Virchows Archiv A Pathol Anat (1992) 420:269–273

Virchows Archiv A
Pathological Anatomy and Histopathology

Technical report

A simple and rapid technique to process formalin-fixed, paraffin-embedded tissues for the detection of viruses by the polymerase chain reaction*

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Received July 8, 1991 / Received after revision September 9, 1991 / Accepted September 10, 1991

Summary. The use of chelating resin in a simple, rapid and efficient pre-treatment protocol to process formalin-fixed, paraffin-embedded specimens for the polymerase chain reaction (PCR) is described and compared to other pre-treatment techniques. With this modified PCR protocol, a variety of human autopsy and biopsy specimens were investigated for presence of DNA of human papillomaviruses, cytomegalovirus or Epstein-Barr virus. These viruses were detected in a productive or non-/low-productive state. Amplimers generated ranged from 88 to 450 base pairs length. Under the specified technical conditions, a considerable range of DNA molecules can be amplified from paraffin-embedded material, some older than 10 years.

Key words: Polymerase chain reaction – Human papillomavirus – Cytomegalovirus – Epstein-Barr virus

Introduction

Studies on formalin-fixed, paraffin-embedded tissue at the molecular level provide new perspectives of retrospective studies. In recent years special efforts have been focused on DNA extraction and subsequent Southern blot analysis. A major problem arising in DNA analyses is degradation of DNA in older and especially in inadequately formalin-fixed specimens (Dubeau et al. 1986; Wright and Manos 1990).

In situ techniques like immunhistochemistry or in situ hybridization represent a promising tool to link molecular biology and morphology with an expanding range of applications. Nevertheless some clinically important questions concerning subtle genomic deviations such as point mutations or subclinical viral infections are difficult to answer using these techniques. Most of the in situ protocols detect viral infections only when the level of viral expression exceeds a threshold of 100 copies per cell (Gupta et al. 1987). Southern blot analysis requires larger amounts of tissue for adequate investigation.

The polymerase chain reaction (PCR) circumvents these technical problems and allows the detection of low-copy-number DNA targets in single paraffin sections. Although the technical protocols applied to PCR in surgical pathology have already been outlined in detail (Barton-Rogers et al. 1990; Jackson et al. 1990; Kiyabu et al. 1989; Shibata et al. 1988), there is some controversy with respect to the prior handling of paraffin-embedded specimens.

Several pre-treatment protocols with or without deparaffination, proteolytic treatment or phenol/chloroform extraction have been described. Yet, possible loss of material, potential hazard to the personnel and, in particular, contamination would be reduced to a minimum if pre-treatment steps could be limited without influence on the success of PCR reactions.

Singer-Sam et al. (1989) described the use of chelating resin (Chelex) as a means of increasing the signal from the PCR of DNA from small numbers of tissue culture cells. Recently, Walsh et al. (1991) used similar procedures including simple boiling of the material in Chelex suspension for DNA extraction for PCR from forensic material. The alkalinity of Chelex suspensions and boiling at 100° C led to efficient membrane destruction and DNA denaturation, while DNA degradation is prevented by the chelating action of the resin which removes metal ions that might act as catalysts in DNA breakdown (Britten et al. 1974; Walsh et al. 1991).

In this study, we used a similar procedure including chelating resin for DNA extraction from single paraffin sections before PCR amplification, and compared it with four other pre-treatment protocols. The simple and efficient Chelex method was successfully applied to the detection of human papillomavirus (HPV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in formalin-fixed, paraffin-embedded biopsy and autopsy specimens.

* Dedicated to Prof. Dr. G. Seifert to his 70th birthday.

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Materials and methods

Materials. Formalin-fixed, paraffin-embedded biopsy and autopsy specimens were retrieved from our files and consisted of the following groups:

1.1. Typical anogenital condylomas with and without known HPV infections as recognized by in situ hybridization analyses.

1.2. Vulvar cancer and bowenoid lesions of the vulva without detectable HPV infection by in situ hybridization.

2.1. Multiple autopsy specimens from heart, lung and kidney of acquired immunodeficiency (AIDS) patients with and without morphological signs of CMV infection.

