Optimisation of DNA and RNA extraction from archival formalin-fixed tissue

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ABSTRACT

Archival, formalin-fixed, paraffin-embedded tissue is an invaluable resource for molecular genetic studies but the extraction of high quality nucleic acid may be problematic. We have optimised DNA extraction by comparing 10 protocols, including a commercially available kit and a novel method that utilises a thermal cycler. The thermal cycler and Chelex-100 extraction method yielded DNA capable of amplification by PCR from every block and 61% of sections versus 54% using microwave and Chelex-100, 15% with classical xylene-based extraction and 60% of sections using the kit. Successful RNA extraction was observed, by β-actin amplification, in 83.7% sections for samples treated by the thermal cycler and Chelex-100 method. Thermal cycler and Chelex-100 extraction of nucleic acid is reliable, quick and inexpensive.

INTRODUCTION

Most pathological specimens are routinely formalin-fixed and paraffin-embedded prior to histological evaluation. Although tissue architecture and proteins are preserved, extraction of nucleic acids may be difficult, yielding degraded DNA only. However, these samples form an invaluable resource for molecular studies with clinical correlation. We have sought to systematically optimise techniques for nucleic acid extraction.

Most techniques use three steps: deparaffinisation, digestion and purification. Dissolution of the wax in xylene and ethanol, reversing the embedding and dehydration of tissue processing, was described in 1985 (1). Alternative methods include melting any wax in a microwave oven (2) or direct digestion of the tissue (3). Digestion of the deparaffinised tissue with Proteinase K improves the yield of DNA. It is reported that prolonged digestion improves the yield of high molecular weight DNA (4), although others report that a 3-h incubation is as effective (2). Isolation of nucleic acids from the digest solution has utilised phenol and chloroform (5). Alternative methods tried include commercially available kits using spin columns or resin-based techniques (6,7).

We have compared these different deparaffinisation and purification techniques in combination in order to optimise DNA extraction from paraffin-embedded colonic tissue in order to generate DNA suitable for PCR. These methods were also compared to a commercially available kit. Using the most

efficient technique we demonstrate that RNA of suitable quality for RT–PCR may also be obtained from samples up to 20 years old.

Three deparaffinisation techniques were investigated: xylene/ethanol (1), microwave oven (2) and heating in a thermal cycler. After digestion, three purification methods were attempted for each sample, phenol/chloroform, simple boiling and Chelex-100. Thus nine different extraction methods were tried (Table 1). For each extraction technique, analysis was performed on a single 20 μm section of colonic tumour, taken from paraffin-embedded tissue, stored for up to 30 years at room temperature.

MATERIALS AND METHODS

Deparaffinisation methods

Xylene/ethanol. 200 μl xylene was added to each 20 μm section, agitated, heated for 15 min at 37°C then spun at 10 500 *g* for 15 min. The supernatant was removed, fresh xylene added, and the step repeated. Two identical washes with 100% ethanol for 30 min, 37°C, spinning at 10 500 *g* for 15 min, was followed by air drying of the tissue pellet. This was resuspended in 100 μl 1% n-lauryl sarcosine in PBS before digestion.

Microwave. Each 20 μm section was agitated in 100 μl 0.5% Tween-20 (Boehringer Mannheim, Germany). After heating in a 650 W microwave oven for up to 45 s, the tubes were spun whilst warm at 10 500 *g* for 15 min and placed on ice. Using a sterile pipette tip the solid wax disc was removed prior to digestion.

Thermal cycler. 100 μl 0.5% Tween-20 was added to each section, agitated and heated to 90°C for 10 min on a Techne Thermal Cycler. Sections were cooled to 55°C, the wax remaining in solution prior to digestion.

Digestion. 2 μl of 10 mg/ml Proteinase K (Sigma, Poole, UK) was added to each tube (final concentration 200 μg/ml) and digested for 3 h at 55°C, with gentle agitation every hour.

Purification methods

Phenol/chloroform. After gentle agitation in 100 μl phenol, samples were spun at 10 500 *g* for 15 min. The upper phase was removed to a fresh tube and 100 μl chloroform added. After centrifugation the upper phase was added to 200 μl 100% ethanol:0.03 M sodium acetate, gently mixed and placed at –80°C

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Table 1. Comparison of DNA extraction techniques and PCR amplification success

		Purification technique					
		Phenol/chloroform		**Simple boiling**		**Chelex 100**	
Deparaffinisation	**Xylene/ethanol**	**15%**		**0%**		**0%**	
			3 of 20		0 of 6		0 of 8
technique	**Microwave**	**22.7%**		**19.2%**		**54.1%**	
			5 of 22		5 of 26		20 of 37
	Thermal cycler	**11.7%**		**51.3%**		**61.3%**	
			2 of 17		19 of 37		100 of 163

The success of the kit-based method (QIAGEN) was 60% (12 of 20 sections).

for 30 min. The precipitated nucleic acid was pelleted by centrifugation at 10 500 g for 30 min and, after washing with 70% ethanol, air dried. The samples were resuspended in 200 µl sterile water. Samples de-waxed by thermal cycler required the solid wax disc to be removed prior to purification by centrifugation whilst hot, cooling on ice and lifting off the wax with a sterile pipette tip.

