SHORT REPORT

Direct polymerase chain reaction amplification of formalin-fixed, paraffin-wax-embedded tissue after automated sequential laser microdissection and pressure catapulting

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A robust method to facilitate rapid laser microdissection and pressure catapulting (LMPC) coupled with direct polymerase chain reaction (dPCR) to eliminate the need for extraction of DNA before a PCR-based assay is described. This sequential LMPC–dPCR method is rapid and decreases the number of processing steps, reducing the chance of tissue loss and contamination.

Genomics and proteomics techniques have become increasingly sophisticated; however, the accuracy of results depends on the purity of the study sample. Tissues are heterogeneous in their composition, and microdissection techniques have evolved over the years from crude manual dissection using a scalpel blade to the use of micromanipulators and laser microdissection.¹ Laser microdissection and pressure catapulting (LMPC) does not require any contact to isolate the cells. A low-power laser is used to create a gap between the cells of interest and the surrounding tissue; subsequently, a higher-power laser pulse is used to catapult the microdissected cells into the cap of a microcentrifuge tube.² LMPC can be readily used to precisely isolate small numbers of cells from formalin-fixed, paraffin-wax-embedded (FFPE) tissue sections. However, there have been technical challenges in the successful use of these samples for downstream molecular assays. We aimed to develop a robust method to facilitate rapid LMPC coupled with direct polymerase chain reaction (dPCR),³ to eliminate the need for extraction of DNA before a PCR-based assay. This would decrease the number of handling steps, reducing the chances of tissue loss and contamination.

MATERIALS AND METHODS

Preparation of tissue slides

Polyethylene naphthalate (PEN) membrane-covered glass slides (PALM Membrane Slides, PALM Microlaser Technologies, Bernried, Germany) were exposed to ultraviolet light in a PCR cabinet for 15 min before use to cross link any contaminating DNA sources. Treatment of PEN membrane with ultraviolet light also ensured adherence of the tissue to the slide throughout the subsequent dewaxing, rehydration and staining steps. Using a microtome, 4 µm tissue sections of FFPE breast carcinoma were floated on to a warm distilled water bath and lifted on to the membrane side of the slides. After drying at 37°C in an incubator overnight, the slides were dewaxed in prewarmed (37°C) Histoclear II (National Diagnostics, Hull, UK) for 10 min, followed by two changes of Histoclear II (at room temperature) for 10 s each. The slides were passed through three changes of 100% ethanol (10 s each) and rinsed in running tap water for 1 min to rehydrate the slides. They were then incubated in freshly filtered Harris haematoxylin

(VWR International, Poole, UK) for 5 min and rinsed in running tap water for 30 s. The slides were dipped 10 times in acid alcohol to remove any excess stain, rinsed in running tap water for 30 s and then differentiated for 1 min in Scott's tap water substitute. The slides were subsequently rinsed in running tap water for 10 s, passed through three changes of distilled water (10 s each) and air dried for a minimum of 15 min.

Preparation of PCR tubes

All PCR tubes were treated with ultraviolet light in a PCR cabinet for 5 min before use. The PCR master mix was made up of 10 µl of Multiplex PCR Reagent (number 206143 Qiagen, Crawley, UK), 8.7 µl of DNase free water and 1.3 µl of forward/reverse primer mix for one fluorescent microsatellite marker (Linkage Mapping Set, Applied Biosystems, Warrington, UK; table 1). A total of 20 µl of master mix was added directly to the cap of each 0.2 ml thin-walled flat-capped PCR tube.

Sequential laser microdissection and pressure catapulting (sLMPC)

The slide was examined at 5× magnification using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss International, Germany) to identify the precise area to be microdissected. The selected area was focused at 40× magnification, and using PalmRobo software from Palm Microlaser System Microlase (PALM Microlaser Technologies), the areas of the section to be catapulted were outlined either by freehand or by using the automated shape-drawing tool. The optimal area of tumour to be microdissected for microsatellite marker amplicons up to 160 bp was 50 000 µm² (approximately 100 tumour cells), but for amplicons above this size, it was sometimes necessary to use up to twice this amount of tissue. The 0.2 ml PCR tube containing the master mix in the cap was loaded on to a holder with the cap facing downwards. The holder was fitted into a swinging arm attached to the microscope and the cap was swung automatically into position over the area to be microdissected (1 mm above the slide). Fine-tuning of the position was achieved with the use of a joystick. The laser settings used were as follows: focus of laser, 38; energy, 70; speed of laser catapulting, 65–70. The selected tissue area and the underlying PEN membrane backbone were demarcated using the laser in the “cut” mode, which allows the tissue to be left in situ, attached to the rest of the section by a narrow stalk. This prevents the tissue lifting from the slide prematurely. A “blast point” was then applied to the stalk, catapulting the tissue into the cap containing the PCR master mix.