2.2. Placentitis specimens, without morphological signs of specific infections.

3.0. Nasopharyngeal carcinomas.

Preparation of paraffin-embedded tissues. Sections (8 µm) were cut with sterile blades and transferred into sterile 1.5 ml tubes before pre-treatment according to the following protocols:

A. Deparaffination of the tissue sections by immersion in 400 µl xylene for 5 min, centrifugation and decantation of the liquid. Repetition of the procedure with 100% ethanol and 70% ethanol. After decantation of the last rinse, the remaining ethanol was removed by drying. The tissue was resuspended in 50 µl TE 8 (50 mM Tris-HCl, pH8, 1 mM EDTA) containing 1% N-lauroylsarcosine (Shibata et al. 1988; Wright and Manos 1990).

B. Deparaffination was carried out according to method A; after drying the tissue was redissolved in 50 µl TE 8 containing 1% lauroylsarcosine. Five microlitres of proteinase K solution (10 mg/ml) was added followed by 3 h of incubation at 55° C. Boiling the tube content for 10 min ended the procedure (Barton-Rogers et al. 1990).

C. Fifty microlitres of sterile H_2O was added to the tube, followed by boiling for 15 min (Jackson et al. 1990).

D. Three to 5 µg of Chelating Resin Sodium Form (Sigma, St. Louis, Mo.) and 100 µl sterile water were added, followed by boiling at 100° C for 15 min at pH 10.1–10.3. After short centrifugation the tube content was separated into three different phases, the lowest containing the chelating resin particles, the one in the middle, tissue and H_2O, and the upper liquid paraffin.

E. Digestion of the section in 100 µl TE 8 containing 1% N-lauroylsarcosine and 100 µg proteinase K according to method B. Extraction of the DNA was carried out as described previously (Barton-Rogers et al. 1990; Jackson et al. 1990) using phenol-chloroform and phenol-chloroform-isoamyl solutions, followed by ethanol precipitation of the DNA and redissolving in 50 µl H_2O at pH 7.6.

After pre-treatment 5 µl of the aqueous phases was used for PCR processing.

PCR amplification and hybridization analyses. Amplification reactions were performed in 50 µl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TRIS-HCl, pH 8.3, 0.01% gelatin, 100–200 µM of each nucleotide (dATP, dTTP, dCTP, dGTP), 0.1–1 µM of each primer and 1 unit *Taq* DNA polymerase (Perkin Elmer Cetus, Überlingen, FRG). The primers used in our study were (1) HPV 6/11 and HPV 16/33 sequences from the E6 region (Dallas et al. 1989); (2) consensus primers MY09 and MY11 (Manos et al. 1989; Perkin Elmer Cetus); (3) CMV primers from the IE region (Jiwa et al. 1989; Genset, Dianova, Hamburg, FRG); (4) EBV sequences from the *Bam* C, *Bam* Nhet-LMP and EBNA-2A region (Saito et al. 1989; Sixbey et al. 1989; Genset, Dianova, FRG); and (5) beta-globin primers (Perkin Elmer Cetus). After addition of 5 µl solution the mixture was overlaid by 50 µl mineral oil and incubated for 5 min at 94° C for DNA denaturation. Thirty-five cycles were carried out, each including 1 min 94° C for denaturation, 2 min 40° C (HPV), 50° C (EBV, CMV) and 55° (consensus primers) for annealing, and 3 min 72° C for chain elongation. All manipulations were done using sterile materials to avoid contamination. The subsequent analyses of PCR products were performed in a separate building excluding contaminations by the PCR products.

Twenty microlitres of the reaction mixture was separated by agarose gel electrophoresis.

The gels were stained with ethidium bromide and Southern blotting was subsequently conducted. For hybridization we used oligonucleotide probes specific to DNA sequences of amplified targets (HPV type 6/11 and 16, Dallas et al. 1989; CMV, Jiwa et al. 1989; EBV, Saito et al. 1989 and Sixbey et al. 1989) labelled with digoxigenin-dUTP or ^{32}P-dUTP by Terminal Transferase (Boehringer Mannheim, FRG). Hybrids were detected by the nucleic acid detection kit (Boehringer Mannheim, FRG) or autoradiography. In all runs positive and negative controls comprising plasmids, cell lines and paraffin-embedded tissues were included.

