopreservation, however, is often not feasible for many field sites. Problems encountered with transporting liquid nitrogen containers, in the field and aboard airplanes, may prevent some researchers from preserving valuable tissue samples accessible during normal field activities.

Cryopreservation is not required for tissues collected for DNA analysis. Several workers (Nietfeldt and Ballinger, 1989; Sibley and Ahlquist, 1981a) have suggested storing tissues in ethanol or isopropanol if they are to be used for DNA extraction. Seutin *et al.* (1991) encouraged the use of DMSO-salt solution for the preservation of tissues rather than ethanol. Several different lysis buffers for DNA preservation have been described in the literature including Queen's lysis buffer (Seutin *et al.*, 1991), Longmire buffer (Longmire *et al.*, 1997), and others (Cockburn and Seawright, 1988). Although comparative studies of storage methods of blood for DNA analysis have been published (Arctander, 1988; Seutin *et al.*, 1991), comparisons of noncryogenic preservation of soft tissues are lacking. The objective of this study was to compare the abilities of three chemical methods of tissue storage to prevent degradation of DNA in the absence of refrigeration or freezing.

MATERIALS AND METHODS

Liver, a soft tissue typically used for DNA extraction, was collected from three white-footed mice, *Peromyscus leucopus*, within minutes after the mice were killed. The liver was cut into small pieces about 4–6 mm in diameter as recommended by C. G. Sibley (personal communication). Minced liver was stored at room temperature in 20 mL polyethylene scintillation vials in one of three chemical preservatives; 95% ethanol (Sibley and Ahlquist, 1981a), DMSO-salt solution (Seutin *et al.*, 1991), or lysis (Longmire) buffer (Longmire *et al.*, 1997). At least three volumes of chemical preservative relative to the volume of tissue was added to each vial and the vial was sealed with parafilm. The ethanol was discarded and replaced with fresh 95% ethanol after 3 days of storage. The DMSO-salt solution consisted of 20% DMSO, 0.25 M sodium–EDTA, and NaCl to saturation, pH 7.5 (Seutin *et al.*, 1991). Lysis buffer contains 2 M tris–HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, and 20% SDS and was prepared following the protocol of Longmire *et al.* (1997). DNA was isolated from fresh tissue and after storage in each of the chemical preservatives for the following periods of time: 1 day, 3 days, 5 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, and 2 years.

DNA was purified by the conventional proteinase K/phenol/chloroform method of Sibley and Ahlquist (1981b) and Blin and Stafford (1976). Tissues preserved either in ethanol or DMSO-salt solution were washed with several volumes of distilled water before being processed further. The tissue was blotted dry on a paper towel, placed in a mortar containing liquid nitrogen and ground to a fine powder once frozen. The fine powder was rehydrated in a microfuge tube with 700 μL of distilled water and the DNA was released from the cells by the addition of 70 μL of 10% SDS and 5 μL of proteinase K (20 mg/mL). The rehydrated tissue powder was incubated at 55°C for 10–48 h with any solid bits of tissue being mechanically reduced with a dounce after a few hours of incubation. Proteins were removed from the samples with two extractions with 700 μL of a 1:1 mixture of buffered phenol and chloroform–isoamyl alcohol (24:1) followed by two extractions with only chloroform–isoamyl alcohol (24:1). The DNA was precipitated with the addition of 20 μL of 0.5 M NaCl and at least 900 μL of cold 100% ethanol. The precipitated DNA was cooled to -70°C for 10 min and pelleted by centrifugation at 9000 rpm at room temperature for 1 min. The pelleted DNA was washed with 1 mL of cold 70% ethanol and air dried. The dried DNA was resuspended in 100 μL of distilled water.