Simple boiling. 100 µl Tris–EDTA was added to each digest solution, heated to 99°C for 10 min, spun at 10 500 g for 15 min and placed on ice. Any hardened wax was removed, the sample re-spun at 10 500 g for 15 min, and top layer removed for later analysis.

Chelex-100. Each digest solution was heated to 99°C for 10 min with 100 µl 5% Chelex-100 (Bio-Rad laboratories) in Tris–EDTA. After gentle shaking, spinning at 10 500 g whilst hot for 15 min and placing the sample on ice allowed wax to harden enough to be removed. The sample was heated to 45°C and 100 µl chloroform added. Following gentle agitation and further centrifugation at 10 500 g for 15 min, the top phase (~180 µl) was removed and stored.

Commercial kit. Each 20 µm section was treated according to the manufacturer's protocol for the QIAamp DNA minikit (QIAGEN Ltd, Crawley, UK). The eluted DNA (~190 µl) was stored for later analysis.

Successful DNA extraction was assessed by PCR amplification of a fragment of the Cytochrome p450 2D6 gene (8) (Primers, 2D6 i4 forward 5'-AAATCCTGCTCTTCCGAGGC-3', 2D6 e3 reverse 5'-GCGCTTCGCCAACCACTCCG-3') 10 µl sample, 1.5 mM MgCl$_2$, 2% DMSO, 0.2 mM each dNTP, 150 ng primers (Cruachem Ltd, Glasgow, UK), 1 U Bio/polythermase (Bio/Gene, Kimbolton, UK). Amplification was carried out for 35 cycles (94°C for 30 s, 58°C for 30 s, 72°C for 30 s) after an initial denaturation step of 94°C for 3 min, on a Techne PHC3 Thermal Cycler. The cycles were followed by a 2 min extension at 72°C and the PCR product was visualised on an 8% polyacrylamide, non-denaturing gel by ethidium bromide staining and UV photography.

RNA extraction was performed on samples deparaffinised/purified by the thermal cycler/Chelex-100 and was assessed by PCR amplification of a fragment of the β-actin gene (9). (β-actin primers; BAC1779U forward 5'-CTCAGGAGGAGCAAT-GATCTTG-3', BAC1369D reverse 5'-CTGGGCATGGAGT-CCTGTGG-3'). A 5 µl sample was added to 0.75 µl reverse

transcriptase (200 U/µl, Promega UK Ltd, Southampton, UK), 0.06 A$_{260}$U oligo(dT)$_{12-18}$ primer (Amersham Pharmacia Biotech, St Albans, UK), 8 µl buffer and 0.2 mM of each dNTP in a final volume of 40 µl. The samples were incubated at 37°C for 60 min followed by denaturation at 94°C for 5 min. An 8 µl cDNA aliquot was used in a 50 µl PCR amplification reaction: 1.5 mM MgCl$_2$, 0.2 mM each dNTP, 75 ng β-actin primers (Oswel DNA service, Southampton, UK) and 1 U Bio/polythermase. The amplification was carried out on a Techne thermal cycler: an initial denaturation/melting step of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final polymerisation step of 72°C for 2 min. Twenty samples were treated with DNase (Amersham Pharmacia Biotech, St Albans, UK) prior to PCR. In a final reaction volume of 40 µl, a 30 µl sample was digested for 12 min, 37°C, with 7.5 U DNase, 2 µl Tris (1 M) and 0.4 µl MnCl$_2$ (1 M) followed by heating to 94°C for 5 min to denature the DNase. The samples were boiled with 20 µl 6% Chelex-100 for 15 min, centrifuged at 10 500 g for 15 min and 20 µl subject to reverse transcriptase treatment followed by β-actin or Cyp 2D6 PCR as described previously.

