DNA extraction from paraffin-embedded
tissues using a salting-out procedure: a reliable
method for PCR amplification of archival material

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Summary. Many techniques have been described for the extraction of DNA from paraffin-embedded tissues. Numerous efforts have been directed at simplification of these methods for rapid analysis using PCR. One disadvantage to some of the simpler procedures is inefficient PCR amplification, and for more involved ones using phenol/chloroform extraction, reduction in the yield of DNA. In the present study we report the use of a novel salting-out procedure that was utilized to extract DNA from 259 separate microdissection specimens of formalin-fixed, paraffin-embedded tissue sections. These sections were derived from 97 patients with tumors of the ampulla of Vater resected between 1965 and 1995 at our institution. The mean DNA yield was 22.75 μg (median 13.2±30.25) and the mean 260/280 absorbance ratio was 1.68 (median 1.70±0.25). All specimens (259/259) were successfully used to amplify K-ras exon 1 by a nested PCR technique. These results indicate that this DNA extraction method produces good yields of quality DNA, even from specimens several decades old.

Key words: DNA extraction, PCR technique, Paraffin-embedded tissues

Introduction

The advent of the polymerase chain reaction (PCR) has revolutionized the biomedical sciences, and in particular, microbiology, pathology, and molecular genetics. Small quantities of DNA can be efficiently amplified for molecular analysis using this technique. The development of DNA extraction procedures from paraffin-embedded tissues has allowed the widespread application of these techniques for the study of retrospective material, which is especially valuable for rare conditions and when DNA is limiting.

Successful PCR amplification from formalin-fixed, paraffin-embedded specimens has been reported using DNA extraction procedures ranging from the simple boiling of tissue sections (Sepp et al., 1994), boiling with Chelex-100 resin (Chen et al., 1991; Walsh and Higuchi, 1991; Stein and Raoult, 1992), sonication and boiling (Heller et al., 1992), microwave irradiation (Banerjee et al., 1995), to more involved procedures utilizing proteinase K digestion and phenol/chloroform extraction (Goelz et al., 1985). Most of these procedures recommend removal of paraffin by successive washes with xylene and then ethanol. This is generally followed by digestion with proteinase K to remove proteins which might lead to DNA degradation or interfere with PCR amplification, then heat inactivation of this enzyme. This supernatant can then be used for PCR directly, or the removal of degraded proteins is attempted, which theoretically should improve PCR amplification by providing a cleaner template. One traditional method is phenol extraction, followed by chloroform/isoamyl alcohol extraction, then ethanol precipitation. The disadvantages of this method are that it involves using organic solvents, it employs multiple centrifugations and tube transfers which increases the potential for contamination, and the yields of DNA may be decreased. One alternative to this method of removing proteins is a salting-out procedure, as has been described for whole blood by Miller et al. (1988). In this method, the addition of a saturated NaCl solution results in precipitation of proteins, leaving the DNA in the aqueous phase. One modification of this procedure has been reported for paraffin-embedded material (Forsthoefel et al., 1992), and another is commercially available as a kit (Oncor Ex-Wax kit, Gaithersburg, MD). The objective of this study was to modify the DNA extraction procedure of Miller et al. (1988) for paraffin-embedded tissues in such a way that it would be inexpensive, result in good DNA yields, and provide DNA suitable for PCR amplification, even from specimens several decades old. These efforts were undertaken specifically for the study of K-ras mutations in neoplasms of the ampulla of Vater collected at our institution.

Materials and methods

Sample Collection

Archival specimens of tumors of the ampulla of Vater which had been completely resected between 1965 and 1995 were identified from the Surgical and Pathology databases at the Memorial Sloan-Kettering Cancer Center. Slides and paraffin blocks were retrieved and carefully reviewed by a single pathologist (D.S.K.) to confirm the diagnosis, and to definitively discriminate ampullary lesions from other periampullary tumors.

