Aegean Pathology Journal **1**, 33–38, 2004
Available on line at www.epd.org.tr/apj.php

Rapid and effective DNA amplification by polymerase chain reaction directly from paraffin-embedded tissue

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Accepted for publication on 14 April 2004

A large number of archival paraffin-embedded tissue banks have been established during diagnostic surgical pathology, forming a precious resource of tissues for retrospective molecular studies of cancer and a variety of other diseases. Extraction of DNA from formalin-fixed paraffin-embedded tissue samples was previously accomplished. However, this procedure is labor intensive, time-consuming and expensive. This study demonstrated the successful use and optimization of a rapid, reliable and effective protocol for DNA amplification directly from paraffin-embedded tissue eliminating the deparaffinization and DNA isolation steps. In the course of this study effectiveness of a commercially available, ready-to-use PCR master mix kit was also tested. Once the presented protocol for DNA amplification is applied correctly, large quantities of paraffin-embedded material stored in our pathology departments will be available for molecular diagnostics and research.

Keywords: Paraffin-embedded tissue, DNA amplification, PCR

Introduction

Recently there has been a growing interest in using polymerase chain reaction (PCR) to investigate the molecular changes in stepwise progression of various diseases including cancer.[1–5] A large number of formalin-fixed paraffin-embedded tissues have been established during the course of diagnostic surgical pathology. They are the easiest to store, transport and a valuable archive for molecular pathology studies. The use of paraffin-embedded tissues has some limitations in molecular pathology. The fixation of tissue samples in formaldehyde leads to extensive cross-linking of all tissue components and therefore the nucleic acids isolated from these specimens are highly fragmented.[6–9] The level of fragmentation depends on the tissue type and the condition of fixation. In general, the average fragment size of a PCR amplicon is 300–400 bases from formalin-fixed paraffin-embedded biopsy tissues.[6] Extraction of DNA from formalin-fixed paraffin-embedded tissue samples was accomplished as early as 1985.[10,11] However, these procedures are labor intensive, time-consuming and expensive.[12–14] Regular DNA amplification protocols using paraffin-embedded tissues start with microtome sectioning and a series of deparaffinization steps, followed by DNA isolation and purification and finally amplification of target DNA by PCR.[12,15] It usually takes 1-3 days to complete and is quite challenging to work through, especially with large patient numbers. The first problem can be cross-contamination between paraffin-embedded tissue samples. Using a fresh microtome blade for each and every paraffin block and minimal pipetting steps in DNA isolation may decrease this possibility. On the other hand, loss of precious material during these long and tedious procedures is more important and may not be replaceable.

The aim of this study is to use and optimize a rapid, reliable and effective protocol for DNA amplification directly from paraffin-embedded tissue eliminating the deparaffinization and DNA isolation steps. In the course of this study we also tested the effectiveness of a commercially available, ready-to-use PCR master mix kit.

Materials and Methods

Paraffin-embedded Tissue Samples

Nineteen archival formaldehyde-fixed and paraffin-embedded tissues previously used for histopathological diagnosis, were obtained from the Department of Pathology, Pamukkale University School of Medicine. (10 different cancer tissue samples embedded in paraffin in the year 2003 and 9 different leiomyoma samples embedded in paraffin between the years of 1996-2004). Diagnostic details of the biopsies were summarized in Table 1.

1-3 mm^2, small tissue samples were cut from paraffin-embedded tissue blocks by hand dissection. In order to eliminate cross-contamination a new-sterile scalpel blade is used for each paraffin block and small tissue samples were placed directly into the 500 µl PCR tubes. 20µl of 1% Triton X-100 (v:v in ddH_2O, Laboratory Grade, Sigma Chemical) was added to the tubes and then samples were incubated at 95°C for 20 min in Hybaid PCR Sprint Temperature Cycling System.

Primers and PCR conditions

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an autosomal locus, forward (5'-CCCCACACACATGCACTTACC-3') and reverse (5'-CCTAGTCCCAGGGCTTTGATT-3') primers (16) were used to amplify a 97 base pair (bp) DNA fragment by PCR.

