

## Forensic Applications of Laser Capture Microdissection: Use in DNA-Based Parentage Testing and Platform Validation

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<b>Aim</b>	To report on the successful use of Laser Capture Microdissection (LCM) as a tool for isolation of human chorionic villi from admixed maternal tissue. Subsequent DNA isolation for forensic short tandem repeat (STR) analysis for parentage testing was performed in two cases of alleged sexual assault of female victims. We also performed validation of the LCM instrument platform, using archival formalin-fixed human fetal products of conception (POC), for which microdissection was utilized to separate maternal (decidua) and fetal (chorionic villus) components.
<b>Methods</b>	To isolate DNA from placental chorionic villi admixed with maternal decidua recovered after spontaneous or therapeutic abortion, LCM was used to separate fetal from maternal cells. In contrast to the relatively crude conventional microdissection performed using a narrow pipette, needle, or scalpel blade, LCM allows cell- or tissue-specific isolation of placental chorionic villi from archival paraffin-embedded tissue sections, leaving the maternal tissue intact.
<b>Results</b>	After polymerase chain reaction (PCR) amplification of villi after LCM of 9-15 STR loci, the quantity and quality of DNA yielded from fetal cells isolated by LCM was sufficient for PCR analysis and successful forensic parentage testing. The validation data obtained on two sets of formalin-fixed archival POC tissues from anonymous donors demonstrated the encouraging reproducibility of these protocols and procedures.
<b>Conclusion</b>	We demonstrated the reliability and utility of LCM for forensic applications when high specificity of a particular analyzed cell population or tissue is required. Care must be taken during routine pathology procedures to avoid contamination of tissues with admixture of extraneous DNA.

In the event of failed or aborted pregnancy after sexual assault, DNA-based forensic paternity testing is necessary to identify the biological father of the embryo or fetus. This is accomplished using the fetal remains or abortus material

as the source of fetal DNA (1). In addition to sexual assault investigations, DNA-based paternity testing is commonly performed after suspicious death or known homicide of pregnant women to provide information useful to identification of possible suspects (2). If recognizable fetal parts cannot be confidently identified for such parentage testing, microscopic examination of formalin fixed, paraf-

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fin-embedded tissue derived from postmortem tissues (ie, after spontaneous or therapeutic abortion) or by genetic amniocentesis are the only ways to reliably distinguish maternal (decidual) vs fetal (chorionic villi) components of the recovered products of conception (POC). DNA isolation and polymerase chain reaction (PCR) amplification of fetal tissue can then be performed, using standard forensic laboratory procedures (3-5).

Laser capture microdissection (LCM) refers to the marriage of existing light microscopic instrumentation and newer technology utilizing pulsed laser beams. Pulsed laser allows targeting of specific regions of tissues to be separated and placed into snap-cap tubes for DNA extraction and analysis. Thus, LCM can be used to separate specific cell types within whole organs or tissue sections without altering sample chemistry or morphology. Separations of cells and tissues are necessary in a wide array of research investigations and LCM has rapidly been incorporated into the daily work of research pathologists (6).

The use of LCM in forensics is not yet widely appreciated and promises to answer the heretofore unmet need for a technology for efficient separation of cells or tissues in forensic mixtures. We report on the use of LCM as a precise tool for the isolation of placental chorionic villi from aborted material and subsequent genetic analysis for paternal determination in two cases of sexual assault. We also present the evaluation of the same LCM instrument platform, based on archival formalin-fixed POC samples, where microdissection was used to separate maternal (decidua) and fetal (chorionic villus) components.

## Materials and Methods

### *Sample Preparation and LCM in Forensic Case Reports*

Recovery of POC material after two alleged sexual assaults was followed by formalin fixation of POC tissue, followed by embedding in paraffin. Multiple 5  $\mu$ m unstained tissue sections were mounted on glass slides. To specifically extract the fetal chorionic villi from the maternal decidual tissue, the PixCell II (Arcturus, Carlsbad, CA, USA) LCM system was applied according to the manufacturer's specifications, as previously described (7-9). In brief, the tissue sample mounted on a glass slide was first studied under a light microscope. Once the cells of interest (i.e.,

