Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR

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Abstract

Aims—To evaluate the ability of four rapid DNA extraction methods to provide DNA for the polymerase chain reaction (PCR) from routinely fixed, paraffin wax embedded archival tissues.

Methods—Eighteen blocks of various tissues, 18 blocks of cervical cancer specimens, and nine blocks of B cell lymphomas were investigated. Both normal and biopsy specimen sized tissues were studied. DNA was extracted using four methods: boiling for 20 minutes in distilled water; boiling for 20 minutes in 5% Chelex-100 resin solution; 3-hour proteinase K digestion; and 3-hour proteinase K digestion, followed by boiling in 5% Chelex-100. Different exons of the p53 gene, human papillomavirus type 16 (HPV 16) sequence, and immunoglobulin heavy chain (IgH) gene rearrangement were amplified from the extracts.

Results—The Chelex boiling, proteinase K digestion, and proteinase K digestion-Chelex boiling methods produced DNA suitable for amplification in all of the 45 samples. Boiling in water yielded insufficient template for the PCR in three of the 45 cases (7%), and in six of 42 positive cases (14%) much fainter bands were observed, mostly when the processed material was either biopsy specimen sized or a B cell lymphoma sample. Fragments of the p53 gene were successfully amplified up to 408 base pairs in water boiled extracts, up to 647 in Chelex boiled preparates, and up to 984 in proteinase K digested and proteinase K digested-Chelex boiled samples, although with decreased sensitivity in the last case. All of the templates were reusable after 3 months of storage at -20°C .

Conclusions—Chelex boiling, proteinase K digestion, and proteinase K digestion followed by Chelex boiling produce suitable templates for the PCR from a large variety of paraffin wax embedded tissues. As the simple 20 minute boiling method in 5% Chelex-100 solution requires minimal manipulation and time, it could be useful, especially in the routine processing of large amounts of material.

specific nucleic acid sequences.¹ The technology provides a powerful means for the rapid detection of infections, particularly those by viral pathogens,²⁻⁵ of gene rearrangements in lymphoproliferative disorders,^{6,7} and of inherited diseases.⁸ Furthermore, the technique can serve as an initial step for PCR based investigations.

An attractive feature of PCR is that, unlike other molecular biological procedures, high molecular weight DNA is not required for successful amplification. As minute quantities of degraded DNA can also serve as the substrate for the reaction, the method is ideally suited to a template extracted from formalin fixed, paraffin wax embedded archival material. The combined advantages of exquisite sensitivity and the ability to use routinely processed materials allow large scale, retrospective studies to be carried out.

The most widespread protocols for DNA extraction from formalin fixed, paraffin embedded tissues utilise shorter or longer proteolytic treatment—ranging from three hours to several days—with or without additional organic solvent purification and ethanol precipitation.⁹⁻¹³ An alternative procedure for tissue processing for PCR involves lysing cells by boiling in distilled water^{3,14,15} or in a solution containing a chelating resin.^{4,5,16} Other methods such as sodium dodecyl sulphate (SDS) incubation^{12,15} or sonication¹⁷ have also been described.

For the large scale, routine processing of archival material, DNA extraction should be simple and rapid, with no influence on the success of the PCR. Moreover, as few steps as possible should be involved to minimise the possibility of contamination. The laborious and hazardous phenol-chloroform extraction should be particularly avoided.

Previous reports have focused mainly on quantitative issues comparing DNA obtained from fresh or fixed tissues,^{5,12,17} and have examined applicable methods separately^{14,16} or have compared them in the amplification of only a few similar target sequences.^{4,12}

We performed a qualitative study, comparing several reported rapid DNA extraction procedures which take less than 4 hours. The ability of these methods to provide DNA suitable for the PCR was investigated. For a more precise comparison, various sets of primers were chosen to amplify a single copy human gene, a viral sequence, and a rearranged immunoglobulin gene. Several practical aspects of the PCR were also examined, taking into consideration the size of the

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The polymerase chain reaction (PCR) is a method for the in vitro amplification of

processed tissue, previous dewaxing, target sequence length, and the reuse of extracts.