The incubation with 1% Triton X-100 was followed by rapid vortexing, then samples were chilled and finally the ready-to-use PCR reaction mixture (Qiagen, Hilden, Germany) was added directly to the tubes. All PCR amplifications were performed in a final volume of 50 µl containing 1-3 mm^2 tissue sample, 20µl 1% Triton X-100, 20 pmol of each primer (GAPDH forward, and GAPDH reverse), and 25 µl of HotStarTaq Master Mix (containing 2.5 units HotStarTaq DNA polymerase, 1x PCR Buffer with 1.5 mM $MgCl_2$, and 200 µM of each dNTP; Qiagen, Hilden, Germany).

Table 1. Outline of paraffin–embedded tissue samples used in this study.

Case Number	Year (embedded in paraffin)	Pathological Diagnosis
1	2003	Squamous cell carcinoma of cervix
2	2003	Endometrial adenocarcinoma
3	2003	Squamous cell carcinoma of larynx
4	2003	Squamous cell carcinoma of skin
5	2003	Invasive ductal carcinoma of breast
6	2003	Undifferentiated large cell carcinoma of lung
7	2003	Adenocarcinoma of prostate
8	2003	Papillary serous cystadenocarcinoma of ovary
9	2003	Moderately differentiated adenocarcinoma of gallbladder
10	2003	Adenocarcinoma of rectum
11	1996	Leiomyoma
12	1997	Leiomyoma
13	1998	Leiomyoma
14	1999	Leiomyoma
15	2000	Leiomyoma
16	2001	Leiomyoma
17	2002	Leiomyoma
18	2003	Leiomyoma
19	2004	Leiomyoma

Thermal cycling was carried out using the following conditions in Hybaid PCR Sprint Temperature Cycling System: initial activation of HotStarTaq DNA polymerase at 95°C for 15 min, followed by 40-50 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, with final extension at 72°C for 10 min. The PCR products were analyzed by 1 % agarose gel electrophoresis and visualized by exposure to

ultraviolet light after ethidium bromide staining using VilberLourmat, Biocapture Image Analysis Software.

All samples were tested three times. Human genomic DNA isolated from serum using the QIAamp Blood and Body Fluid DNA isolation kit (Qiagen, Hilden, Germany) was used in positive control reactions. PCR without DNA or paraffin-embedded tissue was used as negative control.

Results

Target GAPDH DNA fragment was successfully amplified with the presented technique. Figure 1A illustrates one of the three experiments showing formation of strong 97 bp GAPDH gene products using several different paraffin-embedded human cancer tissue types including cervix, endometrium, larynx, skin, breast, lung, prostate, ovary, gallbladder and rectum. Only 10µl aliquots of the PCR products were analyzed on a 1% agarose gel, and results showed strong target DNA amplification signals with no non-specific DNA fragments. When Triton X-100 incubation is omitted from the protocol, no DNA amplification was achieved using the same set of paraffin-embedded tissue samples (Figure 1B).

Since DNA is known to degrade in time, the ability to amplify DNA from different ages of paraffin-embedded tissues was also examined. Figure 1C demonstrates one of the three experiments using different ages of paraffin-embedded leiomyoma samples. Ninety seven bp GAPDH DNA fragment was successfully amplified from all 9 paraffin-embedded leiomyoma tissue samples collected from 1996 to 2004.