Microdissection technique

Paraffin blocks were cut in 10 μm sections, and 10-15 slides were prepared from each tissue block. One slide was stained with hematoxylin and eosin, and specific areas of normal tissue, adenoma, or carcinoma were marked on the stained slide (multiple blocks were usually cut for each tumor in order to obtain components of normal, preinvasive, and invasive lesions). Unstained slides were aligned by morphology to the stained slide using operating loupes (x2.5 magnification), and corresponding areas excised with a scalpel or razor blade. After these areas were harvested from all slides they were placed in microfuge tubes. When multiple components were microdissected from a single slide, the normal component was always dissected first from all slides. Blades were then changed, and dissection of the preinvasive lesion was conducted (if present), then dissection of the invasive component was performed. Microdissected areas were always scraped peripherally, to avoid contamination of other areas. Sample cases were stained and examined microscopically to confirm the specificity of dissection.

Deparaffination procedure

Microdissected tissues were stored in microfuge tubes at 4 ºC for 4-7 weeks. One milliliter (ml) of xylene was then added, and samples gently vortexed for 5 minutes at room temperature. These were centrifuged at 14000 rpm for 5 minutes, and the supernatant removed. This step was then repeated. It should be noted that pieces less than 1-2 mm in size are vulnerable to being lost because they will generally float rather than spin down in xylene. At this point, the tissue sections became relatively transparent. Next, 1 ml of room temperature 100% ethanol was added, and samples were gently vortexed for 2-3 minutes, then centrifuged at 14000 rpm for 5 minutes. The cell pellets became fluffy, white, and granular in appearance. Residual ethanol was then removed by pipetting followed by centrifugation in a vacuum-concentrator for 10 minutes.

Proteinase K digestion

Two-hundred μl of proteinase K digestion buffer were added to each tube (50 mM Tris-HCl pH 8.5, 1 mM EDTA pH 8.0, 0.5% Tween 20); minimal effort was made to resuspend the pellets, which became quite viscous. Proteinase K was then added to bring to a final concentration of 400 μg/ml. Samples were subsequently incubated at 50 ºC for 4-6 hours, and heated to 94 ºC for 10 minutes to inactivate the proteinase K. In general, there was no tissue residue visible after digestion.

Protein precipitation

Fifty μl of a saturated NaCl solution (approximately 6M) were added to each tube, and the samples gently vortexed for 5 minutes. The tubes were then centrifuged at 4000 rpm at room temperature for 15 minutes, and the supernatant removed to a fresh tube. A white protein pellet was visible at the bottom of the tubes after centrifugation.
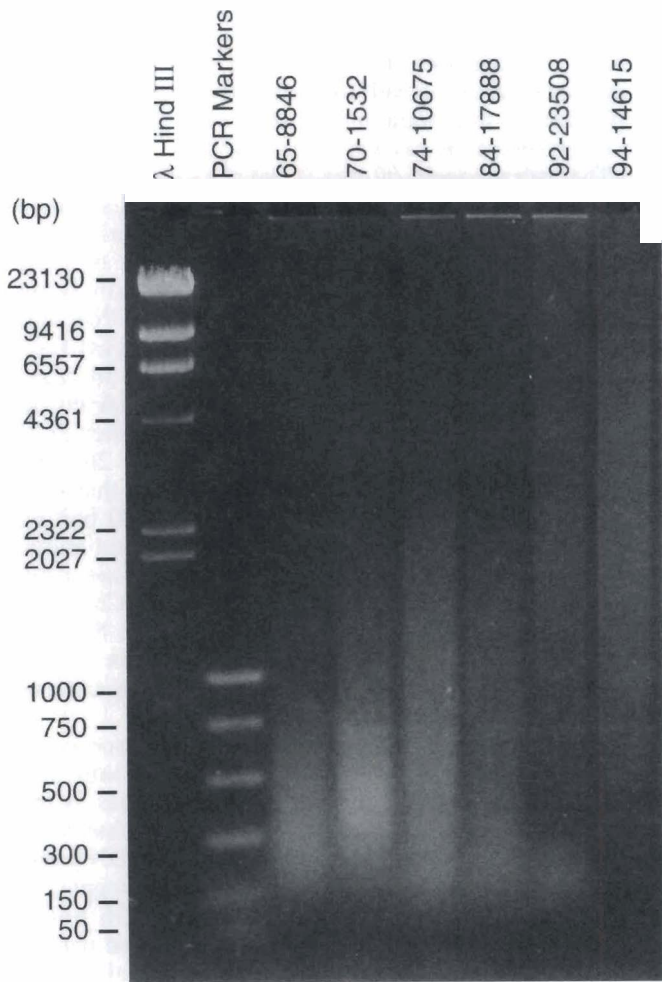
DNA precipitation