Methods

Blocks of formalin fixed, paraffin wax embedded archival tissues were selected from the files of the Department of Pathology, Kagawa Medical School, Japan. Normal sized (average surface dimensions 16 × 18 mm) and biopsy specimen sized (average surface dimensions 1.7 × 2 mm) tissue samples were collected and divided into the following groups:

- (1) Twelve blocks of normal sized and six blocks of biopsy specimen sized material, randomly chosen from various tissues; they represented neoplastic, inflammatory, or hyperplastic processes of the skin, soft tissue, tonsils, oesophagus, duodenum, colon, pancreas, lung, kidney, urinary bladder, meninges, ovary, cervix, normal placenta, and endometrium
- (2) twelve blocks of normal sized cervical cancer samples and an additional six blocks of cervical biopsy specimens (diagnosed as cancer or severe dysplasia), known to harbour human papilloma virus type 16 (HPV 16) DNA by the authors' previous PCR assays
- (3) six blocks of normal sized non-Hodgkin's B cell lymphoma samples (B-NHL) and an additional three blocks of B-NHL biopsy specimens, known to have detectable immunoglobulin heavy chain (IgH) gene rearrangement according to the authors' previous PCR results.

The material was processed between 1988 and 1993. Fixation was in neutral formalin, and the fixation time varied from 24 to 48 hours. Processing was done by standard methods: dehydration in graded alcohols; clearing in xylene; and embedding in paraffin wax.

DNA EXTRACTION

From normal sized tissues a single 5 µm section (average collected volume 1.44 mm³) and from biopsy specimen sized tissues a single 10 µm section (average collected volume 0.034 mm³) were cut and processed according to the following four methods (Methods A–D).

Method A

The section was resuspended in 100 µl distilled water (50 µl for biopsy specimen sized material), with or without pretreatment, and was boiled for 20 minutes (modified from the method of Lench *et al*¹⁵). Pretreatment, if performed, consisted of dewaxing by two washes in xylene at 60°C for 20 minutes, rehydration in absolute ethanol, and desiccation.

Method B

This was similar to Method A, but the section was resuspended in 5% Chelex-100 resin solution (Bio Rad Laboratories, Hercules, California, USA) and was boiled for 20 minutes.¹⁸

Method C

After dewaxing and rehydration, the tissue section was resuspended in 100 µl (50 µl for biopsy specimen sized material) of digestion buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM TRIS-HCl, 0.5% TWEEN 20, pH 9 at 25°C) containing 200 µg/ml proteinase K (Merck, Darmstadt, Germany) and was incubated at 55°C for 3 hours (or at 37°C overnight).¹⁹ The proteinase K was inactivated by boiling for eight minutes.

Method D

This was similar to Method C, but after digestion Chelex-100 was added to a final concentration of 5%, and the mixture was boiled for eight minutes (modified from the method of Gill *et al*²⁰).

After boiling, all the tubes were chilled on ice, and were centrifuged for five minutes at 18 000 × g. An aliquot of 5 µl of the supernatant (10 µl for biopsy specimen sized tissue) was used as the template.

OLIGONUCLEOTIDE PRIMERS

The sequences, target regions, and other characteristics of the primers are shown in table 1. Three types of primer set were used: one for different exons of the p53 gene as a normal single copy human gene, on the randomly chosen tissues; a second type for the open reading frame region E6 of HPV 16 on cervical samples; and a third type for IgH gene rearrangement on B cell lymphomas. Oligonucleotides were synthesised on a