Different sized DNA fragments were separated on 1.2% agarose gels with $1 \times$ TBE buffer (89 mM tris–HCl, 89 mM boric acid, and 2 mM EDTA (pH 8.0), final pH 8.3). DNA was stained with ethidium bromide (0.5 μg/mL) and examined under shortwave UV light. The quality of DNA was determined by the presence of a high molecular weight band ($>12{,}000$ bp) and the relative proportion of low molecular weight (<500 bp) or partially degraded DNA.

The concentration of the extracted DNA was estimated by spectrophotometry (Sambrook *et al.*, 1989). Extracted DNA from tissue stored under each of the three conditions was diluted to approximately 100 ng/μL and each was used to amplify a 400 bp fragment of cytochrome *b* via the polymerase chain reaction (Saiki *et al.*, 1988) using the following parameters: 35 cycles of 94°C denaturing (1 min), 50°C annealing (1 min), and 72°C extension (1 min, 10 s). Amplification reactions were performed in 25 μL volumes with PCR beads (Amersham Pharmacia Biotech), 1 μL of DNA extract (100 ng/μL), and 1.25 μL of a 10 μM solution of each primer, L-14115 and H-14541 (Sullivan *et al.*, 1997). The PCR products were PEG precipitated (Sambrook *et al.*, 1989) and dried in a SpeedVac. Dried samples were rehydrated in 10 μL of distilled water and the yield of double stranded PCR products was estimated by spectrophotometry (Sambrook *et al.*, 1989).

