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DNA extraction from fresh-frozen and formalin-fixed, paraffin-embedded human brain tissue

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ABSTRACT

Both fresh-frozen and formalin-fixed, paraffin-embedded (FFPE) human brain tissues are invaluable resources for molecular genetic studies of central nervous system diseases, especially neurodegenerative disorders. To identify the optimal method for DNA extraction from human brain tissue, we compared methods on differently-processed tissues. Fragments of *LRRK2* and *MAPT* (257 bp and 483 bp/245 bp) were amplified for evaluation. We found that for FFPE samples, the success rate of DNA extraction was greater when using a commercial kit than a laboratory-based method (successful DNA extraction from 76% *versus* 33% of samples). PCR amplicon size and storage period were key factors influencing the success rate of DNA extraction from FFPE samples. In the fresh-frozen samples, the DNA extraction success rate was 100% using either a commercial kit (QIAamp DNA Micro) or a laboratory-based method (sample boiling in 0.1 mol/L NaOH, followed by proteinase K digestion, and then DNA extraction using Chelex-100) regardless of PCR amplicon length or tissue storage time. Although the present results demonstrate that PCR-amplifiable genomic DNA can be extracted from both fresh-frozen and FFPE samples, fresh brain tissue is recommended for DNA extraction in future neuropathological studies.

Keywords: DNA extraction; fresh-frozen human brain tissue; formalin-fixed paraffin-embedded human brain tissue; polymerase chain reaction amplification

INTRODUCTION

Extraction of nucleic acids from a variety of tissue preparations, including fresh-frozen and formalin-fixed, paraffin-embedded (FFPE) human brain tissues, is the prerequisite for molecular biological analysis in the retrospective investigation of diseases. Although several methods have been used for DNA extraction from fresh-frozen and FFPE human brain tissues, only a few studies have compared the methods[1-4]. Human brain tissue is usually stored for years either frozen at –80ºC or in FFPE tissue blocks at room temperature. These samples are an invaluable resource for molecular studies owing to the availability of a pathologically-confirmed diagnosis and clinical information. However, nucleic acids extracted from human brain tissue, especially from FFPE samples, usually have low DNA yields due to abundant lipid[5], and are not always suitable for PCR due to poor quality/degraded template DNA as a result of extensive formalin crosslinking[6]. Most published methods of DNA extraction from FFPE tissue have been optimized using other organs[7, 8], often using surgical rather than autopsy samples, and a variety of lysis buffers[9-11]. Only rarely have postmortem tissues been tested[8, 12]. The European BrainNet group recently recommended QIAamp DNA Micro for DNA extraction from frozen and FFPE brain tissues after comparison with another commercial kit (DNeasy® Tissue)[2].

In order to test previously-published laboratory-based methods, in this study we optimized the different lysis buffers recommended in the literature[9-11] and used the DNA extraction method of heating and Chelex 100[7, 13]. This method was then compared with that recommended

by BrainNet to determine which was more successful in extracting genomic DNA from the samples, as well as the yield of DNA extracted. We also investigated the effects of different formalin storage periods and postmortem delay on the success rate of PCR-amplifiable genomic DNA extraction from postmortem FFPE brain tissue.

MATERIALS AND METHODS

Human Brain Tissue

A total of 102 fresh-frozen and 94 FFPE human brain tissue samples were obtained from 102 donors in a regional brain donor program ethically approved by the Human Research Ethics Committee of the South Eastern Sydney and Illawarra Area Health Service. The cases had been longitudinally followed and had either suffered from a neurodegenerative disease or had no neurological or psychiatric condition (control). The brain was collected at brain-only autopsy and the occipital lobe excised and frozen at −80°C. Postmortem delay ranged from 2.5 to 57 h. The remaining tissue was fixed in 15% formalin for two weeks and standard sample blocks were paraffin-embedded for case characterization. The remaining tissue was stored in formalin.

