

## Technical reports

# Use of magnetic beads for tissue DNA extraction and IS6110 *Mycobacterium tuberculosis* PCR

R Caldarelli-Stefano, L Vago, S Bonetto, M Nebuloni, G Costanzi

### Abstract

**Polymerase chain reaction (PCR) techniques are used increasingly for the diagnosis of *Mycobacterium tuberculosis* infection and can be used on the DNA obtained from both frozen and formalin fixed, paraffin wax embedded tissues. However, the extraction of DNA by means of the conventional phenol/chloroform method is time consuming and requires the use of potentially dangerous chemical reagents. This paper describes a method based upon the use of magnetic beads for the extraction of *M tuberculosis* DNA from both routinely formalin fixed, paraffin wax embedded tissues and frozen tissues. Magnetic bead extracted DNA from brain, lymph node, and lung tissues collected from patients with human immunodeficiency virus and tuberculosis was compared with that extracted using the phenol/chloroform method. The magnetic bead extraction procedure requires less than two hours, including the time necessary to dewax the tissue sections. In all cases, the DNA extracted with both methods was amplified successfully by PCR for the *M tuberculosis* IS6110 sequence. Magnetic bead DNA extraction can be used on both frozen and archival tissues: the method is reliable, simple, sensitive, and rapid; in addition, it does not use hazardous procedures or specialised laboratory equipment and can be used for routine DNA isolation from various human tissues.**

(J Clin Pathol: Mol Pathol 1999;52:158-160)

Keywords: magnetic beads; paraffin wax embedded tissue; DNA extraction for *Mycobacterium tuberculosis*

Over the past few years, the polymerase chain reaction (PCR) has been widely used to identify the genomic sequences of a number of microorganisms from various biological fluids and tissues, including samples processed routinely for histopathological examination, although the fixation and processing steps reduce the efficiency of extraction and the integrity of nucleic acids.<sup>1</sup>

The most widely used method for extracting DNA from formalin fixed, paraffin wax embedded blocks requires tissue lysis with proteinase K, followed by phenol/chloroform extraction,

and ethanol precipitation. However, this procedure is relatively long and exposes the operators to potentially dangerous reagents. Alternative methods for the extraction of DNA from fresh and/or paraffin wax embedded samples have been proposed, based upon different chemical principles.<sup>2-6</sup>

We report here the results of a method based upon the use of magnetic beads (Dynabeads; Dynal, Oslo, Norway) for the extraction of DNA from formalin fixed, paraffin wax embedded tissues and frozen tissues, which has been used previously on human whole blood, bone marrow, and cultured cells.<sup>2</sup>

The process relies upon cell lysis to release the DNA and its subsequent adsorption to the surface of the Dynabeads and does not require specialised laboratory equipment. To compare magnetic bead extraction with a conventional phenol/chloroform method, we amplified the DNA extracted from both formalin fixed and frozen tissues of culture confirmed cases of tuberculosis with a nested PCR (nPCR) method for a sequence from the repetitive insertion fragment IS6110 of *Mycobacterium tuberculosis*.<sup>7</sup>

### Methods

The DNA was extracted from four frozen tissue blocks (three from lymph nodes and one from brain) and 10 paraffin wax embedded blocks (four from lymph nodes, three from lung, and three from brain). All of the samples were obtained at postmortem of 10 human immunodeficiency virus (HIV) positive patients, who died as a result of disseminated tuberculosis, confirmed by the culture of blood, other fluid, or biopsy samples. In all cases, the histopathological diagnosis was confirmed by positive Ziehl-Nielsen staining.

The DNA was extracted from three 5 µm thick sections. To avoid contamination, both the frozen and paraffin wax blocks were cut using disposable blades; the cutting surfaces of the microtome and cryotome were covered with "Parafilm", on which the sections were collected. Paraffin wax block sections not containing biological samples were used as negative cutting and extraction controls.

The paraffin was removed by incubating the sections at 60°C for 10 minutes and with xylene at room temperature for 15 minutes, then washing them with 100% and 70% ethanol.

