
Universal Amplification of DNA Isolated from Small Regions of Paraffin-Embedded, Formalin-Fixed Tissue

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The polymerase chain reaction (PCR) analysis of DNA isolated from formalin-fixed, paraffin-embedded tissue can be difficult because of the inherently poor quality of template DNA available for amplification. As a consequence of the formalin fixation process, DNA is complexed with proteins and is often nicked, giving relatively short fragments. Such samples are often of low DNA concentration and poor quality. A high rate of random failure, the inability to routinely amplify fragments greater than 650 bp and poor storage make paraffin-embedded tissue a less than ideal source of DNA template (2).

We wanted to assay specific cells from very small regions of paraffin-embedded tissue sections for loss of heterozygosity (LOH) using microsatellite markers. Various protocols have been written for the successful extraction of DNA from a small number of paraffin-embedded cells (2,5), but these protocols were not amenable to genomic screening projects because of the difficulties previously described.

Universal genomic amplification is a way to overcome the problems associated with DNA isolated from paraffin-embedded tissue. There have been several protocols reported for the generation of synthetic genomic template, the most notable being Alu PCR (4), degenerate oligonucleotide-primed PCR (DOP-PCR) (8) and primer extension pre-amplification (PEP) (9). The latter has been applied to achieve multiple PCR assays on single cells and has recently been proven useful for extending limited DNA samples (1). The strength of the PEP protocol is the opposite of PCR—it relies on random amplification without any preference for specific sequences.

Control genomic DNA was prepared from blood collected in EDTA using standard salting out procedures and

quantitated spectrophotometrically (3). DNA was isolated from formalin-fixed, paraffin-embedded tissue sections of unknown age, fixation method or fixation time by the following procedure. Regions of cells were identified from hematoxylin and eosin-stained sections, and the selected cells were scratched from a single corresponding, unstained 10- μ m slide section and transferred to 500- μ L PCR tubes. The area of the scratched material ranged from about 1 to 10 mm², depending on the number of cells selected. Samples were heated to 95°C for 10 min under paraffin oil in 20 μ L of 1 \times PCR Buffer II (Perkin-Elmer, Norwalk, CT, USA) with periodic mixing to aid de-waxing of each sample. After cooling briefly, samples were digested with proteinase K (1000 μ g/mL) at 65°C for 90 min followed by enzyme inactivation at 85°C for 15 min. This solution (10 μ L) was then used as DNA template for

universal amplification. Larger samples were purified by chloroform extraction followed by salt/isopropyl alcohol precipitation. DNA pellets were resuspended in 20 μ L TE buffer (2 mM Tris-HCl, 0.2 mM EDTA, pH 8.0).

Universal amplification products were generated using a modification of the PEP protocol (9). Standard PEP reactions were performed under paraffin oil in 60- μ L volumes containing 200 pmol of random 12-mer oligonucleotides, 5 U AmpliTaq[®] DNA Polymerase (Perkin-Elmer), 200 μ M of each dNTP, 5 mM MgCl₂, 1 \times PCR Buffer II and DNA template. Templates consisted of either purified DNA extracted from paraffin-embedded tissue sections, 10 μ L of the proteinase K digest of cells dissected from paraffin-embedded tissue sections or a known quantity of the control DNA. Water blanks were included as contamination checks for each amplification series. In

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parallel experiments, 10 U AmpliTaq DNA Polymerase, Stoffel Fragment (Perkin-Elmer) and 1× Stoffel Buffer were substituted for AmpliTaq DNA Polymerase and PCR Buffer II, respectively.

Thermal cycling conditions were as follows: 92°C for 4 min, 40 cycles at 92°C for 1 min, 25°C for 2 min, 30°C for 30 s, 35°C for 30 s, 40°C for 30 s and 72°C for 2 min, followed by a final extension at 72°C for 15 min. The ramp was set at 0.25°C/s between the 25° and 30°C steps. A slow ramp at low annealing temperature was used to ensure optimal annealing of oligonucleotides to template DNA. All thermal cycling was performed in a 96-well Model FTS-960 Thermal Sequencer (Corbett Research, Sydney, NSW, Australia).

Microsatellite markers were used to assess the products generated in each genomic amplification experiment.