Results

Comparing the pre-treatment protocols A–D described in Materials and methods, the chelating resin technique (D) was demonstrated to be the most efficient one when used on small amounts of formalin-fixed, paraffin-embedded material. The detection of HPV type 6/11 infection in anogenital condylomas is demonstrated in Fig. 1; one condyloma had previously proved to generate enough HPV copies per cell for detection by in situ hybridization.

In comparing the chelating resin technique to DNA extraction procedures (method E) no technique showed superiority in detection of HPV infection (Fig. 2).

The equivalence of both methods (D and E) was shown for HPV type 16 infection in two vulvar carcino-

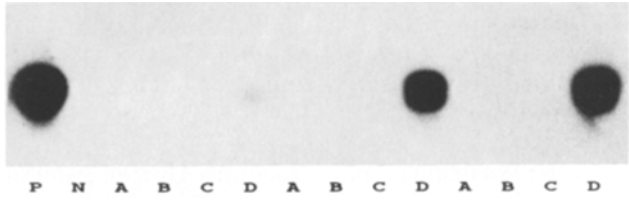

Fig. 1. Polymerase chain reaction (PCR) analysis of paraffin sections of three anogenital condylomas with human papillomavirus (HPV) type 6/11 specific primers using pre-treatment methods A, B, C, D. Southern blot hybridization with a ^{32}P-labelled HPV type 6/11 specific oligonucleotide probe. *N*, Negative control; *P*, positive control (plasmid). Specimens 1–3 were processed by each method (A–D). Amplimers = 120 base pairs length

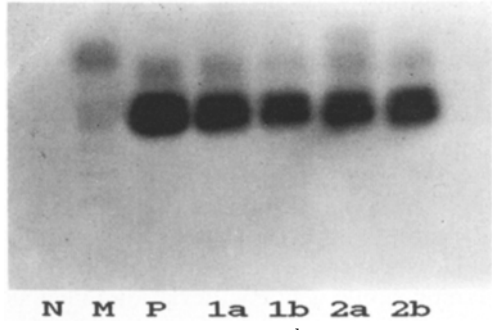

Fig. 2. PCR analysis of paraffin sections of two anogenital condylomas with HPV type 6/11 specific primers using pre-treatment methods D (chelating resin technique) and E (DNA extraction). Southern blot hybridization with a digoxigenin-labelled, HPV type 6/11 specific oligonucleotide probe. *N*, negative control; *M*, molecular weight marker; *P*, positive control; *a*, method D; *b*, method E. Amplimers = 120 base pairs length

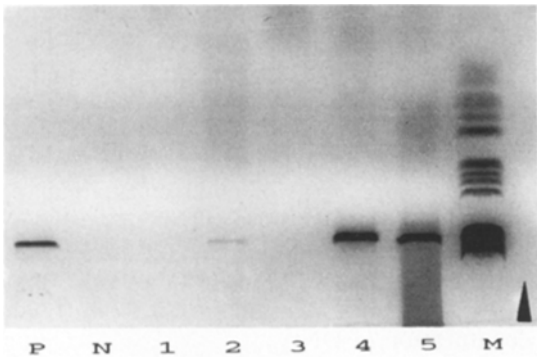

Fig. 3. PCR analysis of paraffin sections of three anogenital condylomas and two specimens of lichen sclerosus et atrophicus of the vulva using HPV consensus primers. Visualization by ethidium bromide staining (the negative is shown). *Lanes 1 and 3*, Lichen sclerosus et atrophicus; *lanes 2, 4, 5*, anogenital condylomas; *N*, negative control; *P*, positive control (CasKi cell line); *M*, molecular weight marker (*Hae* III digested pBR 322 DNA; Boehringer Mannheim, FRG). Amplimers = 450 base pairs length. The running direction is marked by the *arrow*