For this study, 100 mg of tissue from the frozen occipital pole was excised and stored in autoclaved plastic microtubes (1.5 mL) at −80°C until required. Fixed cerebellar tissue, which had been stored in 15% buffered formalin for 1–101 months, was processed through graded ethanols, then xylene and chloroform, prior to embedding in paraffin using an automated processor. Two 10-μm thick FFPE sections were cut on a microtome and stored in autoclaved plastic microtubes (1.5 mL) until required.

Genomic DNA Extraction using the QIAamp DNA Micro Kit

DNA was extracted using the QIAamp DNA extraction kit according to the procedure recommended by the manufacturer. Briefly, 10 mg brain tissue in a 1.5 mL microtube was treated with 180 μL ATL buffer and incubated with 20 μL proteinase K at 56°C overnight. After tissue lysis, 200 μL AL buffer was added with 200 μL ethanol, and the lysate transferred to the QIAamp MinElute column. After washing with AW1 and AW2 buffer, 20 μL AE buffer was added and the eluted genomic DNA collected.

Genomic DNA Extraction using Laboratory-based Lysis Buffers

Preliminary experiments were carried out to compare four lysis buffers for DNA extraction from frozen and FFPE samples from three cases. The lysis buffers were (1) 100 mmol/L NaCl, 10 mmol/L Tris-HCl, 25 mmol/L EDTA, 0.5% SDS, pH 8.4; (2) 0.32 mol/L sucrose, 10 mmol/L Tris at pH 7.5, 5 mmol/L $MgCl_2$, and 1% Triton X-100; (3) 0.1 mol/L NaOH; and (4) 1% Tween 20, 0.1% lauryl sulfate, 1% Nonidet P40, 10 mmol/L Tris-HCl.

Lysis buffer 3 was found to be optimal for fresh-frozen tissue and lysis buffer 4 was optimal for FFPE tissue. These buffers were used to process all further samples. Lysis buffer (200 μL) was added to the 1.5 mL tube, covered and heated to 99°C for 10 min on a thermal cycler (Eppendorff Thermomixer Comfort, Hamburg, Germany). Then 2 μL proteinase K (10 mg/mL) was added (final concentration 100 μg/mL) and incubated overnight at 55°C with gentle agitation for 20 s every hour (the dewaxing step was omitted for FFPE tissue due to the melting of the wax in hot solution, 55°C). Chelex-100 (200 μL 10%; Bio-Rad Laboratories, CA) was added to each tube, and gently inverted 3 times, followed by heating to 99°C for 10 min in the thermal cycler with gentle shaking. A cooling time of 5 min was allowed, and the microtubes were then centrifuged at 13 000 rpm for 15 min at 4°C (modified from the methods[7, 13]). The supernatant was collected, and then 1/10 volume of 3 mol/L sodium acetate (pH 5.2) and 2.5 volumes of ice-cold absolute ethanol were added. The solution was inverted several times and then cooled to −20°C. One hour later, the sample was centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was removed by pouring and blotting the open tube end on Whatman paper. The precipitate was washed in 1 mL 75% ethanol, and then the microtube was centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was removed by pouring and blotting the open tube end on Whatman paper. The DNA pellet was dried at room temperature for 30 min while the microtube was left open. The pellet was resuspended in 100 μL filtered TE buffer by flicking the bottom of tube when the tube was seen to be dry. The DNA was dissolved by incubating the sample at 37°C for 30 min.

Evaluation of DNA Yields

The quality of the template DNA extracted was determined

by running 5-µg samples on a 1% agarose gel, followed by staining with ethidium bromide and photographing under ultraviolet light. The DNA yields were analyzed by a spectrophotometer (Bio-Rad SmartSpec 3000) recommended by the manufacturer.

