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STR profiling of epithelial cells identified by X/Y-FISH labelling and laser microdissection using standard and elevated PCR conditions

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ABSTRACT

During the investigation of allegations of sexual assault, samples are frequently encountered that contain DNA from a female and a male donor. These may represent contributions of DNA from the complainant and potentially, the offender. Many semen stained samples successfully undergo DNA analysis and interpretation using a differential extraction method that separates sperm from the epithelial cells present in the stain. However, for those mixed cell samples that contain only epithelial cells, separation of any male cells from female cells is problematic. This paper describes the application of fluorescent in situ hybridisation (FISH) for the gender identification of epithelial cells and subsequent recovery of target cells using laser microdissection (LMD). The profiling results obtained from samples of known cell numbers using the Identifiler™ multiplex at standard 28-cycle PCR conditions and, when cell numbers are low, the SGM Plus™ multiplex at elevated 34-cycle PCR conditions (also known as Low Copy Number DNA analysis (LCN)) are described.

1. Introduction

The examination of samples associated with an allegation of sexual assault frequently involves the analysis of samples that comprise mixtures of cells. Separating cell mixtures prior to undertaking DNA testing simplifies downstream DNA profile interpretation as profiling results are more likely to originate from single contributors. Furthermore, more complete profiles are likely to be obtained from a minor DNA contributor through the removal of the masking effect of shared DNA results with a major DNA contributor. Methods, such as preferential extraction, have focussed on the separation of sperm from epithelial cells based on physical differences in cell structure [1]. However, it may be necessary to separate cells for DNA profiling when sperm are not present in a cell mixture, such as semen stained genital swabs containing azoospermic semen. Laser microdissection (LMD) technology, which involves microlaser ablation to collect target cells from cellular samples deposited on slides, has been utilised by the forensic community over recent years to isolate sperm from cell mixtures [2,3], foetal cells from maternal tissue [4], nucleated cells from hair follicles [5] and to isolate blood cells from cell mixtures [6,7].

A method which distinguishes between morphologically similar cells, such as epithelial cells, of male and female origin, is fluorescent in situ hybridisation (FISH). In order to differentiate cells based on gender, different coloured fluorescent probes to the X and Y sex determining chromosomes are applied to samples of cells, which are usually fixed onto microscope slides. This X/Y-FISH labelling method has a particular application for those forensic samples where cells of one gender are mixed with a large number of cells from the other gender, such as may occur with azoospermic semen mixed with vaginal epithelial cells [8,9], female cells on post coital penile swabs [10] or condoms [11]. Cells of interest can be identified by X/Y-FISH labelling and then separated, by LMD, from other cells in the sample. The recovered cells are then be subjected to DNA profiling analysis.

A viable DNA extraction method has been developed which allows for the release of DNA from recovered cells coupled with denaturation of cellular proteins and endogenous nucleases [12]. This method enables DNA extraction and PCR to be performed in the same tube, providing time benefits and improved sensitivity. It is also hypothesised that a further benefit would be reduction of the stochastic effects in DNA profiling brought about by unequal sampling of alleles from a DNA extract as, in a one-tube test, all of the DNA from the recovered cells is progressed to PCR.

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Production of DNA profiles from X/Y-FISH LMD cells has, so far, to our knowledge, been limited to ultra-sensitive methods of DNA analysis, such as the Low Copy Number (LCN) technique using 34-cycles of the PCR versus the manufacturer's recommended 28 cycles [13]. Given the relatively small number of forensic laboratories employing an ultra-sensitive DNA profiling method, this has likely limited the application of X/Y-FISH in forensic analysis. This research combines the use of a one-tube extraction and amplification method to samples of known numbers of laser microdissected X/Y-FISH labelled cells to obtain DNA profiles using either the Identifiler™ multiplex at standard 28-cycle PCR conditions or the SGM Plus™ multiplex using 34-cycle PCR conditions. We present data of the profiling success rates using these two protocols and the observed variation in heterozygote balance in these profiles. The theorised effect on stochastic variation from sampling prior to PCR was investigated.

2. Materials and methods

2.1. Sample collection

Epithelial cells were collected from consenting male (n = 3) and female (n = 3) participants, with known Identifiler™ DNA profiles, between the ages of approximately 20 and 50 years old. Buccal epithelial cells were self-collected by participants, by rubbing the insides of their cheeks and gums with sterile swabs for 20 s. Swabs were placed back into the swab casings, which were cut, and placed in a laminar flow hood to dry. Once dry, the samples were placed into a paper envelope and stored at room temperature until sample processing commenced.

2.2. Cell recovery and slide preparation

Cells were recovered from swab heads by agitation in 500 μL of Tris extraction buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8.0) and collected by centrifugation at 10,000 rpm for 10 min. Cells were chemically fixed using either 30 μL of Carnoy's fixative (3:1 methanol:acetic acid) or 1:1 methanol:acetone and re-suspended single source cell pellets were placed onto polyethylene terephthalate (PET) membrane slides (Leica Microsystems, Germany). Slides were stored at room temperature in a laminar flow hood to dry completely and left, at least overnight, prior to X/Y-FISH labelling or Christmas Tree staining.