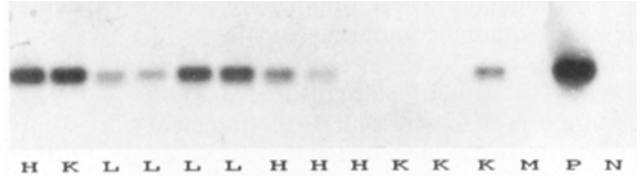

Fig. 4. PCR analysis of paraffin sections of AIDS patients with cytomegalovirus (CMV) primers. Southern blot hybridization with a digoxigenin labelled, specific oligonucleotide probe. M, molecular weight marker; P, positive control (plasmid); N, negative control; K, kidney tissue; L, lung tissue; H, heart tissue. Amplimers = 137 base pairs length

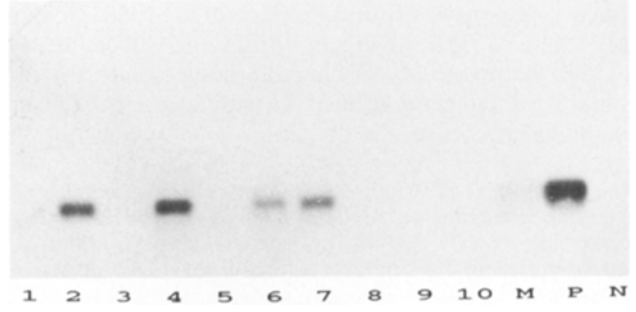

Fig. 5. PCR analysis of paraffin sections of placentitis specimens of different patients with CMV primers. Southern blot hybridization with a digoxigenin-labelled specific oligonucleotide probe. *M*, Molecular weight marker; *P*, positive control (plasmid); *N*, negative control. *Lanes 1–10* = placentitis specimens. Amplimers = 137 base pairs length

mas and one bowenoid lesion. These lesions failed to reveal HPV infection by in situ hybridization but proved to be positive with HPV type 16 specific primers.

"Consensus primers" which were designed to amplify an approximately 450 base pair product from the open reading frame region L1 of several genital HPV types including HPV type 6, 11, 16, 18, 33 were also used on anogenital condylomas. Visualization of the PCR products by ethidium bromide is demonstrated in Fig. 3.

Table 1. Comparison of different pre-treatment protocols (described in Materials and methods) to process small amounts of paraffin-embedded specimens

Method	Time required	Organic solutions required	Manipulations while processing	PCR results
A	1 h	+	++	−
B	4 h	++	++	−
C	30 min	−	+	−
D	15 min	−	(+)	++
E	6 h	++	+++	++

PCR, Polymerase chain reaction

A comparison of the different pretreatment protocols used for HPV detection is given in Table 1.

In order to investigate those specimens without positive results by PCR analyses, beta-globin primers which are expected to amplify a 268 base pair sequence from normal human cells were used in parallel reaction or in the same tube. Cases revealing no amplification product were excluded from this study.

Since the integrity of the DNA may be altered by inadequate fixation using unbuffered formalin and storage, we tested material up to 12 years of age, finding no difference in PCR processing when comparing recently fixed and embedded specimens.

We also compared radioactive (^{32}P) and enzyme immunoassay (digoxigenin) protocols for detection of amplification products by hybridization with specific oligonucleotide probes. As the results using both methods for detection of the same specimens were the same we decided to continue to use the non-radioactive protocol. Figures 1 and 2 demonstrate HPV type 6/11 infection in the same anogenital lesions using radioactive and non-radioactive detection, respectively.

The chelating resin technique was also superior to other methods for CMV detection in formalin-fixed, paraffin-embedded autopsy specimens of AIDS patients. Heart, kidney and lung tissue of 25 AIDS patients was investigated for CMV infection. Seven out of nine patients without any morphological indication of CMV infection revealed an infectious status after PCR analyses. Fifteen of 16 AIDS patients with previously diagnosed CMV infection as recognized by classical morphological criteria or immunocytochemical detection showed CMV infection in at least one of the tissue samples by PCR analyses (Fig. 4).

Twenty specimens of morphologically diagnosed placentitis without proven specific infection and without classical morphological criteria of CMV infection were investigated by PCR. CMV sequences were demonstrated in 4 out of these 20 specimens (Fig. 5).