Evaluation of DNA Quality by PCR Analysis

Amplification of *LRRK2* exon 41 (primer set: forward 5'-gagcacagaatttttgatgcttg-3'; reverse 5'-ttttatccccattccacagcagtac-3'; product size: 257 bp)[14] and haplotyping microtubule associated protein tau (*MAPT*) (primer set: forward 5'-ggaagacgttctcactgatctg-3'; reverse 5'-aggagtctggcttcagtctctc-3'; product sizes: 483 and 245 bp)[15] was performed as previously described to determine the efficiency of the genomic DNA template. Conventional PCR was used for the templates. Human genomic DNA extracted from blood in a previous study was used as a positive control[16].

Comparison of Extracted Genomic DNA Quality by Real-time PCR

The same amount of genomic DNA (200 ng) was extracted from the same cases (fresh-frozen and FFPE samples) using the lab-based or the commercial kit method. DNA was amplified for *LRRK2* on a real-time PCR machine (Eppendorf, Hamburg, Germany) based on a SYBR green approach (SYB green PCR Master Mix, Roche Applied Science, Indianapolis, IN). An average of three Ct values was obtained.

Statistical Analysis

The SPSS-Fisher exact test was used to determine the effect of formalin storage duration on the suitability of genomic DNA for PCR. SPSS *t*-test was used to determine factors (postmortem delay and storage duration) influencing the suitability of genomic DNA for PCR. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Four Lysis Buffers for DNA Extraction using Laboratory-based Methods

Using DNA extracted from fresh-frozen tissue, there were no differences in the DNA yields and the OD_{260}/OD_{280} ratios using the four lysis buffers (data not shown). Lysis buffer 3 had less degradation of the genomic DNA (Fig. 1A) and all genomic DNA extracted using lysis buffer 3 was amplified by PCR (Fig. 1C).

Using DNA extracted from FFPE tissue, there was also no difference in the DNA yields and the OD_{260}/OD_{280} ratios with the different lysis buffers, although the electrophoresis patterns of the four were different (Fig. 1B). Lysis buffer 4 had less genomic DNA degradation and gave more identifiable PCR product bands (Fig. 1D).

Comparison of DNA Extracted from FFPE Human Brain Tissue using the QIAamp DNA Micro Kit *versus* the Optimized Laboratory-based Method

LRRK2 exon41 was successfully amplified in 76% of the DNA samples extracted using the QIAamp kit, but only in 31 out of 94 DNA samples (33%) extracted using the laboratory-based method. With regard to the DNA extracted, there was a significant influence of time of formalin storage on the success of DNA extraction and PCR amplification of *LRRK2* exon 41 (Table 1). Comparing *MAPT* H1 haplotyping with the *LRRK2* exon41 PCR data, the success rate of DNA amplification was significantly lower when the amplicon was larger (Fisher's exact analysis) (Table 1). No genomic DNA was extractable using the laboratory-based method in tissue stored in formalin for >4 years, while ~67% of these cases had extractable genomic DNA usable for PCR with the QIAamp kit (Table 2). There was no significant impact of postmortem delay on the DNA extracted (Table 1) and there were no differences in the quantity of genomic DNA extracted using the different methods (2–5 µg in total).

Comparison of DNA Extracted from Fresh-frozen Human Brain Tissue using the QIAamp DNA Micro Kit *versus* the Optimized Laboratory-based Method

Genomic DNA was successfully extracted from all fresh-frozen samples using either method. All DNA samples were suitable for PCR without influences of storage time or amplicon size, and both methods took a similar time for DNA extraction. The main difference between the methods was that considerably more DNA (250–750 µg) was extracted using the laboratory-based method than with the QIAamp kit (3–10 µg) due to the larger capacity of the laboratory-based method.