Table 1 Oligonucleotide primers used in the study

Primer	Sequence (5'–3')	Target region	Size of PCR product (base pairs)	Source
E81	CCTATCCTGAGTAGTGGTAATCTAC	p53 gene, exon 8	166	Miller <i>et al</i> ²¹
E82	GTCCTGCTTGCTTACCTCGCTTAGT			
E71	CTCCTAGGTTGGCTCTGACTGT	p53 gene, exon 7	131	
E72	CAAGTGGCTCCTGACCTGGA			
E61	CACTGATTGCTCTTAGTCTGGC	p53 gene, exon 6	144	
E62	AGTTGCAAACCAGACCTCAGGCG			
E51	TTCCTCTTCTGCACTAC	p53 gene, exon 5	214	
E52	GCCCCAGCTGCTCACCATCG			
E41	AAAACCTACCAGGGCAGCTAC	p53 gene, exon 4	99	
E42	CTCAGGGCAACTGACCGTG			
H1	ATTAGTGAGTATAGACATTA	HPV 16, ORF E6	110	Shibata <i>et al</i> ³
H2	GGCTTTTGACAGTTAATACA			
Fr3A	ACACGGC(C/T) (G/C) TGTATTACTGT	IgH gene, V region IgH gene, J region	80–120 —	Wan <i>et al</i> ⁶
LJH	TGAGGAGACGGTGACC			
VLJH	GTGACCAGGGT(A/G/C/T)CCTTGGCCCCAG			

ORF = open reading frame; V region = variable region; J region = joining region

Cyclone Plus automated DNA synthesiser (Millipore, Bedford, Massachusetts, USA).

PCR

The PCR was carried out in a Perkin Elmer Cetus Thermal Cycler (Perkin-Elmer, Norwalk, Connecticut, USA). The reaction mixture (in 50 μ l or 25 μ l total volume) contained 10–20 pmol of primers, 1 $\times$ PCR buffer (50 mM KCl, 10 mM TRIS-HCl, 3 mM MgCl₂, pH 9 at 25°C), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.625–1.25 U of *Taq* polymerase (Pharmacia Biotech, Milwaukee, Wisconsin, USA), and the template. The reaction mixture was overlaid with a drop of light mineral oil (Sigma, St Louis, Missouri, USA).

Each PCR cycle consisted of a denaturation step at 94°C for 30 seconds, primer annealing at 60°C (55°C for H1 and H2 primers) for 30 seconds, and extension at 72°C for 1 minute. An initial denaturation step was performed at 94°C for 2 minutes, and in the final cycle the extension step was elongated to 5 minutes at 72°C. Samples were amplified through 35 (for normal sized samples) or 40 cycles (biopsy specimen sized samples).

For biopsy specimen sized B cell lymphoma samples, seminested PCR was performed: a first round of 30 cycles with primers Fr3A and LJH; and a second round of 35 cycles with Fr3A and VLJH, with 1 μ l of the product of the first round of the PCR as the template.⁶

In all runs positive and negative controls were included. The positive and negative controls were as follows: HPV 16 positive DNA (Genemed, San Francisco, California, USA) and DNA extracted from human T cell lymphotropic virus type I (HTLV-I) carrier cell line, respectively, for the HPV 16 PCR; high molecular weight DNA extracted from fresh frozen human lung and distilled water, respectively, for the p53 PCR; and DNA extracted from paraffin wax embedded B cell and T cell lymphoma samples, respectively, for the IgH PCR.

Sterile materials were used throughout, with strict precautions to avoid contamination or product carryover.²² DNA extraction, the PCR, and electrophoresis were performed in separate laboratories.

Table 2 PCR results on different materials prepared by Methods A–D (numbers shown are positive cases/total cases)

Material	Method			
	A	B	C	D
Normal sized:				
Various samples	12/12	12/12	12/12	12/12
Cervix samples	12/12	12/12	12/12	12/12
B cell lymphoma samples	5*/6	6/6	6/6	6/6
Biopsy specimen sized:				
Various samples	4†/6	6/6	6/6	6/6
Cervix samples	6/6	6/6	6/6	6/6
B cell lymphoma samples	3/3	3/3	3/3	3/3
Total	42‡/45	45/45	45/45	45/45

* Fainter bands in four cases

† Fainter bands in two cases

‡ Fainter bands in six cases

Figure 1 3% agarose electrophoresis gel of the PCR products, showing amplification of a 166 base pair fragment of exon 8 of the p53 gene from DNA extracted from normal and biopsy specimen sized paraffin wax embedded tissue by different methods. M: ϕ X 174 DNA *Hae* III digest. 1a–d: normal sized tissue sample of colonic adenocarcinoma processed by Methods A (1a), B (1b), C (1c), and D (1d). 2a–d: biopsy specimen of duodenal polyp processed by Methods A (2a), B (2b), C (2c), and D (2d). N: negative control (distilled water instead of template). P: positive control (high molecular weight DNA as template, extracted from fresh frozen human lung). The sizes of the molecular weight marker and the specific band (in base pairs) are shown to the left and right, respectively.