One-tenth volume of 3M sodium acetate was added to the supernatant, followed by 2.5 volumes of 100% ethanol; 5 μl of mussel glycogen (2 mg/ml) were also added as a DNA carrier to improve DNA recovery. Samples were placed at -20 ºC overnight, then centrifuged at 4 ºC for 30 minutes at 14000 rpm. The supernatant was removed, the pellets were washed with 1 ml of 70% ethanol, and then centrifuged at 4 ºC and 14000 rpm for 15 minutes. The supernatant was again removed, and pellets were dried in a vacuum-concentrator. Pellets range in size from being barely visible to being 3-4 mm in size and fluffy-white in consistency (the latter of which probably contain protein carry-over). Pellets were resuspended in 50 μl of sterile water. One μl was used to measure the absorbance in a UV spectrophotometer at 260 and 280 nm.

PCR amplification and sequencing

PCR was carried out on a DNA thermal cycle (GeneAmp PCR System 960C, Perkin-Elmer Cetus, Norwalk, CT) for K-ras exon 1 using approximately 500 ng of template DNA; the final magnesium concentration was 2.5 mM, primer concentration 2 μM, dNTPs 0.2 mM, and 1 unit Taq polymerase was used in each reaction (Promega, Madison, WI). The primers used were 5'-GACTGAATATAAACTTGTGG-3' and 5'-CTATTGTTGGATCATATTCG-3' (as described by Verlan-de Vries et al., 1986); 35 cycles of amplification were performed, with 1 min denaturation at 94 ºC, 2 min annealing at 58 ºC, and 2 min polymerization at 72 ºC. One μl of this 10 μl reaction mixture was then used as template for a second round of amplification using the nested primers 5'-AAACTTGTGGTAGTTGGAGC-3' and 5'-TCGTCCACAAAATGATTCTG-3' (magnesium concentration 1.5 mM, in a final volume of 20-50 μl; the same thermal cycling conditions were used, except that the annealing and polymerization times were 1 min

