Int. J. Exp. Path. (1993) **74**, 333–337

DNA Amplification by polymerase chain reaction from brain tissues embedded in paraffin

KORALJKA GALL*, JASMINKA PAVELIĆ*, DUBRAVKA JADRO-ŠANTEL†, MARIO POLJAK‡ AND KREŠIMIR PAVELIĆ*

**Laboratory of Molecular Oncology, Department of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 41001 Zagreb, Croatia; †Department of Neuropathology, School of Medicine, University of Zagreb, Kišpatićeva 12, 41000 Zagreb, Croatia; and ‡Institute of Pathology, Medical Faculty, University of Ljubljana, Korytkova 2, 61105 Ljubljana, Slovenia*

Received for publication 19 November 1992
Accepted for publication 8 March 1993

Summary. A method which enables analysis of DNA from archival paraffin embedded normal and malignant brain tissue is described. The demonstration of a 317-bp long β-actin DNA sequence by the polymerase chain reaction (PCR) was used to identify which fixation procedure, deparaffinization time and DNA extraction procedure would give the best results. Tissue specimens 1–39 years old were included in the experiments. Specimens fixed in either 10% formalin, Carnoy's or AMeX fixative were found to be best suited for subsequent analysis by PCR. Paraformaldehyde and acetone compromised amplification efficiency, while Bouin's fixed tissue gave uniformly negative results. Regardless of fixative used, PCR reaction had to be run through at least 40 cycles. Prolonged deparaffinization time and phenol/chloroform extraction of DNA did not influence DNA quality as a template for PCR reaction. Formalin fixed brain tumours can be successfully used for DNA/PCR analysis even if they are up to 39 years old.

Keywords: brain tissue, PCR, paraffin embedded tissue

The possibility of studying DNA obtained from preserved tissue enables valuable retrospective analyses. The most common method for preserving human tissue is its fixation in formalin followed by paraffin embedding. Owing to its ability to amplify relatively impure, fragmented DNA, the use of PCR provides a method of choice for analyses of DNA obtained from such tissues. Moreover, PCR analyses of preserved tissues can be accomplished on a thin section where other methods fail.

Correspondence: Dr Krešimir Pavelić, Laboratory of Molecular Oncology, Department of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, POB 1016, 41001 Zagreb, Croatia.

The integrity of DNA obtained from paraffin embedded tissues depends greatly on the fixative used. The fixatives other than formalin may modify DNA and reduce the amplification (Dubeau *et al.* 1986; Tokuda *et al.* 1990; Ben-Ezra *et al.* 1991; Greer *et al.* 1991). The effects of different fixatives and fixation times on DNA/PCR reaction have so far been studied on only a limited number of different normal human and malignant tissues (Shibata *et al.* 1988a; Tokuda *et al.* 1990; Wan *et al.* 1990; Ben-Ezra *et al.* 1991; Coates *et al.* 1991; Greer *et al.* 1991). However, it would be worth knowing what processing conditions are best to get a good template for PCR reaction for a particular tissue.

No such information is so far available for the tumours of the central nervous system. Therefore, archival specimens of benign meningioma and malignant meningiosarcoma were used as a model system for our studies.

Meningiomas are one of the most common tumours of the human nervous system (Schoenberg *et al.* 1976). There is evidence which indicates genetic predisposition for the disease. Besides monosomy of the chromosome 22, terminal deletion of the long arm of chromosome 22 has been indicated as a possible cause of the disease (Seizinger *et al.* 1987a, b). Since paraffin embedded brain tumour specimens are easily obtained, the possibility of studying DNA from these specimens would be of great value in the elucidation of genetic event(s) resulting in tumorigenesis.

Therefore, in this study we examined how different fixation procedures, DNA extractions and the age of archival specimens influenced the ability of a treated tissue to serve as a template for the DNA amplification in the PCR reaction. The main aim of this study was to find the best possible combination of individual steps (from fixation up to the PCR amplification) in order to prepare DNA from brain tissue embedded in paraffin for amplification and further genetic analyses. The described method is moderately time consuming permitting numerous specimens to be analysed in a relatively short time.

Materials and methods

Surgical extirpations of brain tumours (meningioma, 12 specimens and meningiosarcoma, two specimens), as well as normal brain tissue, fixed (detailed below) and embedded in paraffin, were obtained from the Department of Neuropathology, School of Medicine, University of Zagreb, Croatia and Institute of Pathology, School of Medicine, University of Ljubljana, Slovenia.

