Evaluation of DNA and RNA Extraction Methods

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SUMMARY

This study was done to evaluate various DNA and RNA extractions from archival FFPE tissues. A total of 30 FFPE blocks from the years of 2004 to 2006 were assessed with each modified and adapted method. Extraction protocols evaluated include the modified enzymatic extraction method (Method A), Chelex-100 extraction method (Method B), heat-induced retrieval in alkaline solution extraction method (Methods C and D) and one commercial FFPE DNA Extraction kit (Qiagen, Crawley, UK). For RNA extraction, 2 extraction protocols were evaluated including the enzymatic extraction method (Method 1), and Chelex-100 RNA extraction method (Method 2). Results show that the modified enzymatic extraction method (Method A) is an efficient DNA extraction protocol, while for RNA extraction, the enzymatic method (Method 1) and the Chelex-100 RNA extraction method (Method 2) are equally efficient RNA extraction protocols.

KEY WORDS:
FFPE, DNA extraction, RNA extraction, crosslinking

INTRODUCTION

Formalin-fixed, paraffin embedded (FFPE) tissues represent an extraordinary source of archived and morphologically defined disease-specific biological material enabling the correlation of histological, immunohistochemical and molecular findings with therapy and clinical outcome[1]. FFPE tissue blocks have been used extensively in histopathology evaluation due to their stable format for histological analysis and long period storage capabilities. The extraction of nucleic acids from archival FFPE tissues enables researchers to perform various types of downstream studies including diagnostic and retrospective molecular genetic studies based on DNA amplification by polymerase chain reaction (PCR). The information elicited from FFPE tissues is valuable for better understanding of various types of human diseases[2]. Researchers have been using DNA extracted from FFPE tissues for diagnosis of various infectious agents such as CMV, EBV, HPV, HSV and *Mycobacterial tuberculosis*[3-5].

However, the extraction of high quality nucleic acids from archival FFPE tissues can be difficult and challenging. Formalin is the most commonly used fixative in histopathology, but it causes damage to tissue nucleic acids by crosslinking it to tissue proteins and consequently results in extensive DNA and RNA fragmentation[6]. Therefore, the use of PCR is very difficult with DNA extracted from FFPE tissues, and is usually associated with decreased PCR yields and inability to amplify longer DNA targets[7].

While formalin facilitates preservation of cellular proteins and conserves the tissue structure, it also reduces the recovery and quality of RNA[8]. Extensive crosslinking of RNA with proteins during formalin fixation causes very difficult extraction[8]. Researchers have reported that the enzyme and chemical degradation that occurs before and during the fixation process causes the decrease in yield and integrity of RNA[8]. Formalin also causes the formation of mono-methylol adducts with bases of nucleic acids, especially adenine, which reduces the efficiency of reverse transcription in reverse transcriptase polymerase chain reaction (RT-PCR), and negatively affects the performance of RNA samples in other downstream applications[9,10].

The aim of this study is to evaluate various DNA and RNA extractions from archival (FFPE) tissues. A total of 30 FFPE blocks from the years 2004 to 2006 were assessed with each modified and adapted method.

In the evaluation of DNA extraction methods, we compared four protocols, namely the modified enzymatic extraction method (Method A), Chelex-100 extraction method (Method B)[2], heat-induced retrieval in alkaline solution extraction method (Methods C and D)[11] and one commercial FFPE DNA Extraction kit (Qiagen, Crawley, UK).

As for RNA extraction methods, 2 extraction protocols which are the enzymatic extraction method (Method 1)[12] and Chelex-100 RNA extraction method (method 2)[2] were evaluated.

MATERIALS AND METHODS

FFPE tissue blocks

The FFPE tissue blocks were collected from the Department of Pathology Hospital Universiti Kebangsaan Malaysia, with consent from the proper authorities. The type of tissue samples evaluated comprised endometrial cancer, skin cancer and colorectal cancer.

For each paraffin block, one 10 m thick section was cut using rotary microtome (Leica, Germany) and collected in each sterile microfuge tube, ensuring that an equivalent amount of tissue was placed in all the microfuge tubes.