PCR was performed in 10-μL volumes containing 0.5 U AmpliTaq, 10 pmol of each oligonucleotide, 1 μCi [α -³²P]dCTP (3000 Ci/mmol), 20 μM dNTPs, 1× PCR Buffer and either 1 μL universal amplification product, 1 ng of control template DNA or a water blank as a negative control. DNA and control samples were amplified using standard thermal cycling conditions, and allelic fragments were electrophoresed on denaturing 6%, 19:1 polyacrylamide gels and exposed to X-ray film for autoradiography.

Universal amplification products were generated from control DNA diluted to theoretical single-copy quantity (approximately 3 pg) by amplification using the PEP protocol with two exceptions: (i) a 12-mer random oligonucleotide was used in place of the reported 15-mer, and (ii) only a single round of microsatellite PCR was required. Further experiments determined

that reducing the oligonucleotide concentration to one tenth its original concentration decreased both the level and number of additional nonspecific bands in subsequent PCR amplifications without causing any notable loss in product levels. Decreasing the number of thermal cycles from 50 to 40 achieved a similar result. Two points of interest were noted from these investigations: (i) universal amplification products could only be reproducibly detected from initial DNA quantities equivalent to no less than 4 genomic copies (about 12 pg), indicating a reduction in sensitivity, and (ii) allelic imbalances were observed after amplification of low-copy-number templates, detected by microsatellite PCR as differing allelic band densities. This suggested preferential allelic amplification, having implications for the desired LOH analyses. Investigation of this phenomenon demonstrated that

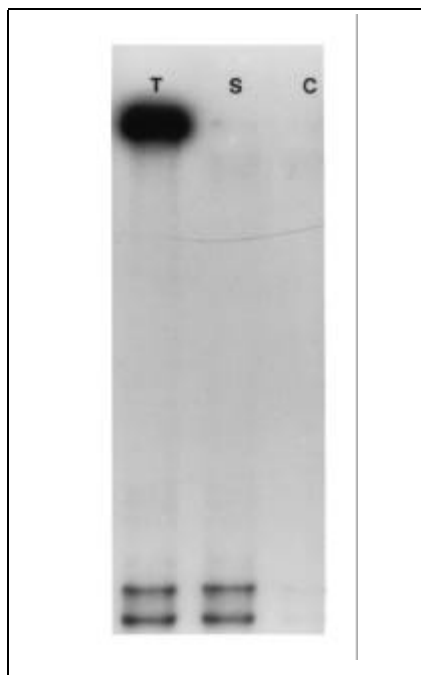


Figure 1. PCR amplification of microsatellites with and without genomic amplification. Lane T shows allelic PCR products from universal amplification products generated using *Taq*; lane S shows allelic PCR products from universal amplification products generated using Stoffel Fragment; lane C shows allelic PCR products generated from DNA template without genomic amplification. All PCRs were performed using equal starting quantities of genomic DNA isolated from a single paraffin-embedded tissue section.

this preferential allelic amplification was random between universal amplifications and constant within single-locus amplifications. Comparison of alleles amplified by microsatellite PCR from universal amplification products generated from cross-titrated control DNAs and equivalent amounts of unamplified, titrated DNAs demonstrated no evidence of bias in universal amplification. Products appear to be an accurate representation of DNA content, maintaining the allelic ratio of the original sample. The observed allelic imbalances at low copy number were attributed therefore to the statistical distribution of alleles in each universal amplification (9). DNA quantities of greater than 50 pg did not appear to be affected by this phenomenon. Care must therefore be taken when considering dilute samples prior to amplification by this PEP protocol.

The relative levels of product generated from DNA samples isolated from

paraffin-embedded tissue were less consistent than for the control DNAs when amplified using *Taq* DNA polymerase. In addition, subsequent microsatellite PCR from *Taq*-amplified universal amplification product templates demonstrated a high-molecular-weight background signal present in the wells of all samples after electrophoresis. These byproducts incorporate significant amounts of isotopic label and so reduce the enzyme activity available for microsatellite PCR. The generation of these byproducts appears to be independent of the presence of DNA, suggesting that it is a complex of oligonucleotide polymerizations. Some microsatellite markers either failed to amplify or gave very poor results compared to control DNAs when amplified using *Taq*-amplified universal amplification product as the DNA template.

Stoffel Fragment's greater tolerance for variations in reaction conditions makes it a candidate to substitute for *Taq*. The level of universal amplification product generated using Stoffel Fragment was comparable to amplifications using *Taq* (Figure 1). Universal amplification with the Stoffel Fragment reliably generated products from DNA samples isolated from paraffin-embedded tissue unlike *Taq*, which suffered random failures. An unexpected effect of substitution of *Taq* with Stoffel Fragment was the absence of the high-molecular-weight byproducts observed for *Taq* amplifications. As a result, Stoffel-amplified products have a reduced background of artifact bands after microsatellite PCR, making it easier to score for LOH. To date, there is no evidence to suggest Stoffel-amplified templates reduce the PCR amplification efficiency for any microsatellite marker.