Three nasopharyngeal carcinomas were investigated for infection by EBV, since several reports stated a coincidence between EBV infection and development of this type of cancer (Fahraeus et al. 1988; Lanier et al. 1981; Weiss et al. 1989; zur Hausen et al. 1970). All specimens

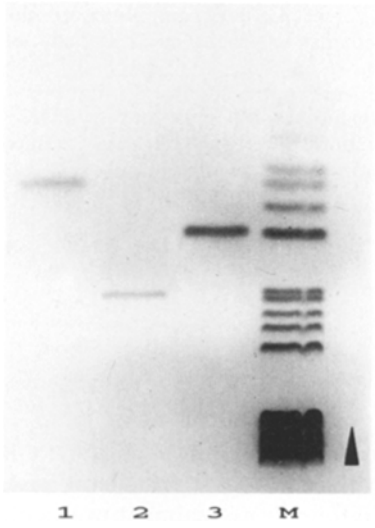

Fig. 6. PCR analysis of paraffin sections of one nasopharyngeal carcinoma using three different types of primers. Visualization by ethidium bromide (the negative is shown). *M*, Molecular weight marker (*Hae* III digested pBR 322 DNA; Boehringer Mannheim, FRG). *Lane 1*, Primers directed against the Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA 2) region of the EBV genome (amplimers = 88 base pairs length). *Lane 2*, Primers directed against the *Bam* Nhet LMP region (amplimers = 181 base pairs length). *Lane 3*, Primers directed against the *Bam* C region (amplimers = 120 base pairs length). The running direction is marked by the *arrow*

investigated with three types of primers, directed against different regions of the EBV genome, revealed EBV-DNA. The resulting amplification products of different lengths are demonstrated in Fig. 6.

Discussion

The use of chelating resin to process formalin-fixed, paraffin-embedded specimens for PCR analyses was investigated with a spectrum of primer pairs designed to generate amplimers of different length.

The method was found to be a simple and efficient pre-treatment protocol, yielding intense and specific signals when used on small amounts of paraffin-embedded material. Other methods failed to reveal the viral sequences and only standard DNA extraction (method E) was found to yield similar signals, but it also required more time for processing as well as organic solutions.

While other authors favour amplification products of up to 100 base pairs length when using formalin-fixed, paraffin-embedded specimens (Wright and Manos 1990), we were able to demonstrate a broader spectrum of possible amplification products. The resulting amplification products in our investigation range from a length of 88 base pairs (EBV) up to a length of 450 base pairs (HPV consensus primers).

Chelating resin is a polyvalent chelating agent producing alkaline conditions in aqueous solutions. It has been used for prevention of DNA degradation when heated by chelating heavy metals which promote the breakdown of DNA (Britten et al. 1974). Further reports also showed its value when added to standard enzyme assays (Raymond and Weinshilboum 1975). The removal of divalent cations like calcium and magnesium probably prevents the unwanted influence of these intrinsic constituents of catalytic reactions. The PCR reaction also depends on the exact concentration of divalent cations and consequently the removal of endogenous magnesium will allow the investigator to adjust magnesium concentrations precisely within the reaction mixture. In addition to the chelating effect, boiling of tissues in alkaline condition leads to cell membrane destruction and, of course, denaturation of DNA.

This PCR technique is able to detect viral infections not only in a productive state, as demonstrated in anogenital condylomas, but also in cases where conventional methods failed to reveal viral infections, as shown in the case of HPV infection in vulvar carcinomas, the bowenoid lesion and in AIDS patients regarding the CMV infection. In the latter group infections of the kidney and heart were observed in high frequency in previously diagnosed CMV-infected AIDS patients as well as in those without morphological or immunocytochemical signs of infection. These findings may be of particular interest in immunocompromised patients.

With respect to the detection of CMV infection in 4 out of 20 cases of placentitis of unknown aetiology it is important to stress that no statement concerning the activity of the infective state can be made. Further sources of placentitis have to be considered.