The QIAamp kit extraction method had lower threshold

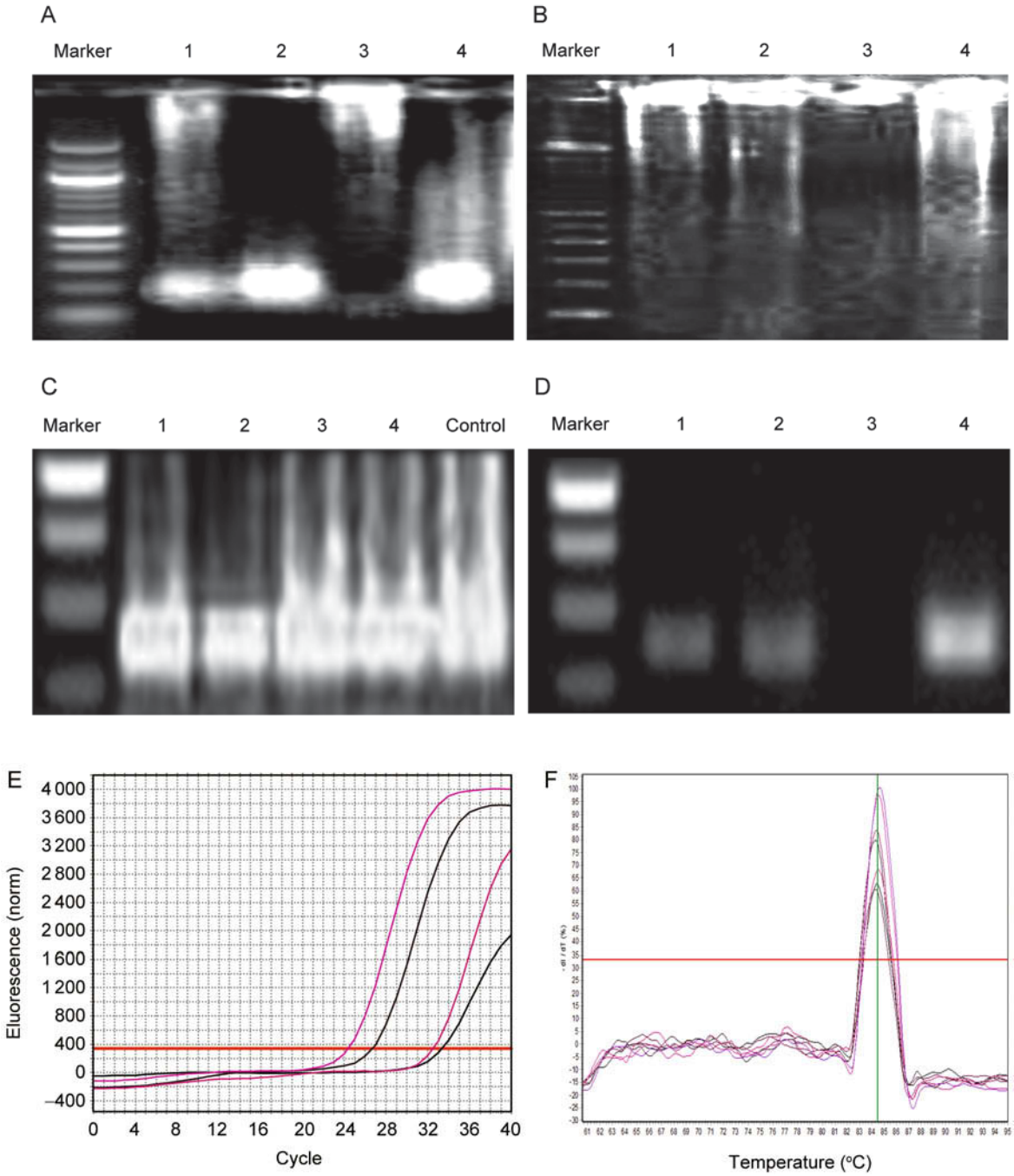

Fig. 1. Comparison of genomic DNA extracted using different lysis buffers in preliminary laboratory-based experiments. A and B: 1% agarose electrophoresis gels of genomic DNA extracted from the same fresh-frozen (A) and the same FFPE (B) human brain tissues using the four lysis buffers (lanes 1–4). C and D: 2% agarose electrophoresis gels of the *LRRK2* G2019S PCR product (257 bp) of genomic DNA extracted from fresh-frozen (C) and FFPE (D) tissue using the four lysis buffers (lanes 1–4). E: Comparison of SYBR green real-time PCR efficiency of DNA extracted from two different tissue sources (fresh-frozen and FFPE) using two different methods (red, QIAamp DNA Micro Kit; black, lab-based method). The Ct values were <28 cycles for all fresh-frozen samples, and >30 cycles for all FFPE samples. F: The PCR products with templates derived from fresh-frozen and FFPE samples had the same melting temperature.

Table 1. Factors influencing DNA extraction from FFPE human brain tissue

PCR-usable	PCR amplicon size (success rate)		#Storage duration (months) (mean ± SD)	#Postmortem delay (hours) (mean ± SD)
	LRRK2 exon 41 (257 bp)	*MAPT* H1 haplotyping 483 bp/245 bp		
Yes	33%	4%	31 ± 21	17 ± 17
No	67%	96%	43 ± 22	20 ± 16
P value	<0.0001		0.013 (t = 2.523)	0.405 (t = 0.837)

#Tested based on *LRRK2* exon 41 PCR products.

Table 2. Proportion of samples usable for PCR examined with time in formalin storage using Fisher's exact test (two-tailed)#

Time in formalin storage (years)*	Extraction using lysis buffer 4 (%)	Extraction using QIAamp DNA Micro Kit (%)
0–2	51	86
2–4	32	80
4–8	0	67
Overall	33	76

*P <0.0001; #tested by *LRRK2* exon41 PCR.

cycle (Ct) values than the lab-based method (24.4 *vs* 26.7 cycles for fresh-frozen, and 32.6 *vs* 33.4 cycles for PPFE tissue) (Fig. 1E), indicating more PCR products when using the QIAamp kit. All DNA extracted from fresh-frozen samples had significantly lower Ct vales than those from FFPE samples (Fig. 1E), suggesting that the DNA samples extracted from PPFE were degraded. The PCR products with the template extracted from either fresh-frozen or PPFE samples had the same melting temperature, indicating specific amplification (Fig. 1F).