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Accepted for publication  
21 January 1999

Table 1 DNA and protein concentrations in paraffin wax embedded and frozen samples extracted by means of two different methods: magnetic beads (MB) and phenol/chloroform (PC)

Sample	MB DNA extraction		PC DNA extraction	
	DNA concentration (µg/ml)	Protein concentration (µg/ml)	DNA concentration (µg/ml)	Protein concentration (µg/ml)
<i>Paraffin wax embedded</i>				
1 Brain	8.9	0.3	89.5	1.7
2 Brain	37.6	0.3	53.7	8.4
3 Brain	36.2	0.3	151	8.1
4 Lymph node	40.1	0.4	252.2	1.1
5 Lymph node	61.6	0.5	255	1.2
6 Lymph node	33.8	0.5	123.4	1.5
7 Lymph node	38.4	0.6	107.5	1.4
8 Lung	35.3	0.3	400.9	1
9 Lung	225.9	0.4	3873	9.4
10 Lung	29	0.2	1083.6	3.8
Mean	48.3	—	638.9	—
<i>Frozen</i>				
11 Brain	38.6	1.4	60.2	1.6
12 Lymph node	15.4	0.1	226.8	1.1
13 Lymph node	19.3	0.3	139.4	1.5
14 Lymph node	21.3	0.2	123.5	1.7
Mean	23.6	—	137.4	—

After air drying, the tissue pellets were hydrated in distilled water and 200 µl DNA DIRECT Dynabeads (Dyna) were added in a 1.5 ml Eppendorf tube and left at room temperature for five minutes to obtain cell lysis and ensure the adsorption of the DNA to the surface of the Dynabeads. After placing the tube in a magnetic separator (MPC; Dynal) for 90 seconds, the supernatant was discarded in such a way as to avoid touching the dark brown pellet. The tube was removed from the magnetic separator, 200 µl of washing buffer was added, and the tube was returned to the separator for about 30 seconds; the supernatant was then discarded and the washing step was repeated twice, to remove any residual contaminants. The DNA-Dynabead complex was resuspended in 40 µl TE (Tris/HCl, pH 7.6, 50 mM EDTA) buffer, mixed using a pipette, and vortexed to obtain a homogenous suspension. The DNA was eluted by incubation at 65°C for 10 minutes, followed by

contact with the magnetic separator for 30 seconds. A 5 µl aliquot of the supernatant was used directly in the PCR reaction.

All of the samples were also extracted using a modified phenol/chloroform method.<sup>8</sup> Briefly, after dewaxing with xylene and ethanol, the sections were digested overnight with proteinase K (300 µg/ml), and the DNA was extracted once with phenol/chloroform and once with chloroform and isoamyl alcohol (24/1), and then precipitated in ethanol.

Spectrophotometric determinations were performed for each sample. Readings were taken at 260 nm, 280 nm, and 320 nm (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden) to measure the amount of total DNA and protein.

nPCR to the IS6110 fragment of the *M tuberculosis* complex was performed as described previously,<sup>7</sup> using ~ 500 ng of extracted DNA. The amplified DNA products were run on a 2% agarose gel, stained with ethidium bromide, and visualised under UV light.

Care was taken to minimise cross-contamination. All PCR reactions were performed using a negative control, containing all PCR components and sterile distilled water instead of DNA, negative cutting samples, and a positive control, containing DNA extracted from a culture of *M tuberculosis*.

## Results and discussion

As summarised in table 1, less DNA was obtained by the magnetic bead extraction method than by phenol/chloroform extraction. In two lymph node samples, cases 6 and 7, the amount of DNA obtained by magnetic bead extraction was very low, ~ 4 µg/ml and 4.5 µg/ml, respectively, with a low optical density (OD) 260:280 ratio. In these two cases, ethanol precipitation was performed to purify the extracted DNA; after washing, the DNA concentration increased to 33.9 µg/ml and 38.4 µg/ml, respectively.

All the DNA obtained was used for PCR amplification. In all cases, nPCR amplified the IS6110 sequence of *M tuberculosis* and yielded an expected band of 122 bp (fig 1). Magnetic bead extracted samples gave slightly stronger and cleaner bands on agarose gel than samples extracted with the phenol/chloroform method, but this was not quantified further. No false positive results were observed.