DOP-PCR was performed on known quantities of control DNA following a published protocol (8). Distinct band patterns were evident within a smear of genomic fragments. A high rate of PCR failure was observed between samples and individual microsatellite loci when DOP-PCR products were used as template DNA. Successful microsatellite amplifications were not reproducible.

PEP may also provide an efficient method of generating genomic probes for use in comparative genomic hybridization. DOP-PCR has been used

successfully to generate probe from limited DNA template (6–8). Application of this protocol to comparative genomic hybridization is currently under investigation.

We have found this protocol to be a reliable and efficient means of generating genomic DNA from paraffin-embedded tissue sections. Multiple microsatellite markers can be reproducibly and reliably amplified from universal amplification products generated by this protocol without the difficulties generally associated with the PCR analysis of DNA isolated from paraffin-embedded tissue.

REFERENCES

1. Casas, E. and B.W. Kirkpatrick. 1996. Evaluation of different amplification protocols for use in primer-extension preamplification. *BioTechniques* 20:219-225.
2. Crisan, D. and J.C. Mattson. 1993. Retrospective DNA analysis using fixed tissue specimens. *DNA Cell Biol.* 12:455-464.
3. Miller, S.A., D.D. Dykes and H.F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16:1215.
4. Nelson, D.L., S.A. Ledbetter, L. Corbo, M.F. Victoria, R. Ramirez-Solis, T.D. Webster, D.H. Ledbetter and C.T. Caskey. 1989. *Alu* polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. USA* 86:6686-6690.
5. Shibata, D., D. Hawes, Z.-H. Li, A.M. Hernandez, C.H. Spruck and P. Nichols. 1992. Specific genetic analysis of microscopic tissue after selective ultraviolet radiation fractionation and the polymerase chain reaction. *Am. J. Pathol.* 141:539-543.
6. Speicher, M.R., S. du Manoir, E. Schrock, H. Holtgreve-Grez, B. Schoell, C. Lengauer, T. Cremer and T. Reid. 1993. Molecular cytogenetic analysis of formalin-fixed, paraffin-embedded solid tumours by comparative genomic hybridization after universal DNA-amplification. *Hum. Mol. Genet.* 2:1907-1914.
7. Speicher, M.R., A. Jauch, H. Walt, S. du Manoir, T. Reid, W. Jochum, T. Sulser and T. Cremer. 1995. Correlation of microscopic phenotype with genotype in a formalin-fixed, paraffin-embedded testicular germ cell tumour with universal DNA amplification, comparative genomic hybridization, and interphase cytogenetics. *Am. J. Pathol.* 146:1332-1340.
8. Telenius, H., A.H. Pelmear, A. Tunnacliffe, N.P. Carter, A. Behmel, M.A. Ferguson-Smith, M. Nordenskjold, R. Pragner and B. Ponder. 1992. Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chromosomes. Cancer* 4:257-263.

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9. Zhang, L., X. Cui, K. Schmitt, R. Hubert, W. Navidi and N. Arnheim. 1992. Whole genome amplification from a single cell: implications for genetic analysis. *Proc. Natl. Acad. Sci. USA* 89:5847-5851.

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UV Irradiation of Polystyrene Pipets Releases PCR Inhibitors

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Many polymerase chain reaction (PCR) inhibitors have been detected, ranging from naturally occurring byproducts of decay (3,4,6) or plant extracts (2) to introduced reaction inhibitors (1,7,8). Lee and Cooper have even documented PCR inhibition from wooden toothpicks used to pick colonies prior to lysis and amplification (5). We have recently found that PCR inhibitors are released upon UV irradiation of polystyrene pipets.

Ironically, this observation was made while instituting changes to increase the efficiency and throughput of our PCR assays. During this process, all PCR preparations were moved into a dedicated PCR preparation (PCR prep) room equipped with a UV lamp, and a 96-well protocol was adopted. After switching to the plate PCR format, we began experiencing sporadic partial to total inhibition of product. Troubleshooting experiments to evaluate obvious variables eventually localized the

problem to the use of individually wrapped, sterile, 5-mL polystyrene pipets (used to make large-volume stocks of dNTPs in water) in the place of micropipettors with polypropylene tips. Notably, all the polystyrene pipets used in these assays had been stored in the PCR prep room and had been exposed to UV light for varying lengths of time.