Fig.1. Products of DNA extraction after 1.0% agarose gel electrophoresis. Molecular weight size standards are shown in the 2 far left lanes. The other lanes are designated by their pathology accession numbers.
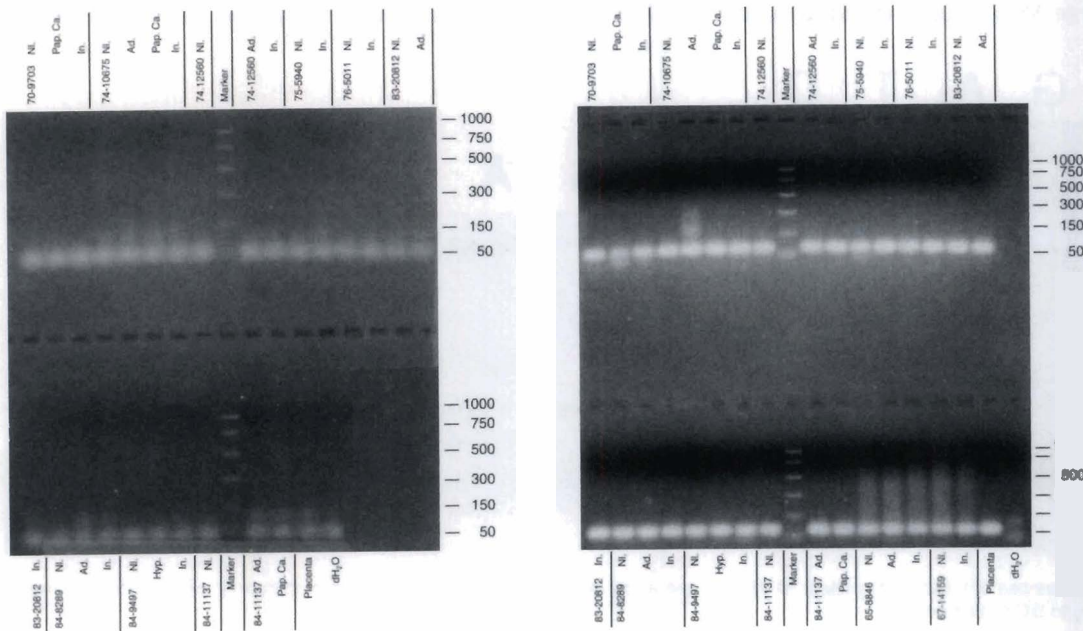
each). PCR products were sequenced using the latter primer according to the Sequenase PCR product sequencing kit protocol (U.S. Biochemical, Cleveland, OH).

Results

DNA yield and absorbance

A total of 259 separate specimens were micro-dissected from 97 patients. The average DNA yield from approximately ten to fifteen 10 μm sections (with areas of microdissection on average 3 x 3 mm) was 22.75 μg, with a median of 13.2 μg (range of 0 to 242 μg, s.d. ±30.25). Even specimens in which no DNA was detectable by spectrophotometry were successfully amplified by PCR. The mean 260/280 nm absorbance ratio in these samples was 1.68, with a median of 1.70 (range 1.02 to 2.70, s.d. ±0.25). Figure 1 shows the sizes of different DNA specimens spanning several decades; the approximate average sizes from 1965 was 150-600 bp, 1970 200-800 bp, 1974 150-2000 bp, 1984 150-1200 bp, 1992 150-5000 bp, and 1994 400-20000 bp.

PCR amplification

After the first PCR amplification, visible products were seen on ethidium bromide stained agarose gels in approximately one-third of cases (Fig. 2a). This provided the rationale for the second round of amplification. After the second amplification, all

Fig. 2. A. PCR products resulting from one round of PCR amplification for K-ras exon 1, after electrophoresis through 2.5% agarose gel. Lanes are designated by pathology accession numbers and the specific histology microdissected. Nl.: Normal; Ad.: Adenoma; In.: Invasive; Hyp.: Hyperplasia; Pap. Ca.: Papillary Cancer. **B.** PCR products resulting from second amplification of K-ras exon 1 using nested primers.

samples showed strong products (Fig. 2b). Positive (placental DNA) and negative (dH_2O) controls were always used, and subjected to the same amplification conditions as the other samples. Amplification was successful even after DNA had been stored for 10 weeks at 4 ºC.

Sequencing of PCR products

Sequencing was successful in all PCR products in which it was attempted (approximately 150 samples). All tumor specimens found to have mutations had both their sense and anti-sense strands sequenced (the former using two different 5' primers for PCR amplification); in all cases, the mutations found on the sense strand were confirmed by sequencing the anti-sense strand using these different primers for amplification of K-ras exon 1 (Fig. 3).