GEL ANALYSIS

The quality of the template DNA was determined by running samples on 1% agarose gel (Wako Chemicals, Osaka, Japan) stained by ethidium bromide. Amplifications were analysed by electrophoresis of a 10 μ l aliquot through 3% NuSieve GTG agarose (FMC Bioproducts, Rockland, Maine, USA), followed by ethidium bromide staining visualised by an ultraviolet light transilluminator. Bands of appropriate size were identified by comparison with DNA markers of known size.

Results

The overall results obtained by the PCR using the different primer sets are shown in table 2.

Figure 2 3% agarose electrophoresis gel of the PCR products, showing amplification of an ~100–120 base pair fragment of a rearranged immunoglobulin heavy chain gene from DNA extracted from two normal sized B cell lymphoma samples by different methods. M: ϕ X 174 DNA *Hae* III digest. 1a–d: parotid B cell lymphoma processed by Methods A (1a), B (1b), C (1c), and D (1d). 2a–d: intestinal B cell lymphoma processed by Methods A (2a), B (2b), C (2c), and D (2d). N: negative control (DNA extracted from a T cell lymphoma sample as the template). The sizes of the molecular weight marker bands (in base pairs) are shown to the left.

Figure 3 1% agarose electrophoresis gel of DNA extracted from a normal sized oesophageal carcinoma sample by different methods. M: λ DNA Hind III digest; (A): boiled in distilled water (Method A); (B): boiled in 5% Chelex solution (Method B); (C): proteinase K digested (Method C); (D): proteinase K digested and boiled in 5% Chelex solution (Method D); P: positive control (high molecular weight DNA extracted from fresh frozen human lung). The sizes of the molecular weight marker bands (in base pairs) are shown to the left.

P53 GENE AMPLIFICATION IN VARIOUS RANDOMLY CHOSEN TISSUES

A 166 base pair fragment of exon 8 or a 99 base pair fragment of exon 4 of the p53 gene was amplified. All of the templates prepared from normal sized tissues by different methods produced a single specific amplification band with the same intensity (fig 1, lanes 1a-d). However, two of the biopsy specimen sized samples (33% of the specimens) prepared by the water boiling method (Method A) failed to show the amplification, and in two other cases (50% of the successful amplifications) the bands were much fainter. Biopsy samples prepared by Chelex boiling (Method

B), proteinase K digestion (Method C) and proteinase K digestion-Chelex boiling (Method D) provided uniformly intense bands (fig 1, lanes 2a-d).

HPV 16 AMPLIFICATION IN CERVICAL SAMPLES

A 110 base pair fragment of the open reading frame region E6 of HPV 16 was amplified. All of the samples prepared by the different methods from both normal and biopsy specimen sized tissues were successfully amplified (data not shown).

IMMUNOGLOBULIN GENE REARRANGEMENT

AMPLIFICATION IN B CELL LYMPHOMA SAMPLES Depending on the given rearrangement of particular clones of B cell lymphomas, an 80–120 base pair fragment was amplified. One normal sized lymphoma specimen (17% of the normal sized B cell non-Hodgkin's lymphomas) prepared by the water boiling method failed to reveal amplification and in four other cases (80% of the successful amplifications) much fainter bands were observed. DNA extracted by the other three methods produced intense bands of appropriate size in all cases (fig 2). The three biopsy specimens investigated by seminested PCR showed similar strong bands, regardless of the method used for extraction.

ANALYSIS OF PREVIOUS DEWAXING

To reduce further the number of steps involved in the extraction, the dewaxing and rehydration steps were omitted from the water boiling and Chelex boiling protocols. No differences were found in the successful amplifications using these extracts (data not shown).