Abbreviations: dPCR, direct polymerase chain reaction; FFPE, formalin-fixed, paraffin-wax-embedded; LMPC, laser microdissection and pressure catapulting; PCR, polymerase chain reaction; PEN, polyethylene naphthalate; sLMPC, sequential laser microdissection and pressure catapulting

Table 1 Details of microsatellite markers used in the dPCR assay

Microsatellite	Chromosome location	Size range (bp)
D17S927	17q12	113–129
D17S1795	17q21.31	136–146
D17S1862	17q24.1–24.2	202–232
D17S836	17q25.2	204–214
D17S831	17p13.3	107–129
D16S3091	16q23.3	166–182
D16S505	16q23.2	137–159
D16S520	16q24.1	149–165
D8S552	8p22	110–124
D8S264	8p23.2	136–160

The “cut” function seemed to be efficient when processing multiple tissue areas. A series of areas could be delineated on the computer in advance and the “cut” function would automatically sequentially cut each area, leaving the tissue stalks intact. Once all the areas had been cut, the tissue samples were individually catapulted and collected in a succession of PCR tube caps.

dPCR amplification

The PCR tube (containing the master mix and catapulted cells) was immediately closed and pulsed briefly in a bench top centrifuge to bring the contents to the bottom of the tube. A fluorescent PCR-based microsatellite assay was used to assess the quality of the PCR amplifications.⁴ A dPCR technique, as described previously,³ was used as this removed any further manipulations that would be required by a standard DNA extraction procedure. When all samples were collected, the tubes were placed in an automated thermal cycler (Model 2400, Perkin Elmer, California, USA). The two-step PCR programme heated the samples initially to 95°C for 15 min, followed by 45 cycles of 95°C for 30 s and 55°C for 1 min. On completion, the tubes were held at 4°C.

Capillary electrophoresis

A volume of 1 µl of PCR product was added to a 0.5 ml microfuge tube (Applied Biosystems) containing 12 µl of formamide (Applied Biosystems) and 0.5 µl Rox 350 size standard (Applied Biosystems). The samples were denatured at 95°C for 5 min in a thermal cycler and the tubes were plunged into ice for 2–3 min before loading into the sample tray of a Prism 310 Genetic Analyser (Applied Biosystems). Electrophoresis was performed by using Performance Optimised Polymer-4 (Applied Biosystems) and fragment analysis was achieved with GeneScan software (Applied Biosystems).

RESULTS AND DISCUSSION

A total of 150 sLMPC–dPCR assays were attempted with the final protocol and 136/150 (90%) samples produced acceptable levels of amplification (allele peak height of at least 1000 units) with the first attempt. Use of a larger area of tissue in subsequent assays, especially where amplicons >160 bp were expected, generated sufficient amplification levels in most of the remaining cases. The assessment of corresponding FFPE normal tissue from each patient confirmed that the allele sizes generated were of the correct sizes for each microsatellite. The FFPE tissue was obtained from standard blocks used for histopathological analysis dating from 1995 to 2004.

The technique of sLMPC–dPCR has several advantages. We found the technique to be robust and performed consistently, with amplicons ranging from 107 to 232 bp.

The sLMPC process is rapid and fully automated. The cutting process worked smoothly despite minor variations in thickness

Take-home messages

- The method described facilitates laser microdissection of archival FFPE tissue.
- It is coupled with a direct PCR (dPCR) method to eliminate the need for extraction of DNA prior to a PCR-based assay.
- The sequential laser microdissection and pressure catapulting LMPC (sLMPC)–dPCR method is rapid and decreases the number of processing steps, reducing the chance of tissue loss and contamination.

of tissue on the slide. Visual confirmation of successful catapulting was achieved by looking into the cap for blue-coloured specks.

Here, the use of a microsatellite-based assay, with accurate allele sizing and normal control tissue for comparison of allele sizes, would readily identify possible cross contamination. The appearance of additional alleles or incorrectly sized alleles would be indicative of this. The use of appropriate negative controls should be considered if a less robust PCR assay is to be used.

The method of sLMPC–dPCR described here allows the consistent, rapid production of several PCR amplicons from a 4 µm FFPE tissue section. After initially pipetting the PCR mastermix into the cap of the sample tube, there is no further pipetting of the tube contents, thus ensuring no loss of sample and minimising the risk of contamination. The ability to pre-select all relevant tissue areas for microdissection before beginning collection with sLMPC results in a greater number of samples being collected in a shorter period of time. Although we have used the technique to microdissect breast tumour sections, the technique could be applied to other PCR-based applications and may be amenable to multiplex PCR assays where tissue samples are limited. With modification, the technique could also be adapted for microarray-based comparative genomic hybridisation or for RNA and protein analyses.

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