All meningioma and normal brain tissues were processed 6 hours after surgical extirpation at the latest or 6 hours post-mortem. We could not get exact information for 10 and 39-year-old paraffin embedded meningiosarcoma tissues. After fixation all samples were embedded in paraffin and classified according to standard morphological criteria. Tissue paraffin blocks were of different ages. All meningiomas were a year old and the two meningiosarcomas were 10 and 39 years old; the normal brain tissue blocks used were 3 months old. The six fixation methods used were (a) neutral buffered 10% formalin (4.1% formaldehyde, 33 mM NaH_2PO_4, 45.7 mM Na_2HPO_4, pH 7.0 for 24 hours; room temperature); (b) 1% paraformaldehyde for 24 hours; room temperature; (c) acetone (−20°C for 24 hours); (d) AMeX (acetone at −20°C overnight, acetone at 4°C for 15 minutes, acetone at room temperature for 15 minutes; methyl benzoate for 30 minutes at room temperature, xylene for 30 minutes at room temperature; (e) Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid for 24 hours; room temperature); (f) Bouin's fixative (75% saturated picric acid, 25% formalin, 5% acetic acid for 24 hours; room temperature). After fixation samples were processed for paraffin embedding. After embedding the blocks were stored in wooden boxes at room temperature.

DNA was isolated as follows. A 5-μm thick section was sliced from each block and placed in a microcentrifuge tube with a sterile needle. Portions of the samples were deparaffinized in one of two ways: in two changes in 1 ml xylene overnight (5 h + 16 h) or in two changes of xylene for 4 hours. During that time the tubes were rotated on a Rotex (30 r.p.m.). The remaining samples were treated with xylene for a shorter time. After the xylene treatment the samples were rinsed twice with 100% ethanol for 30 minutes. The samples were pelleted between each extraction step. After the last ethanol rinse the pellets were dried and resuspended in 0.1 ml of digestion buffer (200 μg/ml Proteinase K in 50 mM TRIS-HCl pH 8.5, 1 mM EDTA, 0.5% Tween 20). The samples were then incubated overnight at 37°C. Proteinase K was inactivated at 95°C for 8 minutes.

To evaluate the effects of additional purification on DNA yield and its susceptibility for PCR reaction, a portion of the sample was extracted with phenol/chloroform after Proteinase K treatment.

For the PCR reaction two pairs of primers were used for human β-actin and the bcl-2 gene.

The primer sequences for human β-actin gene were respectively: 5′-ATCATGTTTGAGACCTTCAACACCCC-3′ and 5′-CATCTCTTGCTCGAAGTCCAGGGCGA-3′. These sequences flanked a 317-bp long region in genomic DNA. The GC content of the amplified sequence was 59.3%. The primer sequences for bcl-2 gene were: 5′-ATGGCGCACGCTGGGAGAACAGGGTA-3′ and 5′-TGGGTGCCTATCTGGGCCACAAGTGA-3′. These sequences flanked a 720-bp long region in the genomic DNA. The GC content of the amplified sequence was 67.8%.

PCR was performed in a Perkin-Elmer-Cetus Thermal Cycler. An aliquot of supernatant from each sample (15 μl, 1 μg) was used for the PCR amplification. PCR was carried out in 100-μl volume by addition of the Taq polymerase (2.5 U, Amplitaq: Cetus), dNTPs (2.5 mM each), primers (300 nM), 10 μl of 10× reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01%