The analyses of nasopharyngeal carcinomas confirmed previous findings (Fahraeus et al. 1988; Lanier et al. 1981; Weiss et al. 1989; zur Hausen et al. 1970) of a high prevalence of EBV-DNA in these tumours. The use of different sets of primers allows not only the detection of infection but also further differentiation concerning the type of EBV involved. The EBV nuclear antigen 2 region was found (Sculley et al. 1988; Sixbey et al. 1989) to bear divergent forms and subsequently provides the possibility of characterizing isolates by investigating their geographical distributation as well as their prevalence in special diseases.

Acknowledgements. The study was supported by the Hamburger Stiftung zur Förderung der Krebsbekämpfung and the Hamburger Krebsgesellschaft e.V. (I 417). The authors thank Prof. Dr. M. Vogel for providing CMV positive placentitis tissue as control.

References

Barton-Rogers B, Alpert LC, Hine EA, Buffone G (1990) Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction. Am J Pathol 136:541–548

Britten R, Graham DE, Neufeld BE (1974) In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 29E. Academic Press, New York, p 376

Dallas PB, Flanagan JL, Nightingale BN, Morris BJ (1989) Polymerase chain reaction for fast, nonradioactive detection of high- and low-risk papillomavirus types in routine cervical specimens and in biopsies. J Med Virol 27:105–111

Dubeau L, Chandler LA, Gralow JR, Nichols PW, Jones PA (1986) Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. Cancer Res 46:2964–2969

Fahraeus R, Fu HL, Ernberg I, Finke J, Rowe M, et al (1988) Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int J Cancer 42:329–338

Gupta J, Pilotti S, Rilke F, Shah K (1987) Association of the human papillomavirus type 16 with neoplastic lesions of the vulva and other genital sites by in-situ hybridization. Am J Pathol 127:117–126

Hausen H zur, Schulte-Holthausen H, Klein G (1970) Epstein Barr virus DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature 228:1056–1058

Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P (1990) Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J Clin Pathol 43:499–504

Jiwa NM, Gemert GW van, Raap AK, Rijke van de, et al (1989) Rapid detection of human cytomegalovirus in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation 48:72–76

Kiyabu MT, Shibata D, Arnheim N, Martin WJ, Fitzgibbons PL (1989) Detection of human papillomavirus in formalin-fixed, invasive squamous carcinomas using the polymerase chain reaction. Am J Surg Pathol 13:221–224

Lanier AP, Bornkamm GW, Henle W (1981) Association of Epstein-Barr virus with nasopharyngeal carcinoma in Alaskan native patients: serum antibodies and tissue EBNA and DNA. Int J Cancer 28:301–305

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM (1989) The use of polymerase chain reaction amplification for the detection of genital human papillomarviruses. Cancer Cells 7:209–214

Raymond FA, Weinshilboum RM (1975) Microassay of human erythrocyte catechol-O-methyltransferase: removal of inhibitory calcium ions with chelating resin. Clin Chim Acta 58:185–194

Saito I, Servenius B, Compton T, Fox RI (1989) Detection of Epstein-Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjögren's syndrome. J Exp Med 169:2191–2198

Sculley TB, Sculley DG, Pope JH, Bornkamm GW, Lenoir GM, Rickinson AB (1988) Epstein-Barr virus nuclear antigens 1 and 2 in Burkitt lymphoma cell lines containing either 'A'- or 'B'-type virus. Intervirology 29:77–85

Shibata DK, Arnheim N, Martim WJ (1988) Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 167:225–230

Singer-Sam J, Tanguay RL, Riggs AD (1989) Use of Chelex to improve the PCR signal from a small number of cells. Amplifications 3:11

Sixbey JW, Shirley P, Chesney PJ, Buntin DM, Resnick PJ (1989) Detection of a second widespread strain of Epstein-Barr virus. Lancet I:761–765

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10:506–513

Weiss LM, Movahed LA, Butler AE, Swanson SA, Frierson HF, et al (1989) Analyses of lymphoepithelioma and lymphoepithelioma-like carcinomas for Epstein-Barr viral genomes by in situ hybridization. Am J Surg Pathol 13:625–631

Wright DK, Manos MM (1990) Sample preparation from paraffin-embedded tissues. In: Innis MA (ed) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 153–156