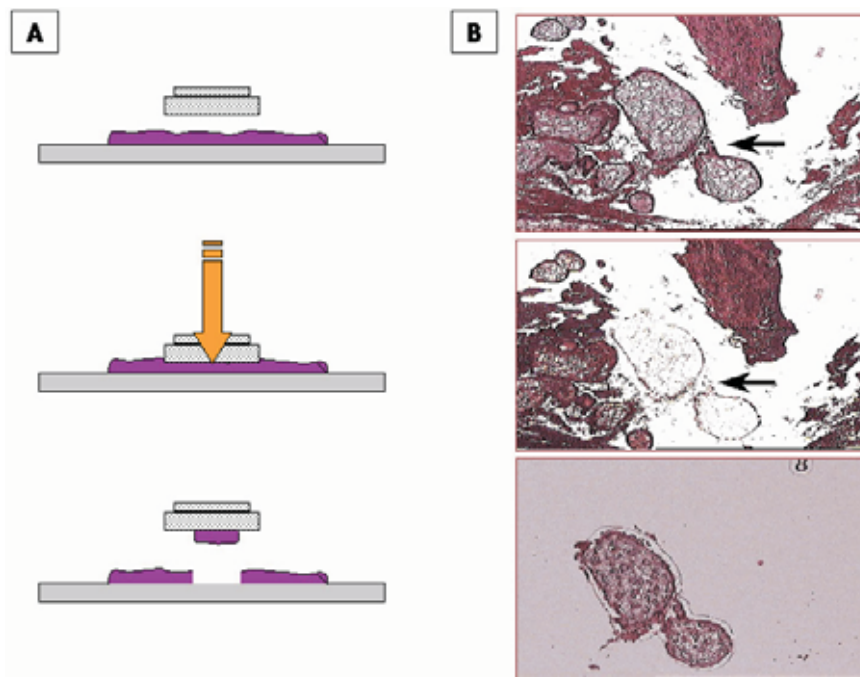
placental chorionic villi) were identified, a LCM cap was placed over the target area and focused infrared diode laser beam was activated. Pulsing of the laser (90 mW of pulse power, 15  $\mu$ m laser spot size) through the cap has caused the thermoplastic film to form a thin protrusion that bridged the gap between the cap and the placental tissue and adhered to the cells (Fig. 1A). Now attached to the cap, the targeted chorionic villi were simply removed from the rest of the placental tissue by lifting of the cap (Fig. 1B).

### *DNA Extraction and PCR*

DNA was extracted from the isolated chorionic material by using standard nucleic acid extraction procedures (5,8). For one of the cases, caps were swabbed with sterile swabs, which were submerged in 5% Chelex (total volume 200  $\mu$ l) and incubated at 56°C for 30 minutes. Samples were then vortexed and boiled for 8 minutes. Volume was reduced by Microcon to 11  $\mu$ l, of which 1  $\mu$ l was used for slot blot (c/w 0.1 ng/ $\mu$ l DNA). PCR-based short tandem repeat (STR) profiling was performed (Orchid Cellmark, Gaithersburg, MD, USA) using the commercial kit Profiler Plus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's specifications. Of the DNA extracted from the chorionic villi, 10  $\mu$ l was used in a reaction volume of 25  $\mu$ l (8). For the second case samples were processed as described below except that Cofiler and Profiler Plus (Applied Biosystems) were employed for the amplification.

### *LCM Platform Validation*

For the validation of the instrument platform, the slides of 10 formalin-fixed archival POC tissues from anonymous donors were obtained from the Department of Pathology, Bellevue Hospital Center, NY. To separate the chorionic villi from the placenta, each tissue type was micro-captured using the PixCell II LCM system (Arcturus), as described above. In these validation experiments, two caps per slide were used to separate recognized decidual (maternal) from chorionic (fetal) tissue. Caps were designated as "A" and "B," respectively. After microdissection, the thermoplastic film was removed from the plastic cap and underwent standard organic DNA extraction using phenol:chloroform:isoamyl alcohol (25:24:1) procedure. The estimation of the quantity of extracted DNA was performed using QuantiBlot analysis (Applied Biosystems), and amplification was per-



**Figure 1.** A) Schematic presentation of a laser capture microscopy (LCM). A tissue section is mounted on a glass slide and LCM cap with a transfer film is positioned above the region of interest (upper scheme). Laser pulse (orange arrow) focally activates transfer film and the cells in the activated area adhere to the cap (middle scheme). Removing of the cap detaches cells of interest leaving the rest of the tissue section intact on a glass slide (bottom scheme). B) Histology of laser captured material. Section of fetal material prior to the LCM procedure – placental chorionic villi visible (arrow) in the center of the field (upper figure). The same section following LCM – empty space observed (arrow) after removal of the chorionic villi (middle figure). Dissected chorionic villi on a LCM cap (bottom figure).

formed using PowerPlex 16 (Promega, Madison, WI, USA), following manufacturer's protocols, validated in the New York City Office of Chief Medical Examiner (NYC OCME) Department of Forensic Biology. The amplified PCR products were analyzed using capillary electrophoresis, specifically the ABI prism 3100 Genetic Analyzer (Applied Biosystems), with adopted and validated protocols. In a follow up experiment, 10 additional formalin fixed archival specimens were extracted.