Our data support the use of a magnetic bead based procedure as a rapid method of extracting DNA from formalin fixed, paraffin wax embedded archival tissues suitable for PCR amplification of IS6110 *M tuberculosis* sequences. The method is based upon cell lysis, DNA adsorption to the beads, magnetic separation of the bead-DNA complex, and extensive washing to remove contaminants and inhibitors. The entire procedure takes less than two hours. Although the quantity of DNA obtained from samples extracted using magnetic beads was less than that extracted using a conventional phenol/chloroform method, the efficiency of nPCR to amplify *M tuberculosis* was the same in all the samples. In only two cases a minimal amount of DNA was obtained, and a further step of purifi-

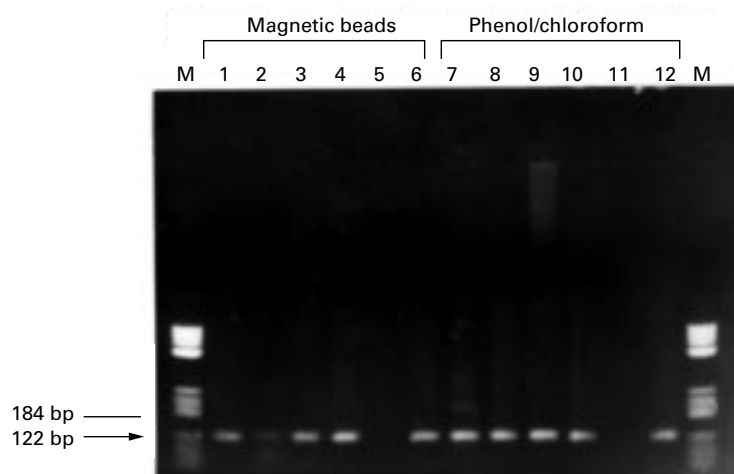


Figure 1 Electrophoresis of 2% agarose gel of nested PCR products of IS110 *Mycobacterium tuberculosis* DNA samples extracted with magnetic beads (lanes 1–6) and phenol/chloroform (lanes 7–12). Lane M: DNA molecular weight marker VIII (Boehringer Mannheim, Monza, Italy); lanes 1, 2, 7, and 8: DNA extracted from brain; lanes 3 and 9: DNA extracted from lymph node; lanes 9 and 10: DNA extracted from lung; lanes 5 and 11: negative controls; lanes 6 and 12: positive controls.

cation was required. Moreover, DNA can be extracted from different type of tissues such as brain, lymph nodes, and lung.

Extraction using magnetic beads is easy and time saving, and does not require potentially dangerous procedures or specialised laboratory equipment and we propose its application for the diagnosis of *M tuberculosis* infection.

This work was supported by a grant from Istituto Superiore di Sanità, Roma, II Progetto Tubercolosi.

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## A simple method for PCR based analyses of immunohistochemically stained, microdissected, formalin fixed, paraffin wax embedded material

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### Abstract

**Microdissection was performed on sections cut from formalin fixed, paraffin wax embedded archival material, which had been subjected to conventional immunohistochemistry. Crude DNA extracts, which were obtained from these microdissected samples by a simple microwave step, were then added directly to amplification reactions. Analyses using a range of polymerase chain reaction (PCR) based techniques, including microsatellite repeat polymorphism analysis at the NM23-H1 locus and sequencing of exons 5, 7, and 8 of the p53 gene, were performed successfully. Universal PCR amplification was also carried out on the microdissected material and probes suitable for use in comparative genomic hybridisation (CGH) were obtained in all cases. This technique will enable a range of effective genetic analyses to be carried out on specific subsets of cells that have been characterised previously by immunohistochemistry.**

(*J Clin Pathol: Mol Pathol* 1999;52:160-163)

Keywords: polymerase chain reaction; comparative genomic hybridisation; paraffin wax embedded immunohistochemically stained material; microdissection

The advent of polymerase chain reaction (PCR) technology has enabled DNA to be studied from a variety of sources, including many that were previously excluded from investigation. Archives containing formalin fixed tissue samples that had been considered

too small for conventional DNA analysis (representing whole subsets of the malignant process—for example, very early lesions or biopsies of advanced metastatic disease), are now available for genetic study.

Similarly, the microdissection of histologically distinct areas within a tissue section has not only enabled samples to be enriched for tumour cells compared with normal cells, which is of great importance in loss of heterozygosity studies, but has also allowed the potential investigation of different subpopulations of tumour cells within a single histological section.