ANALYSIS OF TEMPLATE QUALITY

The DNA extracted by the different methods was analysed on 1% agarose gel. There was evidence of extreme DNA degradation in templates prepared by water boiling (Method A), with the bulk between 100 and 200 base pairs. The DNA obtained by Chelex boiling (Method B) was better preserved, with a distribution in the 100–600 base pairs range. The proteinase K digestion methods (Methods C and D) produced DNA of increased molecular weight with a range from 100 to over 10 000 base pairs, with the bulk between 100 and 4000 base pairs (fig 3).

ANALYSIS OF THE TARGET SEQUENCE LENGTH

Combining forward and reverse primers, homologous to consecutive exons of the p53 gene, amplification products of gradually increasing length were able to be created. Primers E51 and E52 (flanking exon 5) amplify a target of 214 base pairs, while primers E51 and E62 (flanking exon 5 and 6), E71 and E82 (flanking exon 7 and 8) and E61 and E72 (flanking exon 6 and 7) amplify regions of 408, 647, and 983 base pairs, respectively. Two normal sized samples were analysed by this primer network.

Proteinase K digested and proteinase K digested-Chelex boiled templates were successfully amplified up to 983 base pairs (data

Figure 4 2.5% agarose electrophoresis gel of PCR products showing amplification of exon 5, exon 5–6, and exon 7–8 of the p53 gene from DNA extracted from a normal sized cervical cancer sample by different methods. M: ϕ X 174 DNA Hae III digest; 1a–d: amplification of 214 base pair fragment from extracts prepared by Method A (1a), B (1b), C (1c) and D (1d); 2a–d: amplification of 408 base pair fragment from extracts prepared by Method A (2a), B (2b), C (2c) and D (2d); 3a–d: amplification of 647 base pair fragment from extracts prepared by Method A (3a), B (3b), C (3c) and D (3d); 1p, 2p, 3p: positive controls (high molecular weight DNA as template, extracted from fresh frozen human lung). The sizes of the molecular weight marker and the specific bands (in base pairs) are shown to the left and right, respectively.

not shown), although with an accumulation of non-specific products owing to uncompleted amplifications. Chelex boiled material showed a strong signal for 214 and 408 base pair targets, and still produced a weak band on the 647 base pair target. Water boiled material was successfully amplified on the 214 and 408 base pair targets only (fig 4).

ANALYSIS OF THE REUSE OF TEMPLATES

After three months of storage at -20°C , two samples from each group prepared by the four methods were successfully reamplified for their specific targets. However, 408 base pair targets produced weaker bands than freshly prepared samples, and amplification for the 647 base pair fragment was successful only in proteinase K digested and proteinase K digested-Chelex boiled samples (data not shown).

Discussion

Although formalin fixed tissues can also provide high molecular weight DNA, suitable for complex molecular biological investigations,^{9,11} the procedure requires lengthy digestion and purification steps, which would be difficult to perform on a large scale. The major attraction of the PCR, for research and diagnostic purposes in pathology, is its ability to work on poor quality DNA samples obtained by simple methods of extraction.

Four different techniques of rapid DNA extraction, from paraffin wax embedded tissues, were examined, from which PCR results can be obtained within 4–8 hours. DNA produced by the simplest water boiling method failed to be amplified for specific sequences in 7% of the total cases. In addition, in 14% of the positive samples the signal yielded was much weaker than in cases using other methods. Several investigators have also reported a variable degree of failure using this technique.^{4,14,16} It seems likely that the cause of failure is the absence of a detectable amount of the target DNA. Theoretically, the amount of the released target DNA can be small if the size of the processed tissue is small (a biopsy specimen) or if only a portion of the processed material carries the target sequence (monoclonal B cells with a rearranged IgH gene). Indeed, the authors were able to amplify the p53 gene fragment from the water boiled extracts in all of the normal sized tissues but in only 66% of the biopsy specimen sized samples. Furthermore, in half of the latter successful cases fainter bands were observed. The presumed small population of monoclonal B cells also failed to be amplified in 17% of the samples, and yielded fainter bands in 80% of the positive cases, examined on normal sized B cell non-Hodgkin's lymphoma tissues. However, all of the biopsy specimen sized tissues were amplified successfully, probably due to the more sensitive semi-nested PCR, although it is also possible that the clonal population was more numerous in these cases. When 1/2, 1/4, 1/8, and 1/16 subdivisions of the same normal sized lymphoma