DNA extraction methods

Method A:
Deparaffinization was carried out by adding 1ml of xylene to each microfuge tube containing the tissue sections, and

This article was accepted: 12 July 2010
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this was vigorously vortexed for 20 minutes. Centrifugation was then performed at full speed for 5 minutes, and the resulting supernatant was discarded. The deparaffinization step was repeated once again, followed by the addition of 500 l of absolute ethanol, and this was mixed by vortexing. The solution was then centrifuged at full speed for 5 minutes, and the resulting supernatant was discarded. Next, 30 l of acetone was added and the tubes were incubated at 55 C for 20 minutes with the cap opened to evaporate the solvent. After incubation, 200 l of digestion buffer was added, and incubation was done overnight at 55 C. The purification stage was next, and performed by the following steps: addition of 500 l phenol:choloform:isopropanol alcohol at 25:24:1, followed by vigorous vortexing and centrifugation at 12,000 *x g* at room temperature for 10 minutes. The solution at the aqueous phase was transferred to a new 1.5 ml microfuge tube and an equal volume of chloroform was added, followed by mixing by vortexing and centrifugation at 12,000 *x g* for 5 minutes. The upper aqueous supernatant was carefully transferred to a new 1.5ml microfuge tube and 0.1 volume of 3 M sodium acetate was added, followed by vortexing. Then, 1 volume of isopropanol was added to the solution and incubated overnight at -20 C. The solution was centrifuged at 12,000 *x g* at 4 C for 5 minutes to pellet the precipitated DNA. The DNA pellet was washed once with 500 l of 75% ethanol and this was followed by centrifugation at 12,000 *x g* at 4 C for 5 minutes. The supernatant obtained was discarded, the DNA pellet was air-dried aseptically, and resuspended in 50 l of pre-heated AE solution.

Method B:
The tissue section in the 1.5ml microfuge tube was incubated in 100 l of 0.5% TWEEN 20 at 90 C for 10 minutes. The mixture was then cooled to 55 C, followed by the addition of 2 l of 10mg/ml proteinase K, and incubated overnight at 55 C. After incubation, 100 l of 5 % Chelex 100 in TE buffer was added to the mixture and incubated at 99 C for 10 minutes. Gentle agitation was performed on the mixture in the microfuge tube, followed by centrifugation at 10,500 *x g* while the mixture was still hot for 15 minutes. The mixture was placed in ice immediately after centrifugation to remove the hardened wax. After removal of the wax, the mixture was heated up to 45 C and 100 l of chloroform was added. After gentle agitation, the mixture was centrifuged at 10,500 *x g* for 15 minutes. The top phase which contained the extracted nucleic acid was transferred to a new 1.5 ml microfuge tube and stored at -20 C until further application.

Methods C and D:
The protocol for Methods C and D are similar, except that the pH used for the NaOH solution for method C was 12.25 and method D was 12.98. Tissue lysis was carried out by adding 500 l of NaOH, followed by incubation at 100 C for Method C and at 120 C for Method D for 20 minutes. Next, 500 l of phenol:choloform:isopropanol alcohol at 25:24:1 was added and mixed by vortexing, followed by centrifugation at 12,000 *x g* at room temperature for 10 minutes. The resultant aqueous phase was transferred to a new 1.5 ml microfuge tube and an equal volume of chloroform was added, followed by vortexing. The mixture was then centrifuged at 13,400 *x g* for 5 minutes. The upper aqueous phase of the supernatant was transferred to a new 1.5 ml microfuge tube, followed by the addition of 0.1 volume of 3M sodium acetate and agitation by vortexing. After agitation, 1 volume of isopropanol was added and the tubes were incubated overnight at -20 C. The mixture was then centrifuged at 13,400 x g at 4 C for 5 minutes. The resulting supernatant was then discarded and the precipitated DNA pellet was washed with 500 l of 75% ethanol. After centrifugation, the DNA pellet was air-dried aseptically and resuspended with 50 l of ultra pure water. The resuspended DNA was kept at -20 C for further application.