Although performing PCR on DNA extracted from paraffin wax embedded material is now relatively commonplace, most extraction protocols require proteinase digestions of varying lengths.<sup>1,2</sup> Boiling techniques have also been applied in some cases, but with varied and limited rates of success.<sup>3,4</sup> We describe a simple microwave based technique that can be used on microdissected tissue samples that have previously been subjected to standard immunohistochemistry. This method has been applied to both specific PCR (NM23-H1 microsatellite repeat polymorphism analysis and p53 exon sequencing) and degenerate oligonucleotide primed (DOP) PCR for use in comparative genomic hybridisation (CGH) with a high degree of success.

### Methods

#### IMMUNOHISTOCHEMISTRY

We performed immunohistochemistry on formalin fixed, paraffin wax embedded material obtained from diagnostic histopathology ar-

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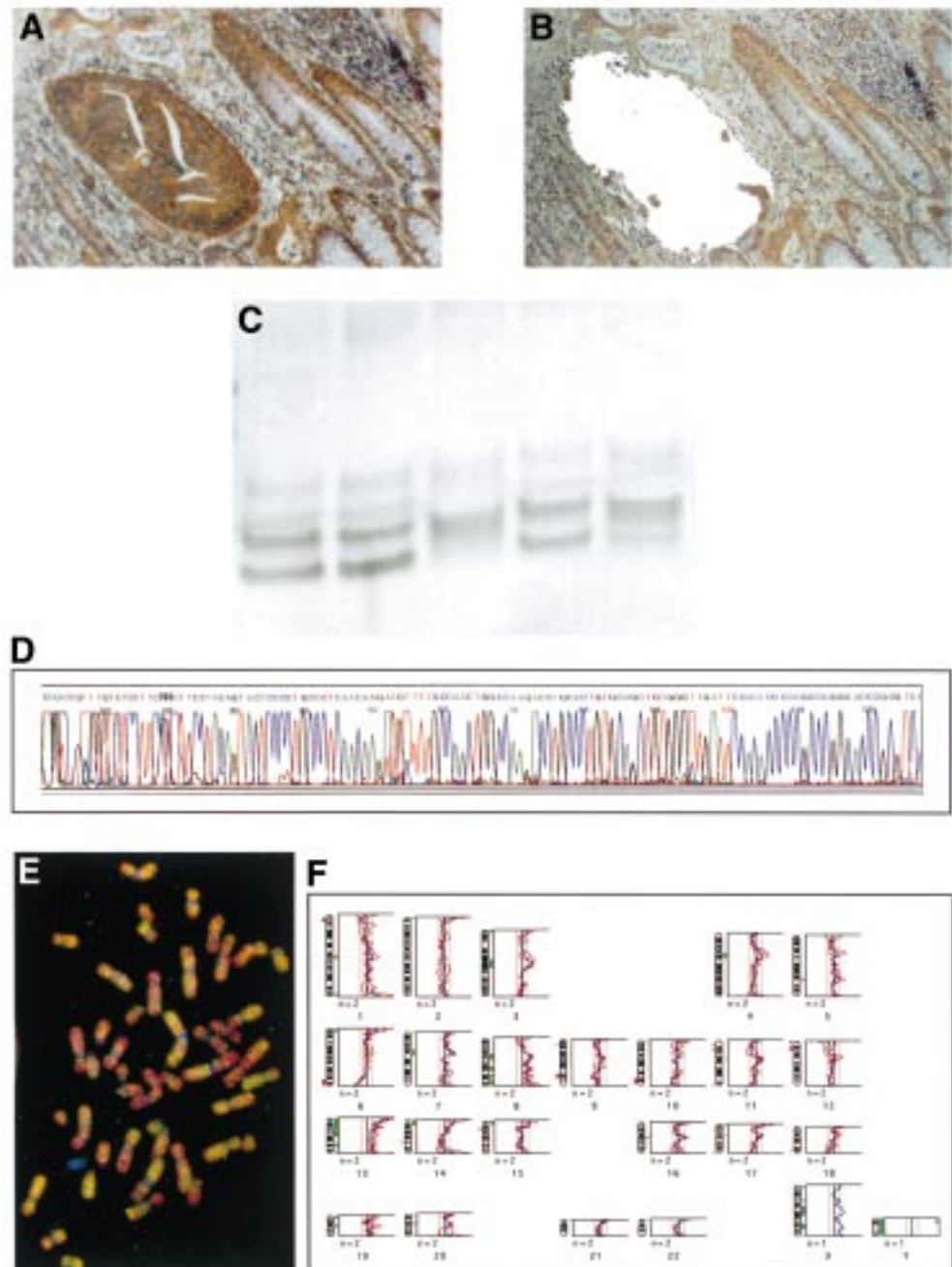
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Accepted for publication  
5 January 1999

chives. The paraffin wax blocks used were between two and 17 years old.