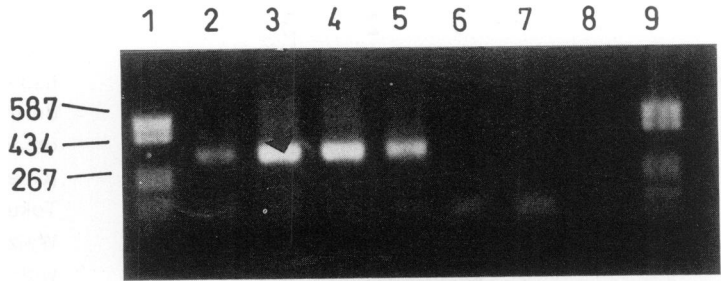

Figure 1. PCR amplification of 317-bp fragment of the β-actin gene from human genomic DNA obtained from paraffin embedded meningioma and normal brain tissue fixed in different ways. Lane 1, DNA molecular weight marker V (Boehringer Mannheim); lane 2, 10% formalin fixed meningioma tissue, 30 cycles; lane 3, 10% formalin fixed meningioma tissue, 40 cycles; lane 4, AMeX fixed normal brain tissue, 40 cycles; lane 5, Carnoy's fixed normal brain tissue, 40 cycles; lane 6, acetone fixed normal brain tissue, 40 cycles; lane 7, paraformaldehyde fixed normal brain tissue, 40 cycles; lane 8, Bouin's fixed normal brain tissue, 40 cycles; lane 9, the same molecular weight markers as in lane 1. The specific amplification product at 317 bp is denoted by an arrow. Although not clearly visible in the photograph (lanes 6 and 7) the 317 product for samples fixed in acetone and paraformaldehyde was present on the gel but at reduced level.

gelatin) and water. Each reaction mix was covered with 50 μl of light mineral oil. The 30 or 40-fold amplification profiles underwent denaturation at 94°C for 1 minute, annealing at 58°C for 2 minutes and extension at 72°C for 3 minutes. PCR products were analysed by agarose gel electrophoresis (1.2% agarose gel stained with 0.5 μg/ml ethidium bromide in TAE buffer).

Results

The demonstration of β-actin sequence in DNA/PCR was used to identify which fixation procedure, deparaffinization time and DNA extraction procedure should be applied to normal brain and meningioma tissue in order to get the best possible results. Examples of the results from amplification experiments are shown in Figure 1. Amplified DNA from 10% formalin fixed meningioma tissue and DNA from either Carnoy's or AMeX fixed normal brain tissue produced equally abundant 317-bp fragments. However, 40 cycles had to be performed to achieve these products. With fewer cycles (30) the fragments were hardly visible. Paraformaldehyde and acetone gave poorer results, whereas the Bouin's fixed tissue gave uniformly negative results. Formalin fixed meningioma tissues showed equally good results after 2 × 120 minutes and overnight deparaffinization treatment. A very weak band was seen after 2 × 60 minutes, while no band at all was observed after the 2 × 30 minutes deparaffinization protocol (Figure 2).

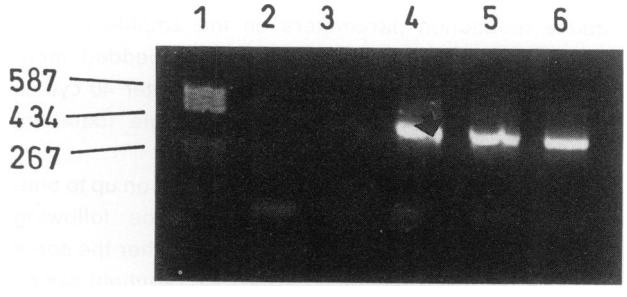

Figure 2. PCR amplification of a 317-bp fragment of the β-actin gene from human genomic DNA obtained from meningioma tissue fixed in 10% formalin and embedded in paraffin. Effect of deparaffinization conditions and phenol/chloroform extraction. Lane 1, DNA molecular weight marker V (Boehringer Mannheim); lane 2, 2 × 30 minutes deparaffinization time; lane 3, 2 × 60 minutes deparaffinization time; lane 4, 2 × 120 minutes deparaffinization time; lane 5, overnight deparaffinization; lane 6, 2 × 120 minutes deparaffinization time + Proteinase K, phenol/chloroform DNA extraction. The specific amplification product at 317 bp is denoted by an arrow.

The results with phenol/chloroform extracted DNA from formalin fixed meningioma tissue indicated that there was no significant improvement in the amplification when compared to Proteinase K DNA extraction only (Figure 2). From the above presented results we concluded that the following parameters permitted successful DNA/PCR amplification: 10% buffered formalin as

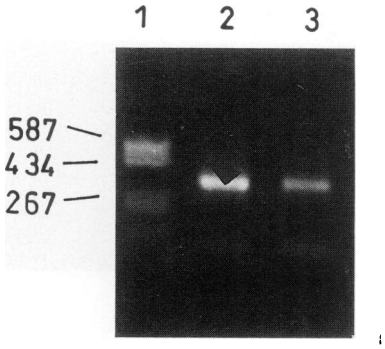

Figure 3. PCR amplification of 317-bp fragment of the β-actin gene from human genomic DNA obtained from meningiosarcoma tissue fixed in 10% formalin and embedded in paraffin. Lane 1, DNA molecular weight marker V (Boehringer Mannheim); lane 2, 10-year-old sample; lane 3, 39-year-old sample. The specific amplification product at 317 bp is denoted by an arrow.

fixative, 2 × 120 minutes deparaffinization time, Proteinase K DNA extraction, 40 cycles.