QIAamp FFPE DNA kit (Qiagen, Crawley, UK):
DNA was extracted from the FFPE tissue sections using the Qiagen kit according to the manufacturer's protocol.

RNA extraction methods:
Method 1:
Deparaffinization was carried out by adding 1 ml of xylene to the tissue section in each microfuge tube, followed by vigorous vortexing for 10 minutes. Next, the mixture was centrifuged at 16,000 *x g* for 5 minutes. The supernatant was discarded and the deparaffinization steps were repeated once, followed by rehydration through subsequent washings with 100%, 90% and 70% absolute ethanol diluted in RNase-free water respectively. The remaining tissue was collected after centrifugation at 16,000 *x g* for 5 minutes after each step. After a 70% ethanol wash, the tissue pellet was dried, followed by the addition of 200 l of RNA lysis buffer [10 mmol/L Tris/HCL (pH 8.0), 0.1 mmol/L ethylenediaminetetraacetic (pH 8.0), 2% sodium dodecyl sulfate (pH 7.3), and 500 g/ml proteinase K]. The mixture was then incubated at 60 C for 16 hours. The RNA was purified by phenol and chloroform purification steps. RNA precipitation was performed by the addition of 0.1 volume of 3 mol/L sodium acetate (pH 4.0), an equal volume of isopropanol and 1 l of 10 mg/ml carrier glycogen, followed by incubation overnight at – 20 C. The mixture was centrifuged at 12,000 *x g* at 4 C for 5 minutes. The supernatant was discarded, followed by washing of the RNA pellet with 500 l of 70% ethanol and air-dried aseptically. The air-dried RNA pellet was resuspended with 10 l of RNase-free water.

Method 2:
Method 2 is a modification of the Chelex 100 DNA extraction protocol by adding further steps to Method B in order to extract RNA from the FFPE sample. 30 l of the extracted nucleic acid obtained from Method B was added with DNase solution [7.5 units DNase, 2 l Tris (1M) and 0.4 l $MnCl_2$ (1M)], and incubated at 37 C for 12 minutes, followed by incubation at 94 C for 5 minutes. After incubation, 20 l of 6% Chelex 100 was added to the solution, and incubated at 100 C for 15 minutes. The solution was centrifuged at 10,500 *x g* for 15 minutes. The supernatant was transferred to a new microfuge tube and stored at -80 C for further application.

Evaluation of DNA yields:
The purity and concentration of the extracted DNA and RNA were determined by Biophotometer (Eppendorf, Hamburg, Germany), according to the manufaturer's protocol.

Reverse Transcription:
Each RNA extract was reverse-transcribed in a final volume

Table I: PCR Primer for this study

Primer	Forward primer	Reverse primer
cytochrome 2D6 gene	p450 5′-AAATCCTGCTCTTCCGAGGC-3′	5′-GCGCTTCGCCAACCACTCCG-3′
-actin gene	5′-CTCAGGAGGAGCAATGATCTTG-3′	5′-CTGGGCATGGAGTCCTGTGG-3′

of 20 l using M-MLV reverse transcriptase (Roche, Mannheim, Germany) with 8.4 l of PCR-grade water added to the 0.2 ml PCR tube, followed by the addition of 2 l of specific primers and 1 l of RNA (2ng/ l). The mixture was incubated for 10 minutes at 65 C. Next, the mixture was added with 4 l of 5x transcriptase reaction buffer, 0.5 l protector RNase inhibitor (40 U/ l), 2 l dNTPs (10 mM/ l), 1 l DTT and 1.1 l reverse trancriptase (20 U/ l). The mixture was incubated for 30 minutes at 45 C, followed by 5 minutes at 85 C. The converted cDNA was used as a template for PCR.