RESULTS

Although high molecular weight DNA ($>12{,}000$ bp) could be recovered from tissues stored for 6 months under all three conditions (Fig. 1), the amount of degradation, as indicated by low molecular weight DNA fragments, was greatest
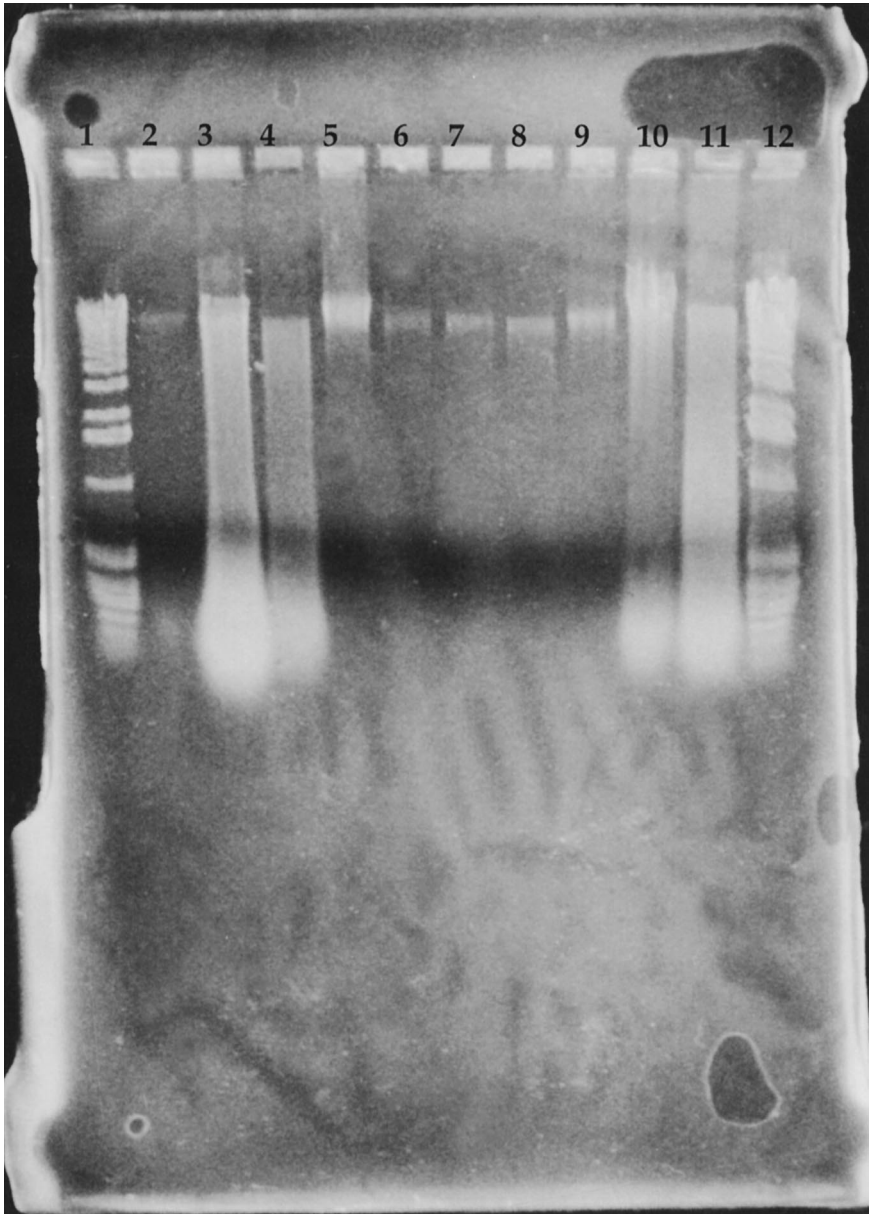
Fig. 1. DNA extracted from tissues stored at room temperature, fractionated on a 1.2% agarose gel with $1 \times$ TBE, and stained with ethidium bromide. Lanes 1 and 12: size marker (1 Kb ladder, Life Technologies); Lane 2: tissue stored in ethanol for 6 months and soaked in lysis buffer for 24 h prior to DNA extraction; Lanes 3 and 4: tissue stored in ethanol for 6 months and 2 months respectively; Lanes 5–7: tissue stored in lysis buffer for 6 months, 2 months, and 3 days respectively; Lane 8–10: tissue stored in DMSO-salt solution for 6 months, 2 months, and 3 days respectively; Lane 11: fresh tissue.
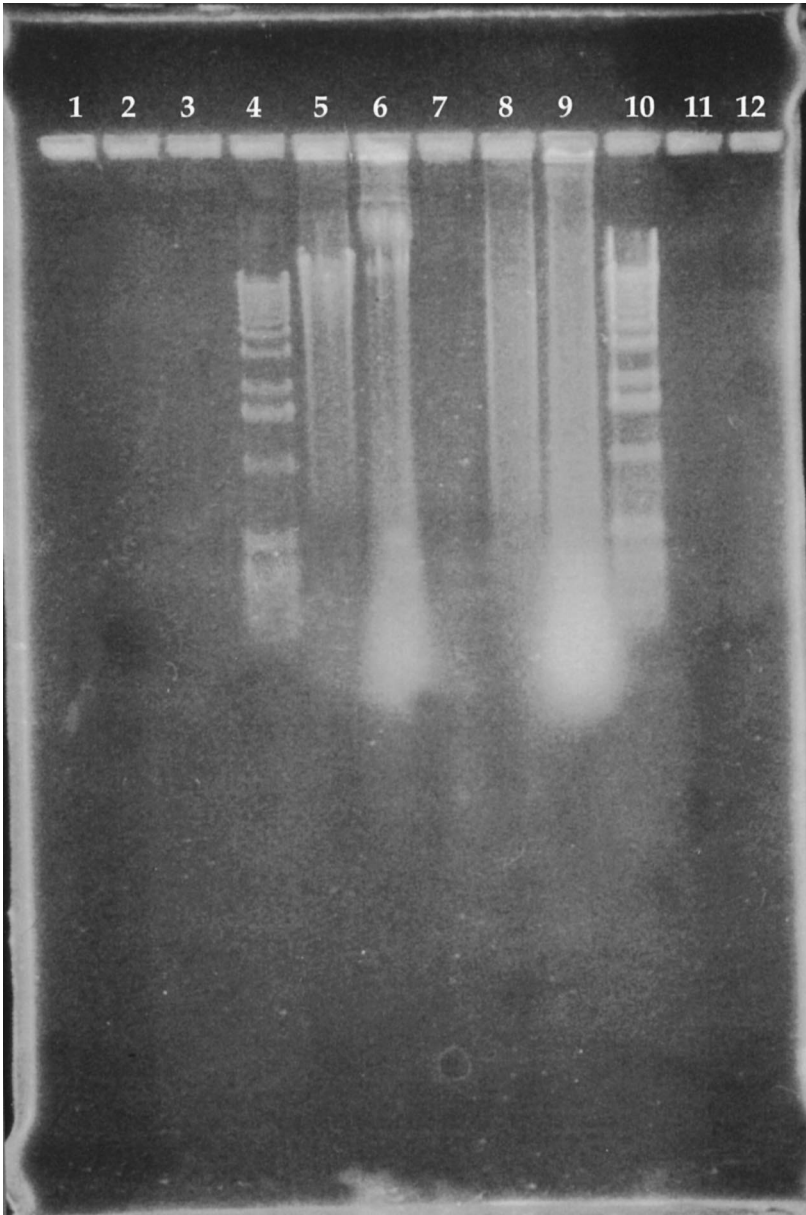
Fig. 2. DNA extracted from noncryogenic preserved tissues after 2 years of storage at room temperature, fractionated on a 1.2% agarose gel with 1 × TBE, and stained with ethidium bromide. Lanes 4 and 10 size markers (1 Kb ladder, Life Technologies); Lane 5: tissue stored in DMSO-salt solution; Lane 6: lysis buffer in which tissue was stored; Lane 7: tissue stored in lysis buffer; Lane 8: tissue stored in ethanol and soaked in lysis buffer for 24 h; Lane 9: tissue stored in ethanol.