The demonstration of a 720-bp long fragment of the bcl-2 gene was used to ascertain the effectiveness of the above mentioned parameters on the amplification of longer genomic fragments. Paraffin embedded meningioma DNA was used as a template. After 40 cycles amplification the 720-bp band was visible (data not shown).

The above experiments were carried out on up to one-year-old paraffin embedded tissues. The following experiment was aimed at elucidating whether the same parameters (from fixation up to the PCR reaction) can be applied to considerably older paraffin blocks (10 and 39 years old). The results with two paraffin embedded meningiosarcoma tissues are presented in Figure 3. Both samples, regardless of age, showed the bands in the same abundance.

Discussion

The possibility of studying paraffin embedded tissues at the molecular level permits valuable retrospective analyses. However, fixation type and fixation time are known to influence the preservation of DNA making it sometimes unsuitable for PCR analysis (Dubeau *et al.* 1986; Tokuda *et al.* 1990; Ben-Ezra *et al.* 1991; Greer *et al.* 1991). Our goal was to establish the optimal conditions under which DNA from preserved tumours of the central nervous system could be used for PCR reaction.

Paraffin embedded meningioma and normal brain tissue fixed either in 10% formalin, Carnoy's or AMeX fixative were found to be best suited for subsequent analysis by PCR. Paraformaldehyde and acetone compromised amplification efficiency, while Bouin's fixed tissues gave uniformly negative results. Our data, considering formalin as fixative, are consistent with those obtained with a variety of the tissues other than brain (Goelz *et al.* 1985; Jackson *et al.* 1989; Rogers *et al.* 1990; Tokuda *et al.* 1990; Wan *et al.* 1990; Greer *et al.* 1991; Weizsäcker *et al.* 1991). There are contradictory results with Carnoy's fixative. While it proved effective with some tissues (Jackson *et al.* 1990), with others it failed (Greer *et al.* 1991). It is encouraging that the AMeX fixation procedure, excellent for the immunohistochemical detection of proteins (Pavelić *et al.* 1991), is also convenient for DNA/PCR enabling the comparison of the results. From these data it seems that fixatives containing either cross-linking agent (formalin), with fixation time up to 48 hours, or acetone or alcohol based, are equally good for preserving DNA for PCR. After deparaffinization, however, the samples have to be subjected to Proteinase K digestion with or without phenol/chloroform extraction before being used in PCR reaction. When just crude, boiled extract of formalin fixed tissue is used, PCR amplification is hindered (Ben-Ezra *et al.* 1991). When the effect of different fixatives on DNA in-situ hybridization was tested, 10% formalin turned out to be again the most suitable (Nuovo & Richart 1989). Besides the fixative used, there are many other factors that could affect the overall efficiency of the DNA amplification. For example, xylene deparaffinization time is also important. In the case of meningiomas either overnight or 2 × 120 minutes deparaffinization were equally efficient. However, 40 cycles had to be performed to achieve PCR products. With fewer cycles (30) the fragments were hardly visible. A method that does not require deparaffinization of the tissue prior to the PCR reaction was described by Coates *et al.* (1991). However, due to the low yield and poor quality of the DNA template the authors were able to amplify only a short fragment (153 bp). Even with an increased number of amplification cycles a longer fragment (355 bp) was not reliable.

The size of fragmented DNA is also a parameter which could predict success of PCR amplification. The tissue fixed in buffered formalin tends to have larger DNA fragments (Greer *et al.* 1991). However, if the pH of buffered formalin is kept neutral and fixation is done at room temperature for 24 hours the purified DNA does not degrade (Tokuda *et al.* 1990). In our case, by the use of paraffin embedded sections of material fixed in neutral buffered formalin for 24 hours, a 720-bp long fragment of bcl-2 gene was easily visible after 40 cycles of amplification. Even longer fragments, up to 1327 bp, were successfully amplified by Greer *et al.* (1991). The reac-

tion efficiency decreased, however, when fixation time had been prolonged to 24 hours (Greer *et al.* 1991). The findings of Tokuda *et al.* (1990) suggest that DNase might also have an important role in degradation of genomic DNA during fixation. All these data suggest that the reliable amplification of a specific sample depends greatly on fixation effects (Impraim *et al.* 1987; Jackson *et al.* 1990).