PCR analysis:
Each DNA extract was assessed by PCR amplification of a fragment of the cytochrome p450 2D6 gene with a product size of 356 bp[2]. Each converted cDNA was assessed by PCR amplification of a fragment of the -actin gene with product of 204 bp and cytochrome p450 2D6 gene[2]. The primer sequences used in the PCR amplification are listed in Table I. The PCR mixture of the final volume of 20 l (Intron Biotechnology Inc, Korea) consists of 13.25 l of ultra pure water, 2 l of 10x PCR buffer, 1.5 l of 10 mM dNTPs, 1 l of forward primer (10 pmoles/ l), 1 l of reverse primer (10 pmoles/), 1 l of DNA template and 0.25 l of DNA polymerase (5U/ l). The PCR thermal cycling for cytochrome p450 2D6 gene primers was carried out with an initial denaturation step at 94 C for 3 minutes coupled to a repeating cycle at 94 C for 30s, 58 C for 30s and 72 C for 30s for 40 cycles, followed by a 3 minute final extension step at 72 C on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The amplification for the -actin gene primers was carried out with an initial denaturation of 2 minutes at 94 C followed by 40 cycles at 94 C for 30s, 60 C for 30s, 72 C for 30s and a final polymerisation step at 72 C for 2 minutes.

Statistical Analysis:
Statistical analysis was performed using the SPSS Version 13, setting the statistical significance level at $p<0.05$. One-way ANOVA and paired-sample T test were used to analyze the FFPE DNA and RNA extraction results.

RESULTS

Thirty different FFPE blocks were used, and 210 sections of 10 m were analysed in this study. Endometrial cancer, skin cancer and colorectal cancer samples used are E1 to E9, S1 to S11, and C1 to C10 respectively.

FFPE DNA Extraction:
Four conventional DNA extraction protocols and one commercial DNA extraction kit (Qiagen FFPE DNA kit) were evaluated. In this study, Method C gave the highest percentage (73.3%) of quality extracted DNA for the range of 1.6 to 2.0 in $OD_{260/280}$, followed by Method A (70.0%), Qiagen FFPE DNA kit (46.7%), Method D (30.0%) and Method B (3.3%). Method B resulted in the lowest percentage for quality of extracted DNA with the range of 1.13 to 1.53 in $OD_{260/280}$. The average DNA concentrations obtained in this study were 28.03 ng/ul (Method A), 369.83 ng/ul (Method B), 13.77 ng/ul (Method C), 33.70 ng/ul (Method D) and 187.20 ng/ul (QIAamp FFPE DNA Kit). One-way ANOVA test showed a statistically significant difference between the purity and concentration of DNA for each extraction studied ($p<0.0001$, One-way Anova). Therefore, Method C significantly produced the highest purity of extracted DNA, and Method B gave the significantly highest yield of extracted DNA. The details of the purity and the yield of the extracted DNA are listed in Table II.

The percentages of Successful amplification of the cytochrome p450 2D6 gene sequence was 60% (18/30) for Method A, 23.3% (7/30) for Method B, 16.7% (5/30) for Method C, 43.3% (13/30) for Method D and 66.7% (20/30) for the Qiagen FFPE DNA kit.

FFPE RNA Extraction:
Method 1 gave the highest percentage (93.3%) of quality extracted DNA for the range of 1.6 to 2.0 in $OD_{260/280}$. The quality of RNA obtained with Method 2 gave an $OD_{260/280}$ reading of not more than 1.45. The average RNA concentration obtained with Method 1 was 371.97 ng/ul and 77.03 ng/ul for Method 2. After reverse transciptase treatments on all of the RNA samples for cDNA conversion, the amplification of the -actin gene sequence on the cDNA was 90% (27/30) for method 1, and 93.3% (28/30) for method 2. No successful amplification of the cytochrome p450 2D6 gene sequence was observed.

DISCUSSION

In the evaluation of the DNA extraction protocol, Method C gave the highest percentage (73.3%) of quality extracted DNA for the range of 1.6 to 2.0 in $OD_{260/280}$, but this method showed a lower rate (16.7%) of successful cytochrome p450 2D6 gene amplification. This could be due to the degradation and fragmentation of DNA during extraction procedures. However, Method A gave good results (70.0%) for the quality of extracted DNA, and it produced the most amplifiable properties (60.0%) compared to other conventional methods.