It was possible that the pipets, stored within a desktop receptacle, were adversely affected by intermittent exposure to the UV lamp (the lamp was on during times when the room was not in use). To test this possibility, two different brands (A and B) of polystyrene pipets were subjected to UV irradiation for 48 h, 2 weeks or 3 weeks. After exposure, the pipets were rinsed repeatedly with 4 mL of H₂O, and the eluates were added to pre-PCR mixtures (Figure 1). Pipets that were tested directly out of the shipping container (0 weeks) did not result in any inhibition of the assay. Increasing inhibition of PCR product formation was noted, however,

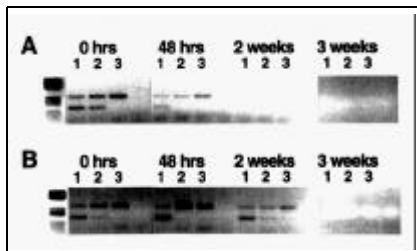


Figure 1. PCR inhibition as a function of UV irradiation of polystyrene pipets. Three samples were processed for PCR using 5-mL polystyrene pipets from two different manufacturers (A and B). The pipets were either used directly after removal from the shipping container (0 h) or after exposure to UV irradiation for 48 h, 2 weeks or 3 weeks. The UV exposure was to a Universal Light Source Lamp G36T6L (San Francisco, CA, USA) at a distance of 0.65 m. To assay each condition, 4 mL of H₂O were pipetted up and down the length of the pipet 10 times; this water was then substituted for the water component in the PCR assay (8). Primer sets amplified DNA from cell lysates (100 cell equivalents per reaction) for HIV-1 gag (lower band) and human β -globin (upper band). Reactions were run for 40 cycles (1st cycle: 95°C for 6 min, 60°C for 2 min, 72°C for 1.5 min; cycles 2–5: 95°C for 2 min, 60°C for 2 min, 72°C for 1.5 min; cycles 6–40: 95°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, final extension: 72°C for 10 min, hold at 27°C); the PCR products were displayed on 2% agarose gels stained with ethidium bromide. In this case, the eluate comprised approximately 61% of the reaction volume, but previous experiments indicate that reactions using as little as 15% eluate were also inhibited (data not shown).

as either brand of pipet was exposed to UV irradiation for increasing lengths of time. To determine whether UV light might generate breakdown products from polystyrene, the absorbance profiles of the eluates were read on a UV spectrometer (Figure 2). Although 48 h of UV exposure were insufficient to cause significant changes in absorbance (data not shown), the eluates from pipets that had been irradiated for two to three weeks contained substance(s) that absorbed in the 200–400-nm range and that were not found in eluates from unexposed pipets.

We conclude that UV irradiation of polystyrene pipets results in breakdown products that are inhibitory to PCR. The chemical composition and necessary concentration of the inhibitors is unknown, but it appears that the inhibitory products are not brand-specific but derived from polystyrene. The minimal irradiation time necessary to generate sufficient inhibitors is also not clear, but constant exposure to UV is not required; three weeks of intermittent but cumulative exposure resulted in eluates with enough inhibitors to prevent PCR product formation in all cases (Figure 1). One colleague observed that after his return from holiday, noticeably fewer of his reactions amplified proper-

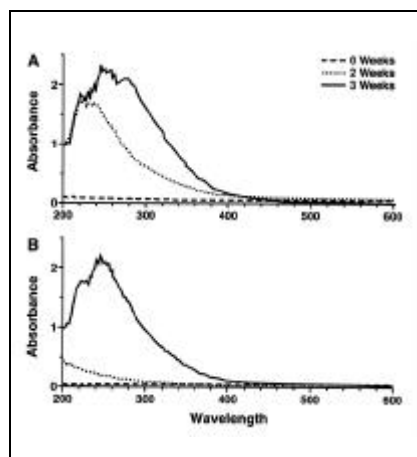


Figure 2. Breakdown products released upon UV irradiation of polystyrene pipets. Polystyrene pipets from two different manufacturers (A and B) were extracted with water, as in the legend to Figure 1, either directly from the shipping container (0 weeks) or after exposure to UV light for 2 or 3 weeks (as described above). The absorbance profiles of the eluates were then examined for on a diode array spectrometer with UV-Vis Software (Hewlett-Packard, Wilmington, DE, USA).