in tissue stored in ethanol. DNA recovered from tissue stored in either DMSO-salt solution or lysis buffer was not degraded after 6 months of storage at room temperature (Fig. 1). After storage for 2 years, high molecular weight DNA could only be recovered from tissues stored in either ethanol or DMSO (Fig. 2). The tissue stored in lysis buffer was now translucent and the extraction from that tissue yielded no DNA (Fig. 2); although DNA could be recovered from the lysis buffer itself. Tissue stored in DMSO demonstrated the least amount of degradation after 2 years of noncryogenic storage (Fig. 2).

In addition, the data from this study suggest that most of the degradation observed in ethanol preserved tissues occurs during the extraction procedures. Extractions from fresh tissues, before placement in any chemical preservative, resulted in DNA extracts containing relatively high amounts of low molecular weight DNA (Fig. 1). Preservation in either DMSO-salt solution or lysis buffer appears to prevent this degradation during the extraction procedures. Little or no low molecular weight DNA was observed in the extracts from tissues stored in DMSO-salt for 3 days to up to 2 years (Figs. 1 and 2), however, low molecular weight DNA was present in the extract of tissue stored in DMSO-salt solution for only 1 day. After 1 day of storage, lysis buffer appears to protect the DNA from degradation for at least 6 months (Fig. 1), however, DNA extracted from the lysis buffer after 2 years of noncryogenic storage did show the presence of moderate amounts of low molecular weight DNA fragments (Fig. 2). DNA extracted from tissues stored in ethanol, however, showed the highest levels of low molecular weight (degraded) DNA (Figs. 1 and 2). Tissue preserved for 6 months, or even 2 years, in ethanol and transferred to lysis buffer 24 h prior to DNA extraction yielded high molecular weight DNA with little or no apparent degradation (Figs. 1 and 2).