## Results

### Case 1

An adolescent victim became pregnant after a sexual assault. She named the alleged perpetrator, a man with whom she was acquainted. The alleged assailant was questioned by the law enforcement officers and denied the sexual assault. A therapeutic termination of pregnancy was performed by routine dilatation and evacuation abortion in the late first trimester. Abortus material

was recovered and embedded in paraffin for routine pathology study at Brigham and Women's Hospital. Histologic sections of the paraffinized tissue block were made for LCM (Fig. 1) and for DNA extraction. A nine locus STR DNA profile was obtained (Orchid Cellmark, Gaithersburg, MD, USA) from the laser capture microdissected chorionic villi, which included the STR alleles of the alleged victim and that of the suspect, consistent with the purported paternity of the named assailant.

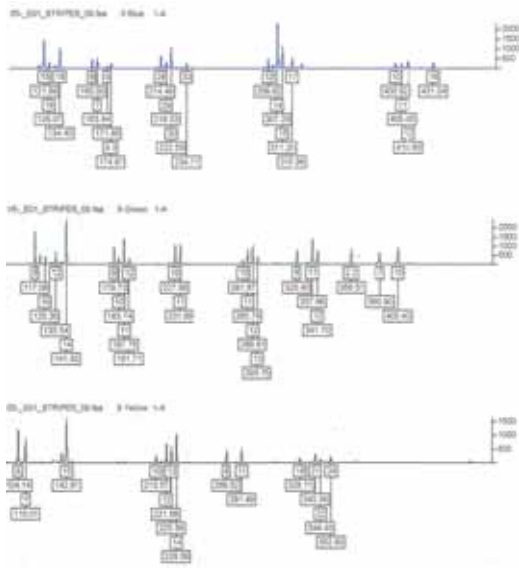
### Case 2

A 13-year-old girl was pregnant after an alleged sexual assault. She named the alleged perpetrator (stepfather), who denied sexual contact. A therapeutic termination of pregnancy was performed in the late first trimester. Multiple fragments of soft POC tissue were embedded in paraffin. One hematoxylin and eosin stained slide was submitted to the New York City Office of the Chief Medical Examiner for DNA analysis. After LCM

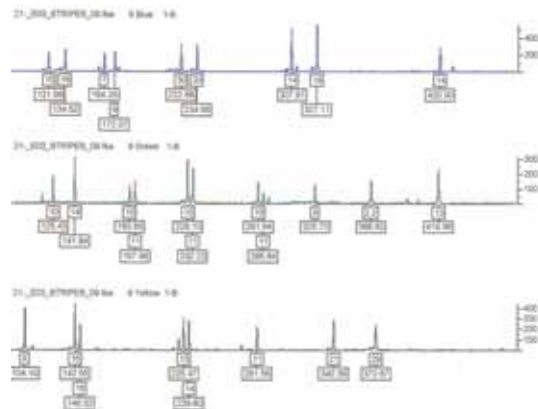
separation of fetal from maternal cells, DNA extraction and STR analysis was performed. The resulting 13-locus STR profile was fully consistent with paternity of the alleged perpetrator. The probability for paternity was calculated to be 99.99% (prior odds = 50%).

### Reproducibility Validation

**PART I: Routine cutting/staining of the products of conception.** Slides from 10 anonymized archival pathology cases were randomly selected for subsequent microdissection of chorionic villi from admixed maternal decidual tissue. Out of 10 pairs of the extracted cellular elements, 4 yielded conclusive DNA results. In each of these paired cases, sharing at least one allele per locus was found by comparison of DNA profiles of maternal (B) tissue with fetal (A) tissue (Fig. 2 and 3). Four cases were inconclusive due to insufficient quantity of DNA, whereas 2 cases could not be used because additional extraneous contaminating DNA was detected, which could not be



**Figure 2.** Electropherograms showing PCR-based DNA profile of POC after routine pathology histopathology cutting/staining procedures. Presence of more than two peaks at multiple loci indicates a DNA mixture derived from two or more contributors. PowerPlex 16 loci presented: D3S1358, TH01, D21S11, D18S51, Penta E; D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D; Amelogenin, vWA, D8S1179, TPOX, FGA. 2-digit numbers below horizontal line indicate the specific STR alleles detected at each locus. The 3-digit numbers (with decimals) indicate the size (in DNA base pairs) of the amplified PCR products. At least one allele at each locus is shared with the profile from microdissected maternal decidua (see Fig. 3).