Discussion

During diagnostic practice, a large number of archival paraffin-embedded tissue banks have been established forming an important resource of tissues for retrospective molecular studies of cancer and various other diseases.[1–5] Paraffin, a colorless/white wax, is produced from petroleum oil and soluble in xylene, benzene, and chloroform, but not in water, ethanol, or acetone. It is widely used in surgical pathology departments, because it preserves the tissue morphology and also paraffin-embedded tissue blocks are the easiest to store and transport. DNA amplification success from paraffin-embedded tissues depends on numerous factors including the type of the fixative used, fixation time, storage time, designed primer of choice, and especially PCR conditions.[13] It is known for some time that nucleic acids, DNA and RNA, extracted from formalin-fixed paraffin-embedded tissue blocks are of lower quality than those recovered from fresh/fresh frozen tissues.[17] There are known organic chemicals used as fixatives that allow superior preservation of DNA, RNA and protein in paraffin-embedded tissue for molecular studies.[9] However, formalin is used routinely as fixative in almost all pathology laboratories worldwide. Thus, for a large-scale retrospective molecular study it is essential to use an effective DNA extraction method utilizing formalin-fixed paraffin-embedded tissues.[15] Formalin fixation can create cross-links between nucleic acids and proteins, DNA adducts with histones, and base modifications in addition to activity of various other factors during storage.[6–9] Extended fixation intervals are associated with decreased PCR yields and a progressive inability to amplify longer templates. A number of studies have reported the successful amplification of the DNA sequences up to 1 kbp long, but it is generally agreed that the fragments below 300 bp appear to be the most appropriate for routine, highly reproducible PCR analysis of paraffin-embedded tissues.[6] Formalin-induced DNA degradation has been studied at different fixation times (3, 7, 16 and 32 days) and shown that the longer the formalin fixation time, the shorter are the amplifiable alleles.[18] On the other hand, with the help of enhancing new molecular techniques and technology it is becoming more and more desirable to identify rapid and cost effective protocols for molecular analyses.[1–5,8,19,20,21] Samples studied with the PCR technique do not require intact chromosomal or viral DNA. A certain amount of damage can be tolerated if the target sequence and also *Taq*DNA polymerase activity are not disturbed.[9] It is also essential that the studied target DNA sequences are not fragmented because of rough extraction methods.[14]