It is already known that slow degradation of the DNA in archival specimens occurs with time (Goelz *et al.* 1985; Dubeau *et al.* 1986; Shibata *et al.* 1988b). Our analysis, however, shows that amplification of 317-bp long DNA sequence is still possible, even after 10 or 39 years of storage.

In conclusion, a method which permits analysis of DNA from archival paraffin embedded normal and malignant brain tissue is described. The information presented may serve as a guide to researchers in deciding which brain tissue processing conditions are best for subsequent PCR analysis.

References

Ben-Ezra J., Johnson D.A., Rossi J., Cook N. & Wu A. (1991) Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. *J. Histochem. Cytochem.* **39,** 351–354.

Coates P.J., d'Ardenne A.J., Khan G., Kangro H.O. & Slavin G. (1991) Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. *J. Clin. Pathol.* **44,** 115–118.

Dubeau L., Chandler L.A., Gralow J.R., Nichols P.W. & Jones P.A. (1986) Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. *Cancer Res.* **46,** 2964–2969.

Goelz S.E., Hamilton S.R. & Vogelstein B. (1985) Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem. Biophys. Res. Comm.* **130,** 118–126.

Greer C.E., Peterson S.L., Kiviat N.B. & Manos M.M. (1991) PCR amplification from paraffin-embedded tissues. Effects of fixatives and fixation time. *Am. J. Clin. Pathol.* **95,** 117–124.

Impraim C.C., Saiki R.K., Erlich H.A. & Teplitz R.L. (1987) Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. *Biochem. Biophys. Res. Comm.* **142,** 710–716.

Jackson D.P., Lewis F.A., Taylor G.R., Boylston A.W. & Quirke P. (1990) Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J. Clin. Pathol.* **43,** 499–504.

Jackson D.P., Quirke P., Lewis F., Boylston A.W., Sloan J.M., Robertson D. & Taylor G.R. (1989) Detection of measles virus RNA in paraffin-embedded tissue. *Lancet* **17,** 1391.

Nuovo G.J. & Richart R.M. (1989) Buffered formalin is the superior fixative for the detection of HPV DNA by *in situ* hybridization analysis. *Am. J. Pathol.* **134,** 837–842.

Pavelić K., Pavelić Z.P. & Preisler H.D. (1991) C-myc detection in bone marrow biopsies. *Leukemia Res.* **15,** 1075–1078.

Rogers B.B., Alpert L.C., Hine E.A.S. & Buffone G.J. (1990) Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction. *Am. J. Pathol.* **136,** 541–548.

Schoenberg B.S., Christine B.W. & Whisnant J.P. (1976) The descriptive epidemiology of primary intracranial neoplasms: the Connecticut experience. *Am. J. Epidemiol.* **104,** 499–501.

Seizinger B.R., de la Monte S., Atkins L., Gusella J.F. & Martuza R.L. (1987a) Molecular genetic approach to human meningioma: loss of genes on chromosome 22. *Proc. Natl. Acad. Sci. USA* **84,** 5419–5423.

Seizinger B.R., Rouleau G., Ozelius L.J., Lane A.H., St. George-Hyslop P., Huson S., Gusella J.F. & Martuza R.L. (1987b) Common pathogenetic mechanism for three tumor types in bilateral acoustic neurofibromatosis. *Science* **236,** 317–319.

Shibata D.K., Arnheim N. & Martin W.J. (1988a) Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J. Exp. Med.* **167,** 225–230.

Shibata D., Martin W.J. & Arnheim N. (1988b) Analysis of DNA sequences in forty-year-old paraffin embedded thin tissue sections: A bridge between molecular biology and classical histology. *Cancer Res.* **48,** 4564–4566.

Tokuda Y., Nakamura T., Satonaka K., Maeda S., Doi K., Baba S. & Sugiyama T. (1990) Fundamental study on the mechanism of DNA degradation in tissues fixed in formaldehyde. *J. Clin. Pathol.* **43,** 748–751.

Wan J.H., Trainor K.J., Brisco M.J. & Morley A.A. (1990) Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. *J. Clin. Pathol.* **43,** 888–890.

Weizsäcker F., Labeit S., Koch H.K., Oehlert W., Gerok W. & Blum H.E. (1991) A simple and rapid method for the detection of RNA in formalin-fixed, paraffin-embedded tissues by PCR amplification. *Biochem. Biophys. Res. Comm.* **174,** 176–180.