Sections (5  $\mu$ m) were cut and mounted on APES coated slides. A different part of the microtome blade was used to cut each block. We dewaxed sections individually in two washes of xylene (five minutes each) and rehy-

drated them individually through a graded alcohol series to prevent possible cross contamination. Endogenous peroxidase activity was quenched by a 20 minute incubation in a 0.3% solution of hydrogen peroxide in methanol. We then performed standard immunohistochemistry, using a Vector stain Elite kit



**Figure 1** Genetic analysis of paraffin wax embedded tumour material. (A) Photomicrograph of paraffin wax embedded section of primary colorectal adenocarcinoma stained immunohistochemically for NM23-H1, detected with diaminobenzidine and counterstained with haematoxylin. (B) Paraffin wax section shown in (A) after microdissection of a focus of positively stained tumour. (C) Silver stained polyacrylamide gel electrophoresis of PCR products from the NM23-H1 locus. Amplification was carried out on microdissected material. Normal lymph node in lane 1 shows heterozygous bands. One primary tumour sample shows loss of the smaller allele (lane 3), two other primary tumour samples are heterozygous (lanes 2 and 4), and allelic imbalance can be seen in the tumour sample in lane 5. (D) Partial sequencing electropherogram of the exon 5 region of the p53 gene. The sequencing reaction was carried out on microdissected material from a colorectal tumour. (The exon 5 sequence begins at position 31.) (E) Target metaphase spread from comparative genomic hybridisation (CGH) of degenerate oligonucleotide primed PCR (DOP-PCR) amplified microdissected material from a colorectal tumour. (F) CGH copy number karyotype from the target metaphase shown in (E). Clear gains, illustrated by green bars to the right of the karyogram, are shown at 5q, 8q, 3q, and Xq. Clear losses, illustrated by red bars to the left of the karyogram, are shown at 1p, 6q, 9p, 9q, 10q, 1 lp, 12p, and 14q. (Apparent gains or losses at centromeric or telomeric regions must be interpreted with considerable caution.)

(Vector Laboratories, Burlingame, California USA), using commercial monoclonal antibodies. Primary antibodies against the protein products of the p53 oncogene (DO7; Novocastra, Newcastle upon Tyne, UK) and the putative metastasis suppressor NM23-H1 (anti-nm23-H1/NDPK-A; Novocastra) were used. Immunoreactivity was visualised with diaminobenzidine and the sections were counterstained with haematoxylin.

Immunohistochemistry using the anti-p53 antibody required an additional antigen retrieval step. This was performed by microwaving sections in 0.01 M trisodium citrate solution for eight minutes at 800 W and then allowing the solution to cool to 50°C. To aid microdissection and to minimise tissue damage, especially to small biopsy samples, the temperature was not allowed to exceed 85°C throughout antigen retrieval, which contributed to the maintenance of tissue architecture and adhesion to the slide. Vectabond (Vector Laboratories) coated slides were occasionally used for small tissue sections.

#### MICRODISSECTION

After immunohistochemistry, sections were kept covered with deionised water (Sigma, Poole, Dorset, UK) in a humidified chamber and never allowed to dry out until required for microdissection. Microdissection was performed on a Leitz Laborlux microscope fitted with a MPS46/52 photographic system at  $\times 100$  total magnification. Excess water was drained from the slide and the area surrounding the tissue section was dried carefully. We then added 100  $\mu$ l of 1 $\times$  TE buffer (10 mM Tris, 1 mM EDTA) to the tissue, creating a bubble of liquid over the section. Areas of interest were microdissected using a disposable microlance 3 needle mounted on a 10 ml syringe, allowed to float into the liquid, and harvested with a pipette. Multiple sampling from one section required extensive washing in deionised water to prevent cross contamination of microdissected samples. We took photomicrographs (fig 1A and B) before and after each microdissection was carried out. These provided a record of each sample, which could be used for independent assessment by a pathologist of both the extent of normal tissue contamination and the immunohistochemical status of each sample.