DISCUSSION

DNA extraction from archival frozen and FFPE human brain tissues taken at postmortem has increasingly been used to inform the molecular biology of disease and age-related processes affecting the brain. In most instances, this is the only way to analyze such processes in the human brain. While there are several published methods for extracting DNA from fresh-frozen and FFPE tissues[17-19], most have been devised in tissues from other organs.

Here, our data showed that DNA extraction from fresh-frozen tissue is successful using either the commercial QIAamp DNA Micro Kit or an optimized laboratory-based method. We therefore conclude that the optimal method for high-yield DNA extraction is the use of fresh-frozen samples and the optimized laboratory-based method, which consisted of boiling samples in 0.1 mol/L NaOH, digestion in proteinase K, and purification in Chelex-100.

Our study also confirmed that the QIAamp kit is better for FFPE tissue, which is the most likely available type of archive material. DNA extraction from FFPE tissue usually includes three steps: dewaxing (with xylene or by heating), digestion, and purification[9, 20]. The QIAamp kit omits the dewaxing step, consistent with previous experiments that showed it is not necessary[21]. This omission appears to protect paraffinized DNA strands in FFPE tissue from rapid degradation during the extraction process. Our analysis showed that postmortem delay did not affect either the extraction effectiveness or the efficiency. But the extraction of DNA from FFPE tissue was compromised if it was stored in formalin for >4 years prior to use, and the successful PCR amplification rate was reduced if the amplicon was larger.

Our data indicate that archival FFPE human brain tissue has genetic value if genomic DNA is extracted with the QIAamp DNA Micro Kit. Genomic DNA extracted in this way is useful for a number of applications, while caution is required when DNA is used as a template for quantitative gene expression or for amplifying large fragments.

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