Discussion

Salting-out of protein contaminants during DNA extraction has been shown to be an effective method with good yields from samples of whole blood (Miller et al., 1988). Cheung et al. (1993) have described a salting-out DNA microextraction procedure which can be applied to both plant and animal species, using a 2M NaCl extraction solution. A salting-out procedure for DNA extraction from paraffin-embedded material has also been reported, in which the authors were able to identify a deletion in the Duchenne muscular dystrophy gene in a patient who had died several years earlier (Forsthoefel et al., 1992). These authors were unsuccessful in amplifying crude lysates after proteinase K digestion (without salting out), and suggested that this was an important component of the procedure. The purpose of this study was to determine the efficacy of modifying the procedure of Miller et al. (1988) to extract

DNA from a large number of microdissected, formalin-fixed, paraffin-embedded tissues collected over several decades. Furthermore, the yields and absorbance ratios of extracted DNA, as well as the ability to perform PCR amplification and sequencing of a well characterized oncogene such as K-ras was to be analyzed.

The average yield of over 20 μg per sample suggests that this method of microdissecting multiple sections from each tumor allows for recovery of sufficient amounts of DNA to perform a large number of PCR reactions. Such yields should be viewed with some caution, since single-stranded and degraded DNA will result in higher absorbance values (per weight) than intact double-stranded DNA (Sinden, 1994). In cases where DNA is limiting, total genomic DNA could theoretically be amplified by the random primer PCR technique (Peng et al., 1994). The yields and 260/280 ratios obtained in this study are comparable to that seen by Chen and collaborators for their Chelex-100 boiling method (Chen et al., 1991, 1993). Bramwell and Burns (1988) described a yield of 12 μg per mg of tissue using a phenol/chloroform extraction procedure, and Chen and Clejan (1993) reported values ranging between 8-97 μg per mg of tissue. Therefore, even rare tumors can be meaningfully studied by collecting many specimens which have been archived over several decades.

One of the drawbacks of using archival tissue is the gradual degradation over time. An important and uncontrollable variable in retrospective studies is the length of fixation. Dubeau et al. showed that with fixation times of 12 hours in formalin, 57% of DNA was spoolable (high molecular weight), which dropped to only 35% if fixation was performed for 36 hours (Dubeau et al., 1986). Fixation in 10% buffered neutral formalin for 30 days resulted in loss of the ability to amplify a 536 bp product which was successfully amplified after 8 days of fixation (Greer et al., 1991). The type of fixative is also important, with fixation in
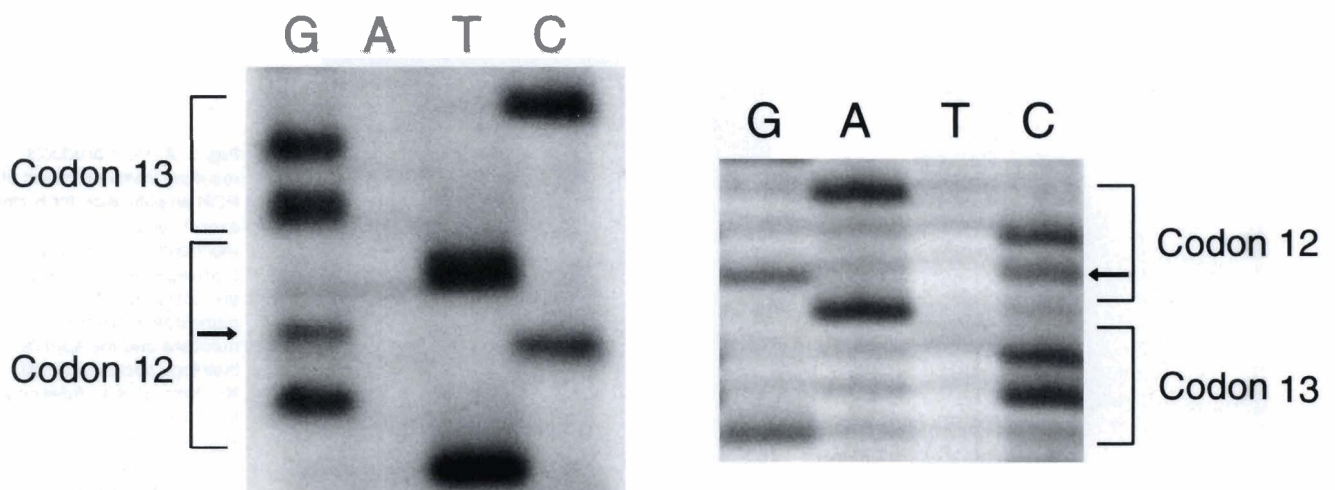

