achieved by a few days incubation at room temperature (5) or 2–4 h incubation at 42°C in the cell/PEG/Li-mixture.

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Microdissection by Exclusion and DNA Extraction for Multiple PCR Analyses from Archival Tissue Sections

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The study of genetic alterations is a crucial facet of human cancer research. However, the molecular analysis of genetic changes has been limited by the diversity of cell types present in specimens. The genomic DNA present in non-neoplastic cells may complicate the results of molecular analysis depending on the ratio of neoplastic to non-neoplastic genomic DNA and the sensitivity of the assays.

With the development of PCR technology that can amplify small quantities of DNA, several kinds of microdissection-based methods have been established. One category uses ablation with UV radiation to destroy or remove the unwanted regions, with the remaining target tissue then collected mechanically (7). The possibility of damage to the target DNA is the main disadvantage of this method. A second category uses a hand-held tool to separate cells of interest from the histologic section. The tool can be a pipet (11), micropipet tip (10), needle (4), blade (5) or adhesive gum (8) that is either hand-held or connected to a micromanipulator arm (6). These techniques are not sufficiently precise to allow microdissection of diffuse tumors because internal stromal

cells are generally not discarded, and so the ratio of tumor cells to normal cells is not optimized. A third category uses a laser-based technique called laser capture microdissection (3) or UV laser microbeam (1), but this sophisticated method is quite costly and not accessible to all laboratories.

We present here a protocol for microdissection of archival paraffin-embedded tissue using a manual approach based on the exclusion of unwanted normal cells and followed by DNA extraction. This procedure allows 8–10 reactions to be routinely performed on lesions as small as 1 mm in diameter, even in cases of diffuse tumor infiltration. Its application will be demonstrated in the study of loss of heterozygosity (LOH) on chromosome 18q.

Sections of formalin-fixed, paraffin-embedded tissues were cut at 7 µm, collected on clean untreated glass slides, deparaffinized twice with xylene and rinsed twice with methanol. The first section was stained with hematoxylin and eosin and used for

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histological control. Other sections were stained with 0.01% toluidin blue for 30 s, washed, air dried and either directly microdissected or stored in a closed box at room temperature.

A pathologist selected areas of specific histological interest on the stained section. To recognize the selected area, the slide was inverted and the area of interest circled with a felt pen. Microdissection was performed using wide-field microscopy (such as an inverted microscope for cell culture) with a surgical scalpel blade; it consisted of the removal of unwanted (nontumorous) cells by gentle scraping. When the area of interest was entirely microdissected, the slide was washed with distilled water under light pressure until unwanted cells were completely eliminated. Where necessary, scraping and washing were repeated until only the selected cells remained. A glass capillary was saturated with TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] to slightly moisten the selected cells that were detached from the slide by gentle scraping.

The tissue fragments adhering to the tip of the glass capillary were transferred to a microcentrifuge tube. To minimize loss of material, the capillary tip was broken off and left in the tube during the DNA extraction. The cells were immediately resuspended in 27 μ L TE buffer, 3 μ L digestion buffer [500 mM KCl, 150 mM Tris-HCl (pH 8.0), 15 mM MgCl₂ and 1.5% Tween® 20] and 0.5 mg/mL of proteinase K and then incubated for at least 24 h at 55°C. An equal volume of a 10% Chelex® 100 resin solution (Bio-Rad Laboratories, Hercules, CA, USA) was added; the mixture was incubated 1 h at 55°C and then boiled for 10 min. After quick centrifugation, the supernatant containing the DNA was extracted with phenol, followed by phenol/chloroform, and co-precipitated in ethanol with 10 μ g of glycogen, either at -20°C overnight or more rapidly for 1 h at -20°C followed by 1 h at -80°C. The extracted DNA was resuspended in 20 μ L 10 mM Tris-HCl (pH 8.0) solution and kept at -20°C until used.

Two microsatellite markers from chromosome 18q, D18S69 and D18S58, were chosen for LOH analysis (9). PCR amplifications were carried out for 40

cycles with 2 μ L extracted DNA in a total volume of 20 μ L. An aliquot of 5 μ L of each PCR product was mixed with 2 μ L formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.025% xylene cyanol), denatured for 10 min at 90°C and loaded on a 6% denaturing polyacrylamide gel containing 8 M urea. After electrophoresis in 0.5 $\times$ TBE buffer (45 mM Tris-borate and 1 mM EDTA) at 500 V for 90 min, gels were stained for 20 min in the dark with a SYBR®

Gold gel stain (Molecular Probes, Eugene, OR, USA) diluted 1:10 000 in 1 $\times$ TBE buffer and visualized with UV light using a charge-coupled device (CCD) camera (2).