**Figure 3.** Electropherograms showing PCR-based multi-locus STR DNA profile of decidual tissue after routine cutting/staining procedure of products of conception – PowerPlex 16 loci presented: D3S1358, TH01, D21S11, D18S51, Penta E; D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D; Amelogenin, vWA, D8S1179, TPOX, FGA. The 2-digit numbers below horizontal line indicate the specific STR alleles detected at each locus. The 3-digit numbers (with decimals) indicate the size (in DNA base pairs) of the amplified PCR products (Note shared alleles with the DNA profile in Figure 2).

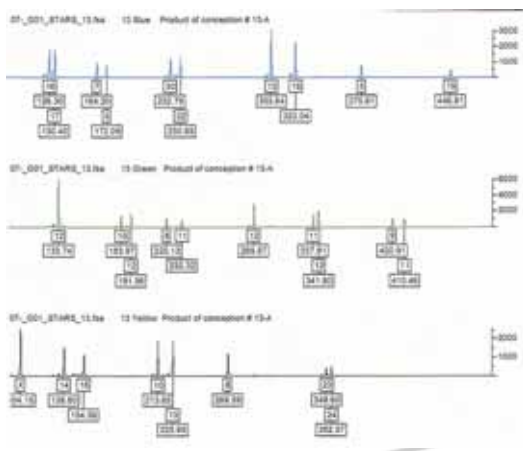
accounted for in either the maternal or fetal sample.

In some cases, DNA mixtures of maternal and fetal profiles were found either on cap “A” and/or cap “B”. Since both tissue types were present in these POC pathology specimens, this was not unexpected, as intermediate trophoblast cells of fetal origin normally infiltrate maternal decidua – thus the presence of fetal cells admixed with maternal decidua is an expected finding. In addition, dislocated cells from extraneous sources could become lodged in the admixed tissue during routine fixation and embedding procedures. Therefore, in all cases showing DNA profiles indicating multiple sources the LCM and DNA extractions were repeated.

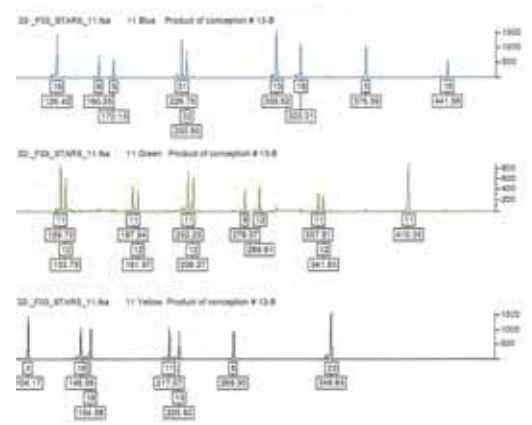
In addition to DNA profiles showing admixture of maternal and fetal tissue, DNA mixtures containing foreign DNA of unknown origin were also occasionally observed. Such contamination by extraneous DNA was possible during slide preparation (i.e., microtome cutting), and is well recognized in histology laboratory practice (10). A follow up experiment was designed to improve results by decreasing the contamination with extraneous DNA.

*PART II: Modifications of the cutting/staining procedures of the products of conception.* Another set of 10 pathology cases was randomly selected to repeat the microdissection step. In this second series, disposable blades were used for the microtome and were discarded after each use. In addition, the working surface of the microtome blade was wiped with 10% bleach and alcohol before and after each cutting and individual floating chambers were used for each case. During the staining procedure, separate sets of jars were used for each cutting such that each individual cutting required 19 individual glass dishes. These modifications were successful in that each of the 10 cases in the second series yielded results which would be suitable for forensic DNA-based paternity analysis (Figs. 4 and 5). These anti-contamination measures applied in the validation process succeeded in eliminating the presence of foreign DNA. As expected, mixtures between fetal and maternal genetic material were detected, but this did not interfere with the interpretation of DNA profiling results.