The DNA extracted from tissue stored for 2 years under each of the three noncryogenic methods produced PCR products; however, considerable variation in the yield of double stranded PCR product was observed (Table I). The highest yield of PCR product was observed from DNA extracted from DMSO preserved tissues, whereas DNA from ethanol preserved tissue produced the lowest yield. Soaking of ethanol preserved tissue in lysis buffer for 24 h before extracting DNA resulted in DNA that yielded larger amounts of PCR product. This preextraction

Table I. Concentration of PCR Products Produced From 100 ng of DNA Extracted From Tissues Stored for 2 Years at Room Temperature

Storage method	Concentration of PCR product
DMSO	530 ng/μL
Longmire buffer	360 ng/μL
Ethanol	185 ng/μL
Ethanol with lysis buffer treatment	340 ng/μL

treatment with lysis buffer doubled the amount of PCR product obtainable from DNA extracted from ethanol preserved tissues. The DNA isolated from the treated ethanol preserved tissues produced yields of PCR product similar to yields from the DNA isolated from the lysis buffer used to store the tissue for 2 years.

DISCUSSION

Although cryopreservation may be the preferred method of tissue storage for many applications (Dessauer *et al.*, 1996), the logistics required for cryopreservation prevent its utilization in many field situations. Liquid nitrogen or dry ice is not widely available (a partial listing of sources is provided in Dessauer and Hafner, 1984). Although both may be shipped by air, they are classified as Restricted Articles by the International Air Transport Association, and the shipper must comply with all pertinent regulations (see Dessauer *et al.*, 1996). The difficulty in obtaining and transporting cryopreservatives may prevent many colleagues from collecting and preserving tissues at their field sites, if they are not directly interested in the tissues as a source of macromolecules.

Ethanol or isopropanol have been suggested (Nietfeldt and Ballinger, 1989; Sibley and Ahlquist, 1981a) for the storage of tissues to be used for DNA extraction. Ethanol was found to be a moderately efficient preservative of DNA in tissue stored at room temperature. It is important to cut the tissue into small pieces to increase the surface area and to cover the tissue with several volumes (three or more) of ethanol. The quality of preservation is also improved by changing the ethanol in the storage container after the first 2 or 3 days of storage.

Tissue preserved in ethanol yielded relatively large amounts of low molecular weight DNA. Several workers (Houde and Braun, 1988; Seutin *et al.*, 1991) have also found that tissues preserved in ethanol yielded primarily highly degraded DNA fragments. Much of the degradation of the DNA in tissues stored in ethanol, however, appears to occur during the extraction procedure rather than during storage. Storage of tissues in either DMSO-salt solution or lysis buffer (both containing EDTA) appears to protect the DNA from degradation during the extraction process, whereas tissue preserved in ethanol (without EDTA) yielded relatively large amounts of low molecular weight DNA.

Mammalian tissues stored in ethanol, but transferred into lysis buffer for 24 h prior to extraction of DNA, yielded high molecular weight DNA with relatively small amounts of low molecular weight fragments. The practice of storing tissues in ethanol or isopropanol and extracting the DNA in a lysis buffer (Cockburn and Seawright, 1988) seems to be a common procedure used by invertebrate biologists (Freitas-Sibajev *et al.*, 1995). The same effect can probably be obtained by storage of the tissue in ethanol with the addition of EDTA (about 100 μM per liter) as suggest by C. G. Sibley (personal communication). The DNA extracted from ethanol preserved tissues, even with treatment in lysis buffer prior to extraction, yielded less

high molecular weight DNA than tissues stored in DMSO-salt solution and thus produced less PCR product per 100 ng of extracted DNA. The yield of PCR product was similar for DNA extracted from ethanol and lysis buffer preserved tissues.

Ethanol has the advantages of being readily available at most field sites, not requiring any special precautions for use in the field, and being the least expensive ($0.05/sample) of the noncryogenic methods examined. Ethanol is highly flammable and may explode if ignited in an enclosed area (boiling point 78.5°C; flash point 13°C; autoignition temperature 363°C). Although it is classified as a Restricted Article by the International Air Transport Associations, limited quantities (5 L by passenger aircraft and 60 L by cargo aircraft) may be transported by air. Once a tissue is thoroughly preserved, the bulk of the ethanol may be discarded just prior to shipping to reduce the total volume of ethanol. Once the tissue has reached its final destination, it should be covered with three or more volumes of ethanol in a tightly sealed container. These advantages must be weighed against the increased expense ($0.23) and additional time required for treatment with lysis buffer prior to DNA extraction and the yield of partially degraded DNA that produces a lower yield of PCR products.