TE buffer containing the microdissected tissue fragments was transferred from the slide to a 0.5 ml plastic tube and centrifuged at 12 000  $\times g$  for 15 minutes. The supernatant was

removed and the tissue pellet was resuspended in 20  $\mu$ l of fresh 1 $\times$  TE buffer. Samples were overlaid with 50  $\mu$ l of mineral oil and microwaved at 800 W for seven minutes. After resuspension, an aliquot of TE buffer containing dispersed material from a microdissected tissue sample was then added to the mastermix for PCR amplification.

#### PCR

We amplified the NM23-H1 locus using the primer pair 5'-TTG ACG GGG GTA GAG AAC TC-3' and 5'-TCT CAG TAC TTC CCG TGA CC-3', as described by Hall and colleagues.<sup>5</sup> We amplified p53 exons 5, 7, and 8 using primer pairs 5'-GGA ATT CTG TTC ACT TGT GCC CTG ACT TTC AAC-3' and 5'-GCA ACC AGC CCT GTC GTC TCT CCA-3' (exon 5); 5'-ACA GGT CTC CCC AAG GCG CAC TGG-3' and 5'-GGG CAC AGC AGG CCA GTG TGC AG-3' (exon 7); and 5'-AGG TAG GAC CTG ATT TCC TTA CTG CC-3' and 5'-GGA ATT CTG AGG CAT AAC TGC ACC CTT CCT CT-3' (exon 8). Locus specific PCR was performed under the conditions described by Kogan *et al.*<sup>6</sup> For NM23 amplification, we used 2 U of Bioline Taq (Bioline, London, UK). For the amplification of p53 exons, we used 5 U of Boehringer "Expand" Taq (Boehringer UK, Lewes, Sussex, UK) in a 100  $\mu$ l reaction volume. DOP PCR was carried out using degenerate primers according to the methods of Speicher and colleagues, using 5 U of Taq (Bioline).<sup>7</sup> To avoid contamination, we set up DOP reactions in an isolated, still air environment, using single use aliquots.

#### SEQUENCE ANALYSIS OF PCR PRODUCTS

Sequencing was performed using the Applied Biosystems ABI373A DNA sequencer and dye primer cycle sequencing with Amplitaq<sup>®</sup> DNA polymerase (P E Applied Biosystems, Beaconsfield, Buckinghamshire, UK).

#### COMPARATIVE GENOMIC HYBRIDISATION

The DOP-PCR product (test) and total human (reference) DNA of the same sex were labelled by nick translation with "spectrum green" and "spectrum red" (Vysis UK, Richmond, Greater London, UK), respectively. The fragment length of the labelled DOP products, as established by agarose gel electrophoresis, was somewhat shorter than that used conventionally in CGH,<sup>8</sup> with a maximum of between 500 and 1000 bp, and the nick translation reaction was adjusted to produce total human DNA in the same range. Test and reference probes were combined with an excess of unlabelled human COT-1 DNA and hybridised to normal human metaphase target spreads for four days at 37°C. After post-hybridisation washes and counterstaining, target images were captured with a cooled CCD camera and then analysed using the Quips CGH analysis program (Vysis). In preliminary experiments, CGH was carried out on two cell lines (Daudi and K562), initially using DNA extracted directly from fresh cells. Material from each cell line was then fibrinogen clotted, formalin fixed, and paraffin wax embedded by

Table 1 Detection by comparative genomic hybridisation of chromosome regions gained or lost in the Daudi or K562 cell lines

	Daudi		K562	
	Gain	Loss	Gain	Loss
Total DNA	7	ND	1p, <b>1q</b> , <b>6p</b> , <b>6q</b> , <b>7p</b> , <b>7q</b> , 9q, 11p, 16q, 19, <b>21q</b>	1p, 3q, 5q, 8, <b>9p</b> , <b>10q</b> , <b>13q</b> , 14q, <b>18q</b>
DOP-PCR	7	ND	<b>1q</b> , <b>2p</b> , <b>6p</b> , <b>6q</b> , <b>7p</b> , <b>7q</b> , <b>21q</b> , <i>Xp</i> , <i>Xq</i>	<b>8</b> , <b>9p</b> , <b>10q</b> , <b>13q</b> , <i>15q</i> , <b>18q</b>