The successful PCR rate of method A (60.0%) is comparable to that of the commercial QIAamp FFPE DNA Extraction kit which has a 66.7% successful PCR rate (60% versus 66.7%, $p>0.05$, paired-sample T test). This result showed that Method A is equally efficient as the Qiagen kit. However, Method A is laborious, has a long extraction time, and is prone to cross contamination[2,13]. Nonetheless, this study

Table II: DNA and RNA Extraction Results

Sample	Year	Purity (OD260/280)						Yield (ng/ul)						Successful Amplification of PCR					
		DNA				RNA		DNA				RNA		DNA				RNA	
		Method A	Method B	Method C	Method D	Method 1	Method 2	Method A	Method B	Method C	Method D	Method 1	Method 2	Method A	Method B	Method C	Method D	Method 1	Method 2
C1	2006	1.72	1.28	1.63	1.09	1.67	1.16	32	251	13	44	156	77	Yes	Yes	Yes	Yes	Yes	Yes
C2	2006	1.55	1.31	1.58	1.92	1.66	1.09	28	457	16	10	243	32	No	No	No	No	Yes	Yes
C3	2006	1.59	1.26	1.58	1.58	1.71	1.16	29	467	9	38	346	92	Yes	No	No	No	Yes	Yes
C4	2006	1.67	1.37	1.57	1.53	1.7	1.18	46	543	7	33	422	76	Yes	No	No	Yes	Yes	Yes
C5	2006	1.67	1.23	1.55	1.94	1.67	1.16	33	155	3	16	218	59	Yes	Yes	No	No	Yes	Yes
C6	2006	1.82	1.31	1.84	1.53	1.74	1.18	43	772	12	36	377	70	Yes	No	No	No	Yes	Yes
C7	2006	1.72	1.37	1.63	1.32	1.56	1.21	35	519	7	100	60	61	No	No	No	No	Yes	Yes
C8	2005	1.8	1.4	1.66	1.82	1.69	1.28	35	747	18	49	802	139	Yes	Yes	No	Yes	Yes	Yes
C9	2005	1.76	1.13	1.5	1.66	1.68	1.2	49	173	7	26	107	35	Yes	Yes	No	Yes	Yes	Yes
C10	2005	1.84	1.9	1.71	1.46	1.73	1.41	48	364	12	34	458	68	Yes	No	No	Yes	Yes	Yes
E1	2006	1.94	1.34	1.76	1.05	1.69	1.35	21	48	13	43	260	66	Yes	No	No	No	Yes	No
E2	2006	1.88	1.47	1.64	1.55	1.7	1.36	62	856	64	51	211	346	Yes	No	No	Yes	Yes	Yes
E3	2006	1.84	1.33	2.28	1.55	1.72	1.11	20	162	7	15	251	35	No	Yes	Yes	No	Yes	Yes
E4	2006	1.81	1.43	1.56	1.33	1.75	1.19	25	372	28	48	956	62	Yes	No	No	No	Yes	Yes
E5	2005	1.96	1.27	1.69	1.32	1.84	1.24	29	86	4	32	67	26	Yes	Yes	No	Yes	Yes	Yes
E6	2005	1.98	1.24	1.91	1.51	1.83	1.12	22	66	2	21	193	58	Yes	Yes	No	No	No	No
E7	2005	2	1.17	1.83	1.55	1.85	1.14	26	235	9	10	308	98	Yes	No	No	Yes	No	Yes
E8	2006	2.13	1.43	1.71	1.51	1.82	1.36	24	504	52	20	796	53	Yes	No	No	No	Yes	Yes
E9	2006	2.18	1.53	1.66	1.8	1.67	1.18	16	899	34	51	1123	63	Yes	No	Yes	No	Yes	Yes
S1	2004	2.1	1.33	2.02	1.47	1.81	1.24	25	678	11	19	576	108	No	No	No	No	Yes	Yes
S2	2004	1.7	1.21	1.97	1.88	1.83	1.15	26	188	10	17	526	74	No	No	No	No	No	Yes
S3	2004	1.86	1.31	1.94	1.35	1.8	1.14	17	348	10	56	354	60	Yes	No	No	No	Yes	Yes
S4	2004	1.93	1.26	1.7	1.24	2.11	1.33	12	60	7	48	15	26	Yes	No	No	Yes	Yes	Yes
S5	2004	1.9	1.28	1.7	1.6	1.79	1.2	18	106	8	26	295	104	No	No	No	Yes	Yes	Yes
S6	2004	1.77	1.18	1.87	1.34	1.73	1.21	16	174	5	45	71	34	No	No	No	No	Yes	Yes
S7	2004	1.52	1.26	1.66	1.74	1.67	1.18	27	326	8	25	606	111	No	No	No	No	Yes	Yes
S8	2004	1.39	1.15	1.68	1.46	1.87	1.22	30	134	10	16	69	43	No	No	No	Yes	Yes	Yes
S9	2004	1.77	1.13	1.69	1.59	1.8	1.15	13	203	8	17	261	64	No	No	No	No	Yes	Yes
S10	2004	2.38	1.47	1.66	1.63	1.84	1.24	16	805	12	35	177	81	No	No	Yes	Yes	Yes	Yes
S11	2004	1.56	1.23	1.7	1.53	1.81	1.13	18	397	7	30	855	90	No	No	Yes	Yes	Yes	Yes