RESULTS

Twenty-four different blocks were used (age 1–30 years) and 336 sections of 20 µm analysed. Four blocks were 21–30 years old, six were 11–20 years old, six were 5–10 years old and eight were 1–4 years old. There were no differences in the ages of the blocks between the nine protocols of deparaffinisation and purification employed (data not shown). DNA was successfully extracted from every block and from 154 of 336 sections (45.8%). After an initial series of experiments, further samples were processed using the more efficient techniques.

Xylene and ethanol was used to deparaffinise 34 sections. Three of these sections, and only those subsequently purified using phenol and chloroform, yielded DNA capable of PCR amplification (8.8%) (Table 1).

Eighty-five sections were deparaffinised using microwave and Tween-20. Thirty sections (35.3%) had demonstrable DNA. The success of DNA amplification after microwave treatment and purification using phenol/chloroform or simple boiling was 22.7% (5 of 22) and 19.2% (5 of 26), respectively. Chelex-100 was the most effective purification technique of samples heated with the microwave; 54.1% (20 of 37) of these samples yielded DNA.

The thermal cycler method was used to process 217 sections. A PCR product was obtained in 121 (55.8%) of these. Chelex purification after thermal cycler processing appeared more efficient than simple boiling with Tris–EDTA; 61.3% (100 of 163) versus 51.3% (19 of 37) and significantly more effective than phenol, chloroform purification; 61.3% versus 11.7% (2 of 17), $P = 0.0001$ (Fisher's exact test). Using the thermal cycler and Chelex-100, amplifiable DNA was successfully extracted from sections less than 5 years old in 65.5% of samples (72 of 110), in 54.5% (10 of 22) of samples 5–10 years old and 51.6% (16 of 31) samples older than 10 years (comparisons not statistically significant). Therefore, the age of the specimen may affect the probability of success of DNA extraction and amplification.

Amplification after Chelex-100 purification [57.7% (120 of 208)] was also significantly more efficient than simple boiling purification [34.8% (24 of 69)], $P = 0.00075$, or purification with phenol/chloroform [57.7% versus 22.2% (10 of 45)], $P = 0.00001$ (Fisher's exact test).

The QIAamp DNA mini kit was used to process 20 sections. A PCR product was visualised in 12 (60%).

RNA extraction, as assessed by β-actin PCR amplification, was positive for 72 of 86 samples extracted by the thermal cycler/ Chelex method (83.7%). However, the age of the tissue section significantly affected the efficiency of β-actin amplification: all samples less than 10 years old whereas only 48.3% of samples older than 10 years gave a PCR product, $P < 0.00001$. No RNA was extracted from samples older than 20 years. Pre-treatment with DNase did not affect the ability to amplify a β-actin product. No expression of CYP2D6-DNA specific product was demonstrated after DNase treatment confirming the β-actin product to be generated from RNA, not anomalous DNA species as has previously been suggested (10).

DISCUSSION

Extraction of nucleic acid from archival tissue allows retrospective analysis and correlation of clinical end points or histological appearance structures with molecular biological markers. Many different techniques of varying success have been published (1–3,5). Although fixation time and type may influence DNA preservation (11) our samples had all been fixed in buffered formalin at room temperature for 24–48 h. The main obstacle in preparing DNA suitable for PCR amplification is removal of paraffin wax and purification. The techniques involving melting and removal of paraffin used here are more effective than methods employing organic solvents to dissolve the wax. The most efficient method uses a thermal cycler to melt the wax and boiling with Chelex-100 to purify any DNA. This is as effective as the commercially available kit that was used.

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We have developed a quick, straightforward, effective technique of extracting DNA and RNA from formalin-fixed, paraffin-embedded tissue. Although other groups have described methods using Tween 20 or Chelex-100 (3,7) for DNA extraction with 50% success of samples examined, using a thermal cycler and Chelex-100 we can extract PCRable DNA from 61% of samples analysed (Table 1). By testing nine different extraction protocols we have demonstrated that this technique is the most efficient in producing PCR quality DNA and is significantly more effective than traditional methods using organic solvents (61% versus 15%; $P = 0.0001$) (4). It is effective in extracting DNA from specimens of all ages (1–30 years old) and is as efficient as the QIAamp kit (60%). This method also extracted RNA from 83.7% of samples examined and from 100% of samples less than 10 years old. These figures are similar to published data using phenol and chloroform (12,13).

Thermal cycler and Chelex-100 extraction of DNA is safer, simpler, more effective and cheaper than the other methods or commercial kit described. The technique does not use any organic solvents and the entire extraction procedure uses only two Eppendorf tubes for each sample, negating the need for the transfer of supernatants between tubes lowering the use of consumables in the laboratory and reducing the possibility of contamination. This simplifies and speeds the entire process, yielding PCR quality DNA and RNA in less than 4 h.

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