To optimize the PCR amplifications, we adapted the DNA extraction to the microdissected material. The amplification of material from simple scraping resulted in approximately 40% of samples and allowed only one reaction on each microdissected area. Thus, with this easy and rapid method, only one

Figure 1. Colorectal carcinoma from paraffin-embedded tumor sections. (A) Hematoxylin and eosin stained section corresponding to the control (original magnification, 20 $\times$). (B) Consecutive section stained with toluidin blue after scraping. Arrows show some of the scraped unwanted tissues. (C) Same section as B, after washing of both scraped material and toluidin blue present on the slide. (D) Magnification (40 $\times$) of framed part from C.

Figure 2. Examples of no loss (NL), loss of heterozygosity (LOH) and microsatellite instability (MI) on chromosome 18q in paired normal (N) and colorectal tumor (T, Ta and Tb) tissues. In case 2, a diffuse tumor sample, LOH detection was performed after either direct microdissection (Ta) or microdissection by exclusion (Tb). Arrows indicate regions of either allelic loss or microsatellite instability.

genetic alteration can be studied for each area of interest on a given slide. When a digestion step with proteinase K was performed, about 60% of DNA samples were successfully amplified. The use of Chelex (a product known to be a chelator of metals that are potential inhibitors of *Taq* DNA polymerase) after the proteinase K digestion step allowed virtually all samples to be amplified. However, PCR amplification of the extracted DNA was not reproducible after a few rounds of freezing and thawing, probably because of product instability in the digestion and Chelex mixtures. After the Chelex step, phenol extraction and ethanol precipitation were added to circumvent this problem.

Using this complete extraction procedure, DNA amplifications have been successful with DNA extracted from tissue fixed in sublimed formaldehyde, in Bouin's solution and buffered formalin. From paraffin-embedded tissue, the

extracted DNA can be preserved at -20°C for several months and was sufficient to perform 8–10 reactions. Thus, using our DNA extraction protocol, we were able to study several genes in the same area on the same slide and to apply different techniques for gene analysis such as single-strand conformational polymorphism or LOH analysis.

The microdissection by exclusion technique that we developed allows the isolation of tumor areas as small as 1 mm² surrounded by stromal cells and muscular cells. The case shown in Figure 1 was chosen specifically because the tumor infiltrates the stroma and makes sampling of the tumor difficult by currently described microdissection approaches. Figure 1 illustrates two of the major advantages of the microdissection by exclusion. First, during the scraping procedure, the tumor cells remain adherent, whereas the unwanted tissues, represented by black points,

were detached from the slide (Figure 1B). The scraping was performed in 10–20 minutes. The second and more important advantage of this method is that it allows the microdissection procedure to be controlled. Indeed, after washing scraped sections (Figure 1C), independent monitoring by a pathologist of the complete removal of normal tissue is possible before collection of tumor tissue, and continued removal of normal tissue remains possible. Depending on the size of the area selected and the histology of the tumor, we succeeded in collecting between 95% and 100% of tumor tissue. We recommend that surrounding tissue be generously discarded to guarantee a larger working area for cell collection and thus reduce contamination.

We have tested our protocol of microdissection by exclusion in several different tissues (colorectal, esophagus, endometrium and lymph node) and ap-

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plied LOH analysis where the presence of normal DNA is a limiting factor for any interpretation of results. Results obtained following analysis of allelic loss of chromosome 18q in three cases of colorectal carcinoma are shown in Figure 2. An analysis of the first two cases was achieved with the microsatellite marker D18S69 and in the third case with D18S58. In comparison with the pattern of the normal sample, the first case did not exhibit LOH, the second case did demonstrate LOH and the third case showed microsatellite instability. To highlight the improvement achieved with microdissection by exclusion and its application to genetic alteration studies, a diffuse lesion (Figure 1) was analyzed by standard microdissection (4) and by microdissection by exclusion (Figure 2; case 2). The LOH was not detectable by the standard method (Figure 2; Ta) because of the presence of non-neoplastic cells conferring a normal pattern to the sample, where use of the exclusion method rendered the LOH obvious (Figure 2; Tb).

In conclusion, the experiments described here highlight the advantages of microdissection by exclusion, followed by an adapted DNA extraction for many fixed archival tissues. Optimization of all the DNA extraction and purification steps allowed us to perform multiple reactions on the same microdissected sample. This permits PCR-based studies of clonal diversity of various carcinomas even when diffuse. The microdissection by exclusion method described here appears to be more precise than other current approaches, especially for diffuse lesions. Indeed, contamination by normal cells can be completely avoided and controlled, repeated scraping is possible and photography before sampling can be performed to document the tumor cells studied. The principle of the microdissection by exclusion is similar to UV laser microdissection (1); however, our methodology avoids the use of a sophisticated and costly apparatus.

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