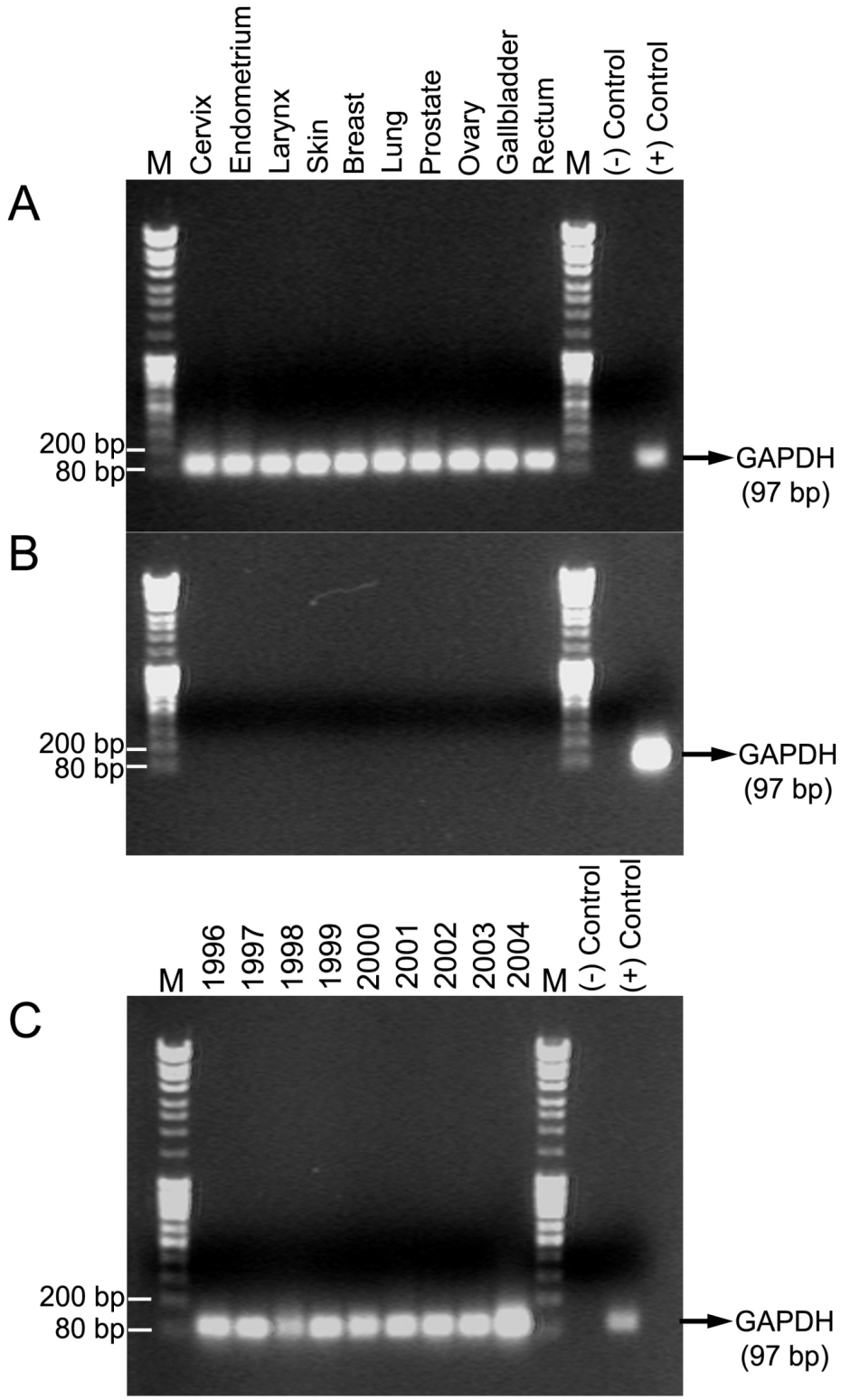

Figure 1. 97 bp fragment of the GAPDH gene amplified from (A) 10 different paraffin-embedded human cancer tissue types, (B) the same set of tissue human cancer tissue samples without Triton X-100 incubation and (C) different ages of paraffin-embedded leiomyoma samples. M, Molecular weight marker. Negative controls (PCR omitting DNA). Positive controls (PCR using human genomic DNA isolated from serum). See text and Table 1 for details.

DNA amplification by PCR directly from paraffin-embedded tissue has many advantages. This procedure eliminates the long and tedious overnight steps including deparaffinization, DNA extraction, and DNA purification. It is a less time consuming and more efficient protocol, which reduces the risk of cross-contamination by limiting the number of steps required.

In our study the targeted GAPDH gene product has successfully been amplified using this protocol with high amplification efficiency from 10 different paraffin-embedded human cancer tissue types (Figure 1A) and from 9 different aged paraffin-embedded leiomyoma tissue samples (Figure 1C). These results clearly show that even 1-3 mm^2 small tissue samples were sufficient to amplify the 97 bp fragment of GAPDH gene. Triton X-100 is a nonionic detergent frequently used in biochemical applications to solubilize proteins. Incubation of the samples with 1% Triton X-100 solution was clearly enough to lyse the cells in our experiments. When Triton X-100 incubation was omitted from the protocol, no DNA amplification was achieved using the same set of human cancer tissue samples (Figure 1B). Although Triton X-100 has been suggested for direct PCR from paraffin-embedded tissue before, no applications for the use of ready-to-use PCR mixtures have been reported yet.[22] This is especially important for the surgical pathology departments because with the use of these commercially available kits, there is no need to have a sophisticated, high technology molecular pathology laboratory. Clinical application of molecular biology methods and examination of large quantities of materials can be achievable with reasonable resources. Amplification of DNA from fixed, paraffin-embedded tissues using this protocol may have many applications including detection of loss of heterozygosity[23], microsatellite instability,[24] gene-specific mutations[25–27] and early diagnostic identification of infective agents, such as Human Papilloma Virus[28,29], Human Cytomegalovirus[30], Helicobacter pylori[31] and Epstein-Barr Virus[32,33]. Once the presented protocol for DNA amplification is applied correctly, large quantities of paraffin-embedded material stored in our pathology departments will be available for molecular diagnostics and research.

Acknowledgements

Authors thank to Dr. Mehmet Öztürk, Bilkent University Department of Molecular Biology and Genetics, for providing Triton X-100 used in this study. This work is supported in part by Pamukkale University, Scientific Research Grants Fund (to N.L. Satiroglu-Tufan).

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