has indicated that it is a reliable protocol to extract DNA from archival FFPE samples. Method A is one of the most frequently used and most efficient methods to extract DNA from archival FFPE sample[11,14]. This method has the advantage of obtaining high purity DNA from FFPE samples[15].

In the evaluation of RNA protocols, both methods in this study gave good result for the -actin gene amplification. Absence of amplicons from the cytochrome p450 2D6 amplification indicated that successful -actin amplifications had been generated from cDNA, and not from contaminants of the genomic DNA. Method 1 and Method 2 are equally efficient in extracting usable RNA for PCR as the statistical analysis shows that Methods 1 and 2 are not significantly different (90% versus 93.3%, $p>0.05$, paired-sample T test). This study thus shows that RNA can be extracted by using either one of the two RNA extraction protocols.

Other researchers have reported that the use of FFPE samples for retrospective studies requires the use of primers that generate smaller amplification products[16]. This suggests that the DNA extracted from FFPE samples are highly fragmented, and lower amplimer size products will have a higher success rate using PCR. Besides that, formalin-fixed tissues undergo degradation most probably due to inadequate neutralization of the formalin, which causes acid depurination and prevents PCR amplification[15]. It has been reported previously that most of the DNA obtained from FFPE samples will have successful DNA amplifications of up to 300bp[6].

Researchers have also claimed that the most successful extraction methods for RNA and DNA from FFPE tissues involve the use of proteinase K that solubilizes tissue proteins and reverses monomethyl nucleotide modification[17]. Proteinase K digestion is important to release RNA and DNA from crosslinked protein and nucleic acids by digesting the protein portion of crosslinked molecules down to the level of tetrapeptides[18]. Proteinase K does not attack the actual methylene bridge that forms the crosslink as it does not involve a peptide bond, but the heating step is responsible for breaking the actual crosslinks[9].

CONCLUSION

Even though DNA and RNA are relatively damaged during the fixation process but they can still be used in various types of downstream applications if suitable extraction methods are employed. In the evaluation of DNA extraction methods from FFPE tissues, Method A is shown to be a reliable extraction method, and is as comparatively efficient as the commercial FFPE DNA extraction kit. For RNA extraction, both Method 1 and Method 2 proved to be equally efficient in extracting RNA from the FFPE tissues.

ACKNOWLEDGEMENT

The authors would like to thank the Research University Grant Scheme 2007, and the Faculty of Medicine and Health Sciences, University Putra Malaysia for the research facilities provided. Special gratitude is extended to Professor Dr. Hayati Abdul Rahman of the Department of Pathology, Hospital Universiti Kebangsaan Malaysia, for her permission in allowing us to use the FFPE tissue samples.

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