Fig. 3. Sense and anti-sense sequences of tumor from patient 94-18812. The arrow designates the site of mutation in codon 12, resulting in a change from the wild-type GGT (glycine) to GCT (alanine).

10% neutral-buffered formalin, ethanol, or acetone resulting in higher molecular weight DNA and improved PCR amplification. The use of Zamboni's fixative, Clarke's fixative, paraformaldehyde, formalin-alcohol-acetic acid, and methacarn are inferior to these agents, but are better than Carnoy's, Zenker's, and Bouin's fixatives (Greer et al., 1991). The bulk of DNA extracted by Goelz et al. (1985) was in the 100 bp to 1500 bp range, with older specimens having a smaller average size than more recent ones. The influence of the age of specimens on DNA quality has not been well described, perhaps because one cannot retrospectively control for length of fixation and the various fixatives used over different periods for archival material. However, degradation of DNA probably continues even when the tissue is embedded in paraffin. Furthermore, it has been suggested that formalin fixation renders the DNA more rigid and therefore more susceptible to mechanical shearing during extraction (Mies et al., 1991). The age of surgical specimens did not influence our ability to generate relatively short PCR products, as it was equally successful in specimens from 1965 as it was for those from 1995. This may be due to the practice at Memorial Sloan-Kettering of buffering formalin solutions at least as far back as the 1970s. Figure 1 illustrates that the average length of DNA species decreased with the age of the specimen.

The small average length of DNA species puts some limitations on the size of PCR products which can be amplified. Honma et al. found that 78% of specimens could successfully be used to amplify a 120 bp product using nested primers, while only 39% were successfully amplified using primers that yielded a 374 bp product (Honma et al., 1993). In the present study, a second round of amplification using nested primers allowed for 100% amplification of the 80 bp product, while only about one third of specimens appeared to amplify the 108 bp product (from the outer set of primers). Both of these products are relatively short, suggesting that the differential amplification is not only a factor of the average size of the DNA fragments, but more importantly related to the number of target molecules present. Presumably, the first round of amplification results in a small number of the expected size products, which are often barely visible on ethidium bromide stained agarose gels (Fig. 2a). The second round markedly amplifies these specific fragments, which then become easily visible on ethidium stained gels. Thus, this round increases specificity (Haff, 1994), and Figure 2b also demonstrates the elimination of the primer-dimers seen after the first amplification, a phenomenom which has been reported by Coates et al. (1991).

One concern with the use of a highly sensitive technique such as nested PCR is the fidelity of PCR amplification. Estimates of error rates for Taq polymerase have ranged from 1 in 5,000 to 1 in 20,000 errors per nucleotide per cycle. These are further influenced by dNTP concentration, MgCl concentration (Chen et al., 1991), buffer pH, and the length and

number of cycles used for PCR (Eckert and Kunkel, 1991). Fidelity appeared to be quite good in this study, where all mutations of K-ras were confirmed by sequencing of both sense and anti-sense strands, which were produced using different sets of primer pairs for each strand. Significant misincorporation events would have been expected to result in occasional changes in the K-ras mutations between sense and anti-sense strands.