The second component of our validation study was performed on a new set of 10 paraffin embedded pathology POC tissues. Among the second set of 10 samples, 4 were among the initial set



**Figure 4.** Electropherograms showing PCR-based Multi-locus STR DNA profile of fetal chorionic villi derived from POC after modified cutting/staining procedure. Strict anti-contamination measures applied in the validation process eliminated the presence of foreign DNA. PowerPlex 16 loci presented: D3S1358, TH01, D21S11, D18S51, Penta E; D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D; Amelogenin, vWA, D8S1179, TPOX, FGA. The 2-digit numbers below horizontal line indicate the specific STR alleles detected at each locus. The 3-digit numbers (with decimals) indicate the size (in DNA base pairs) of the amplified PCR products.



**Figure 5.** Electropherograms showing PCR-based Multi-locus STR DNA profile of maternal decidua derived from POC after modified cutting/staining procedure. Strict anti-contamination measures applied in the validation process eliminated the presence of foreign DNA. PowerPlex 16 loci presented: D3S1358, TH01, D21S11, D18S51, Penta E; D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D; Amelogenin, vWA, D8S1179, TPOX, FGA. The 2-digit numbers below horizontal line indicate the specific STR alleles detected at each locus. The 3-digit numbers (with decimals) indicate the size (in DNA base pairs) of the amplified PCR products.

of 10 and the DNA profiles matched, demonstrating the reproducibility of the method.

## Discussion

Our results are in accord with recently published case reports describing use of microdissection procedures for paternity testing on human abortus material (8,11).

Fetal placental chorionic villi, in direct contact with cells of the maternal uterine wall, have been used for forensic paternity testing from the very beginnings of forensic DNA typing (12). However, early attempts to use chorionic villi for paternity testing were burdened with various technical obstacles. In particular, many early abortions (ie, after sexual assault) were performed by destructive surgical procedures, which result in, at worst, failure to obtain fetal cells, or at best, admixture of fetal and maternal cells (2,4). The interpretation of forensic DNA mixtures, while routine, is often complex, requiring careful analysis and alternative statistical methods (13). This limitation was only partially diminished by the introduction of more advanced techniques into genetic typing such as PCR-based STR-analysis (1) and single locus probes (14). In spite of these improvements,

conventional microdissection of hematoxylin and eosin-stained sections – usually performed by a needle or a blade – was not always successful in diminishing the extent of maternal tissue contamination. Consequently, co-amplification of fetal alleles would often fail due to preferential amplification of the abundant maternal DNA (8). To overcome this obstacle, more advanced microdissection method that would enable exclusive dissection of specific target cells was clearly needed (15).

LCM – a technique originally developed for the isolation of specific tumor cells for cancer research – enables such highly precise targeting and selection of specific cells of interest for subsequent genetic and/or molecular analysis (16,17). In contrast to the conventional microdissection described above, which may collect many non-targeted cells, the heat of the laser beam melts the plastic film, causing it to adhere only to the targeted cells. After adhering to the film, targeted cells are being lifted away, leaving the rest of the tissue section intact (16,17). Applied in these cases, LCM allowed us specific isolation of placental chorionic villi from paraffin-embedded sections, leaving the maternal tissue on a slide (Fig. 1B).

In two alleged sexual assault cases reported in this article, the laser microdissection (LCM) approach was successful in separating the fetal and maternal components from single microscopic slides. In both cases, archival pathology slides material and traditional sampling techniques would likely have resulted in DNA mixtures. However, in both cases the combination of LCM with highly sensitive PCR-based STR-analysis enabled paternity determination from small number of cells precisely isolated from histological sections of placental chorionic villi.

In addition to success with forensic casework, the Arcturus instrument was employed to study the reproducibility of separation and DNA recovery of fetal tissue from 10 archival pathology blocks. The initial experiments using 10 slides cut from such blocks displayed several samples with contaminating extraneous DNA. The routine histology laboratory practices are not ideal for subsequent PCR testing because such procedures include no specific steps to prevent DNA admixture from occurring during the process itself. In contrast to routine procedures employed in pathology departments, a second set of 10 archival pathology

samples was prepared using a revised cutting and staining procedure and all such samples yielded reproducible DNA results. Of course, in criminal casework, it will not always be possible to control this, since archived histology slides might be the only available evidence.

Our results demonstrate the reliability and utility of LCM in forensic DNA-based paternity testing when a specific cell population is required. The possibilities for LCM and related separation methods offer exciting potential for forensic analysis of other types of biological evidence.

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