Seutin *et al.* (1991) encouraged the use of DMSO-salt solution for the preservation of tissues and found that it was as efficient as cryopreservation (at −70°C). This study also found DMSO-salt solution to be a highly efficient preservative of DNA in tissue samples at room temperature for up to 2 years. In addition, DNA extracted from tissue stored by this method produced the highest yield of PCR product observed. This method has the advantages of highly efficient preservation of DNA that is suitable for PCR amplification, moderate cost ($0.68/sample), and no additional steps needed in the isolation procedures.

DMSO must be used with some basic precautions, primarily avoiding skin contact (David, 1972; Mason, 1971). In addition to inducing primary irritation of the skin, DMSO can transport toxic compounds found in the samples, or on the skin, into the body. If contact occurs, the affected area should be washed with clear running water. At room temperature, DMSO is noncorrosive and nonexplosive (boiling point 189°C; flash point 95°C; autoignition temperature 215°C). No special regulations apply to the transport of DMSO by air, however, Seutin *et al.* (1991) recommended that it be bottled in nonbreakable, clearly labeled containers.

The different lysis buffers described in the literature including Queen's lysis buffer (Seutin *et al.*, 1991), Longmire buffer (Longmire *et al.*, 1997), and others (Cockburn and Seawright, 1988), all contain tris, EDTA, and either SDS or lauroylsarcosine. Although these substances are harmful if swallowed or inhaled, all are nonflammable, noncorrosive, and nonexplosive. No special regulations apply to the transport of tris, EDTA, SDS, or lauroylsarcosine by air.

This study found the Longmire buffer was an efficient preservative of DNA in tissues stored in room temperature for up to 6 months. Although no DNA could be extracted from tissue after 2 years of storage in lysis buffer, high molecular

weight DNA was easily recoverable from the buffer solution. However, the lysis buffer used to store tissue for 2 years also contained moderate amounts of low molecular weight DNA (Fig. 2). The DNA extracted from the lysis buffer produced considerable more PCR product than that stored in ethanol but not as much as that stored in DMSO. An advantage of the use of Longmire buffer and possibly other lysis buffers is the lysis of the tissue and the accumulation of the DNA in the buffer solution with time. This self-extraction substantially reduces the cost and labor of the extraction procedure, however, this is the most expensive ($3.41/sample) of the noncryogenic storage methods.

In summary, high molecular weight DNA may be routinely extracted from soft tissues preserved in any of the three noncryopreservatives examined. Tissue stored in lysis buffer for up to 6 months yielded mostly high molecular weight DNA but DNA was released from the tissue with prolonged storage. High molecular weight DNA was recovered from the lysis buffer used to store tissues for at least 2 years, however, moderate amounts of degraded DNA was also present. Although high molecular weight DNA was recovered from ethanol preserved tissues, an additional procedure had to be used to protect the DNA from degradation during the extraction procedure. Tissues stored in ethanol should be transferred into lysis buffer for 24 h prior to extraction to greatly reduce the degradation of DNA during the extraction process. The DNA from tissue stored for years in either lysis buffer or ethanol, even when treated in lysis buffer prior to extraction, experienced some degradation and produced lower yield of PCR products. Tissues stored in DMSO-salt solution yielded mostly high molecular weight DNA that was suitable for PCR amplification and relatively small amounts of low molecular weight DNA. Although DMSO-salt solution would appear to be the method of choice given its moderate cost and efficiency of preservation of DNA, the lack of availability at many field sites may limit its utilization.

ACKNOWLEDGMENTS

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