Studies requiring higher molecular weight DNA, such as Southern blotting, suffer from a low fraction of appropriately sized molecules when DNA is extracted from paraffin-embedded tissues. One means of selecting for higher molecular weight DNA is to spool the DNA on glass rods at the time of reprecipitation. This strategy leaves lower molecular weight, unspoolable DNA behind. Dubeau et al. (1986) showed that with fixation times of 12 hours in formalin, 57% of DNA was spoolable, which dropped to only 35% if fixation was performed for 36 hours. Success with Southern hybridizations is also improved when the probes used are relatively short in length (Goelz et al., 1985; Mies et al., 1991). Since the advent of numerous microsatellite markers which can be amplified by PCR (Litt and Luty, 1989; Weber and May, 1989), loss of heterozygosity or "allele instability" studies can effectively be performed by PCR rather than using Southern hybridization (Huang et al., 1993). This is clearly better suited to the shorter DNA fragments extracted from paraffin-embedded tissues.

The 260/280 absorbance ratio (A260/280) appeared to be acceptable, with a mean of 1.68. This was slightly lower than the optimal range of 1.8 to 2.0. Proteins maximally absorb UV light at 280 nm, and therefore lower A260/280 ratios indicate the presence of residual proteins (Adams et al., 1992). Thus, the salting-out procedure did not completely remove all of the proteins in the extraction solution. This could have been related to the centrifugation step during salting-out. Early extractions done at 2500 rpm often resulted in larger, white pellets after DNA precipitation, which were suspicious for residual protein. This problem was reduced as the speed of centrifugation was increased to 4000 rpm, without an apparent effect on DNA yield. The significance of this is difficult to determine, for most other extraction methods have not reported A260/280 ratios. One exception is the study of Chen and Clejan (1993), in which the mean A260/280 ratio was 1.80 for 14 specimens extracted by boiling with Chelex-100 resin and purification over Sephadex G-50 columns (Chen and Clejan, 1993). It should be noted that even specimens with visible pellets or poor 260/280 ratios could be amplified without difficulty using the nested primer technique in this study, and therefore the significance of the A260/280 ratio is unclear.

Many methods of DNA extraction from paraffin-embedded material will result in adequate template for PCR amplification. Some authors therefore suggest that the simplest, most rapid extraction methods should be used (Sepp et al., 1994). Some groups have shown that

relatively simple methods of DNA extraction, such as boiling with Chelex-100, gave results as good as those seen with conventional phenol/chloroform extraction methods (Walsh and Higuchi, 1991). Another group has reported that the proteinase K step significantly increases their ability to amplify DNA from paraffin-embedded tissues, and when followed by Chelex-100 precipitation of proteins gave a superior result to that of phenol/chloroform extraction (de Lamballerie et al, 1994). von Weizsacker et al. (1994) were only able to amplify DNA from 23 of 67 paraffin-embedded liver biopsy samples for the ß-globin gene (354 bp product) using the supernatant following proteinase K digestion, which they concluded was due to degraded DNA rather than PCR inhibitors (von Weizsacker et al., 1994). The conclusion we have drawn from these studies is that although amplification is possible with relatively simple and rapid methods, it is not as reliable as more labor intensive procedures. Since archival tissues are often limited and microdissection is labor-intensive, we feel that efforts to improve template quality will pay off if multiple genetic studies of these specimens are to be performed. Therefore, digestion of proteins bound to DNA is important to ensure that DNA is available for amplification, and precipitation of these proteins is important to remove potential inhibitors to PCR amplification. The salting out procedure used here allowed us to successfully amplify DNA from all archival cases.

In this study, we have shown that a modification of the salting-out procedure originally described by Miller et al. (1988) for whole blood can be effectively applied to formalin-fixed, paraffin-embedded tissues on a large scale. The DNA extracted from 258 microdissection specimens in this study was of high quality (with a mean A260/280 ratio of 1.68), good quantity (mean yield of 23 μg), and was an excellent template for PCR (all cases were successfully amplified for K-ras exon 1). This success was significantly enhanced by performing two rounds of amplification using nested primers, and continued to be successful for at least 10 weeks following DNA extraction. This method is inexpensive, avoids organic solvents for removal of proteins, and has proven highly reliable for extraction of quality DNA from to formalin-fixed, paraffin-embedded cases.

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