tissue were processed, the samples prepared by the water boiling method produced clearly weaker bands on the 1/8 and 1/16 portions (data not shown). In contrast, the authors did not experience any difference when amplifying HPV 16 on normal or biopsy specimen sized cervical samples, where the target number seems to be above the limit that can cause discernible differences (cervical cancer cells may contain one to 50 copies of HPV per cell).²³

Preparing the tissue samples by boiling in Chelex-100 solution produced enough DNA to be amplified at the same intensity as from those samples prepared by proteinase K based methods, regardless of the tissue type, size, or target sequences used. Chelex-100 is a chelating resin that preferentially chelates polyvalent metal ions. Sample processing by Chelex has been successfully employed for a small number of cells,¹⁸ for forensic type materials,²⁴ and for paraffin wax embedded tissues.^{4,5,16} The effect of Chelex, which prevents the degradation of DNA, is thought to be due to chelating metal ions which may act as catalysts in the breakdown of DNA at high temperatures in low ionic strength solutions.^{18,24} Indeed, templates prepared by Chelex boiling in the present study were shown to be better preserved by electrophoretic examination than those prepared by the water boiling method (fig 3, lanes A and B). It is also possible that Chelex may bind other substances—blood derived products—which could inhibit the PCR itself.²⁴

In the present study, 3 hour proteinase K digestion was also capable of yielding a sufficient amount of template DNA to amplify any of the targets in all of the cases. Although increasing the time of the digestion substantially improves the yield of DNA,¹² for routine purposes this does not seem to be essential. However, to avoid phenol-chloroform extraction and to use the supernatant fluid directly, it is advisable to apply non-ionic detergents in the digestion buffer, instead of sodium dodecyl sulphate which was reported to inhibit *Taq* polymerase.^{19,25} In this experiment, adding Chelex to the proteinase K digested samples (Method D) proved to have no noticeable influence on the sample quality.

These results also indicate several limits regarding the size of the products which can be amplified from various extracts. Water boiled samples are extremely degraded and were found to allow amplifications up to only 400 base pair fragments in the present study. With a better preservation of the target DNA, it was possible to amplify fragments up to about 650 base pairs from Chelex boiled material and up to about 1000 base pairs from proteinase K digested material, although with compromised specificity. Nevertheless, this amplification product size is unlikely to be used for diagnostic purposes.

Dewaxing seems to have no effect on the successful release of DNA from paraffin wax embedded tissues. No difference was found when tissues were boiled with or without previous deparaffinisation, which is in

agreement with other observations.^{4,14} Although tissues were dewaxed before proteinase K digestion, other investigators have eliminated this step without any effect on the yield or quality of DNA obtained.^{9,12}

All extracts were reamplified for their relatively short (80–166 base pairs) specific targets, after storing them at –20°C for three months. However, weakening in the product intensity was observed for the 408 base pair fragment, and failure to reamplify the 647 base pair fragment was noted from Chelex boiled samples. This decreased amplification ability was also reported in stored proteinase K digested samples, and is likely to be due to degradation or breaking of the DNA.¹³

In conclusion, the simplest water boiling method may fail to produce a sufficient quantity and quality of template from formalin fixed, paraffin wax embedded tissues, particularly when the starting material is small, the target molecules are expected to be of low copy number, or longer fragments are to be amplified. DNA prepared by Chelex boiling is of a quality which can be amplified as successfully, in all practical aspects, as that extracted by proteinase K digestion. As the procedure is simpler, requires less time and less manipulation—thus decreasing the possibility of contamination—it would be helpful for the large scale, routine treatment of paraffin wax embedded samples for the use of PCR.

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