2.3. X/Y-FISH labelling

X/Y-FISH was performed using the CEP® X SpectrumOrange™ Y SpectrumGreen™ DNA Probe Kit (Vysis, Des Palines, IL, USA) following the manufacturer's instructions. The slides were immersed in a denaturing solution (70% Formamide in 2× SSC pH 7.0–8.0) within a Coplin jar in a water bath at 73 °C ± 1 °C for 5 min. The slides were dehydrated in an ethanol series by soaking for 1 min in each of 70%, 85% and 100% ethanol then placed on a 42 °C hot block to dry for 2 min. Ten microlitres of probe solution was added to the sample area on each slide. A cover slip was applied and sealed with rubber solution. Slides were incubated in a humidified chamber overnight at 42 °C. Following hybridisation the coverslips and rubber solution were removed and the slides were washed in 0.4× SCC at 73 °C for 2 min and 2× SCC/0.1% NP-40 at room temperature for 1 min. Slides were air dried in the dark, before 10 μL of DAPI II counterstain and then coverslips were applied.

2.4. Christmas Tree staining

For comparison, additional slides were stained with CTS using reduced times for nuclear fast red and picroindigocarmine staining, so as to minimise any deleterious effect of the chemicals but still providing effective visualisation of cells, as described in Meredith et al. [12].

2.5. Laser microdissection

The slides were examined on a Leica LMD6000 laser microdissector (Leica Microsystems, Wetzlar, Germany) at 25× and 40× lens magnification using appropriate DAPI/green/orange filters for the detection of fluorescent signals. Male cells were confirmed by the presence of one orange and one green signal within the DAPI II stained nucleus, while female cells were defined as having two orange signals within the nucleus.

Samples of X/Y-FISH labelled cells were collected by laser microdissection, with the number of cells in each sample ranging from 2 to 150. These cell sets were collected into the caps of Oxygen 0.2 mL flat top, long hinged, microcentrifuge collection tubes (Raylab, New Zealand) containing an extraction solution, as described below. Following collection of the selected cells, the tubes were centrifuged at 13,000 rpm for 1 min to move samples from the cap into the main body of the tube.

2.6. One-tube extraction and amplification

DNA extraction and amplification was carried out according to the method of Meredith et al. [12]. Epithelial cells were recovered into the caps of tubes containing a solution consisting of Tris extraction buffer, Tween 20 at 0.2%, v/v and 0.1 mg/mL Proteinase K (PK). Different quantities of reagents were used depending on the DNA profiling kit. The two profiling kits selected for use in this study are ones that have been validated for casework analysis in the authors' laboratory, at the cycle numbers described below. Cells intended for amplification with the AmpF*l*STR Identifiler™ multiplex (Applied Biosystems, Life Technologies™, Carlsbad, CA) were extracted in a final volume of 10 μL, and cells amplified by LCN AmpF*l*STR SGM Plus™ (Applied Biosystems, Life Technologies™, Carlsbad, CA) were extracted in a final volume of 20 μL. Samples were incubated in a thermal cycler for 1 h at 56 °C and inactivation of the PK was achieved by heating the sample at 95 °C for 10 min before cooling to 4 °C. Samples were stored at 4 °C prior to amplification of the DNA.

For the Identifiler™ amplification reactions, the whole 10 μL extract was used and the reaction was undertaken in the same tube as DNA extraction. The DNA, in a total volume of 25 μL, was amplified at 28 cycles in a silver block 9700 thermal cycler (Applied Biosystems, Life Technologies™, Carlsbad, CA), according to the manufacturer's instructions. For the LCN SGM Plus™ reactions, half of the 20 μL extract was transferred to a new tube and two replicate amplifications were each carried out in a total volume of 50 μL, in a silver block 9700 thermal cycler, according to the manufacturer's instructions, but at 34 cycles.

A total of 30 samples, comprising six replicates each of 2, 4 10, 20 and 30 cells, were profiled using LCN SGM Plus ™. Seventy three (73) samples were profiled using the Identifiler ™ multiplex. These comprised sets of 15 cells (n = 10), 25 cells (n = 8), 30 cells (n = 10), 40 cells (n = 10), 50 cells (n = 22), 75 cells (n = 8) and 100 cells (n = 3).

2.7. Data analysis

Amplified products were separated on a 3130*xl* Genetic Analyser (Applied Biosystems, Life Technologies™, Carlsbad, CA) and analysis of DNA profiles was undertaken using the GeneMapper™ *ID* version 3.2.1 (Applied Biosystems, Life Technologies™, Carlsbad, CA) software. A peak detection threshold of 50 RFU was applied to all