Analysis was carried out on either total DNA extracted from cultured cells or on microdissected paraffin wax embedded material amplified by DOP-PCR. Abnormalities of those chromosome regions shown in bold were detected in both DNAs. Those shown in italics were detected only in DOP-PCR products and those shown in roman only in total DNA. DOP-PCR, degenerate oligonucleotide primed polymerase chain reaction; ND, not detected.

standard methods. We performed DOP-PCR on material microdissected from tissue sections for each cell line and the resulting PCR products were used for CGH. We compared the CGH results from the fresh and fixed microdissected cells to establish the veracity of the DOP-PCR amplification. When microdissected material from clinical samples was amplified, some PCR products included a large proportion of fragments that were less than 300 bp long after the labelling reaction. In such cases, material greater than 300 bp in length was cut out from the agarose gel and recovered by microfugation through glass wool for use as a probe.

### Results

We successfully applied a number of different PCR based analyses to small pieces of paraffin wax embedded material that had been stained previously by immunohistochemical methods. It proved possible to detect loss of heterozygosity at the NM23-H1 locus using a microsatellite repeat polymorphism<sup>5</sup> (fig 1C), and to sequence specific exons of the p53 gene accurately (fig 1D), thus providing genetic information to complement the results obtained by immunohistochemical staining for a specific protein. These methods were equally effective on material obtained from sections stained with haematoxylin and eosin.

Microdissected material from both cell lines and clinical material was also amplifiable by DOP-PCR, yielding products that could be used successfully for CGH (fig 1E and F). A comparison of the results of the CGH experiments carried out on cell lines with and without universal amplification produced concordant results (table 1).

### Discussion

Effective microdissection was carried out without the need for complex and expensive equipment and it would therefore be possible to use this approach in any laboratory, although full competence would require practice by the operator. The microdissected material was subsequently processed for PCR analysis by fast, straightforward methods and could be applied to a number of different reactions. All three PCRs worked well on tissue sections that had been subjected previously to immunohistochemistry, including use of the anti-p53 antibody, which required an antigen retrieval step. For successful microdissection, tissue adherence to the slide was very important, as was the maintenance of tissue architecture. For these reasons, the antigen retrieval step was monitored carefully because the standard methods, which involve temperatures up to boiling point, often resulted in the reduction of tissue structure and adhesion.

The use of the PCR buffer described by Kogan *et al* probably facilitates the PCR of material extracted by microdissection, because a range of other buffers failed to provide satisfactory results.<sup>6</sup>

A sufficient amount of probe for labelling by nick translation was produced for each of the 18 tumours analysed. This high rate of success obtained for DOP-PCR of paraffin wax

embedded, microdissected material was considerably greater than the 50% reported previously,<sup>7</sup> and in each case the amplified DNA was labelled successfully by nick translation. In those cases with a high proportion of short fragments, the removal of material shorter than 300 bp greatly improved hybridisation. When a comparison was made between CGH carried out on directly extracted cell line DNA and on DOP-PCR products from paraffin wax embedded material, the Daudi cell line revealed identical copy number changes. However, differences were seen in K562, which were consistent with an analysis carried out on a cell line with an oligoclonal karyotype and which included a variable number of marker and double minute chromosomes.<sup>9</sup> Microdissection will sample only a limited number of cells, whereas the results of CGH carried out on total DNA will be derived from all amplifications or deletions present in each clone throughout the sample.<sup>8</sup> Therefore, CGH results acquired from small amounts of microdissected material should be interpreted with considerable caution, because it cannot be assumed that these will be representative of the whole section. Indeed, the sampling of multiple areas from within a specimen will enable new questions to be asked about tumour heterogeneity. DOP-PCR products from paraffin wax embedded material are currently being used in a series of CGH experiments that explore genetic change during metastatic progression.

The ability to examine those genetic alterations detectable by PCR or CGH in the context of specific gene expression or inactivity, as demonstrated by the simultaneous application of immunohistochemistry, will be a powerful tool for investigating gene function within subpopulations of cells. Immunohistochemistry using specific tumour markers may also be used to identify neoplastic cells in microdissected tumour samples, thus allowing a more accurate picture of genetic alteration in malignant tissue.

We acknowledge support from Yorkshire Cancer Research.

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*Mol Path* 1999 52: 158-160

doi: 10.1136/mp.52.3.158

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