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Simplification of complex DNA profiles using front end cell separation and probabilistic modeling

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ABSTRACT

Forensic samples comprised of cell populations from multiple contributors often yield DNA profiles that can be extremely challenging to interpret. This frequently results in decreased statistical strength of an individual's association to the mixture and the loss of probative data. The purpose of this study was to test a front-end cell separation workflow on complex mixtures containing as many as five contributors. Our approach involved selectively labelling certain cell populations in dried whole blood mixture samples with fluorescently labeled antibody probe targeting the HLA-A*02 allele, separating the mixture using Fluorescence Activated Cell Sorting (FACS) into two fractions that are enriched in A*02 positive and A*02 negative cells, and then generating DNA profiles for each fraction. We then tested whether antibody labelling and cell sorting effectively reduced the complexity of the original cell mixture by analyzing STR profiles quantitatively using the probabilistic modeling software, TrueAllele® Casework. Results showed that antibody labelling and FACS separation of target populations yielded simplified STR profiles that could be more easily interpreted using conventional procedures. Additionally, TrueAllele® analysis of STR profiles from sorted cell fractions increased statistical strength for the association of most of the original contributors interpreted from the original mixtures.

1. Introduction

One of the biggest challenges with DNA evidence is the presence of cell populations from multiple contributors which can result in decreased statistical strength of STR profile interpretation and, potentially, loss of evidence. Many methods have been developed to separate contributor cell populations prior to DNA profiling including microfluidic manipulations [1], laser capture microdissection [2], and flow cytometry based techniques such as fluorescence activated cell sorting (FACS) [3,4]. However, one limitation of these approaches is that they have largely been demonstrated on mixtures containing only two contributors and/or have been applied to fresh or uncompromised mixture samples. Although probabilistic genotyping systems can perform analyses on mixtures that contain three or more contributors which are superior to human analysis [5,6], limits remain as to the number of contributors that can be successfully disentangled [7]. This is particularly in true for mock casework samples that display stochastic imbalances that impact low level contributors, and create allelic and locus drop-out [8]. Therefore, there is still considerable need for front-end techniques that can reduce the complexity of mixtures with three or more individuals prior to DNA analysis and facilitate the generation of single or near single source STR profiles.

The purpose of this study was to test a workflow for resolving complex biological mixtures that combines front-end cell separation with probabilistic genotyping of the simplified sorted cell fractions. A similar approach has been previously demonstrated with laser capture microdissection as the front end separation approach for enhanced interpretation of buccal cell mixtures containing two contributors in equal ratios [9]. We have built upon this work by processing two-, three-, four- and five-contributor mixtures where only one cell type, blood, is present. Front-end separation was accomplished using antibody probe labelling and Fluorescence Activated Cell Sorting (FACS), a high-throughput, non-destructive cell separation technique previously described for forensic applications [3,4,10,11]. The abundance of antigen targets on white blood cells and average DNA yield make this a useful sample system for investigating this workflow. Additionally, complex blood mixtures may be encountered in forensic casework following homicides with multiple victims, mass disasters, or terrorism incidents.

We employed fluorescently labeled antibody probes targeting the

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A*02 allele of the Human Leukocyte Antigen (HLA) Complex to selectively label individual contributor cell populations in a mixture that were recovered from dried whole blood stains. Cell populations were then physically sorted into two fractions, A*02 positive and A*02 negative (referred to as 'P2' and 'P3', respectively), each of which contained a simplified subset of contributors from the original mixture. The unsorted and sorted fractions were subjected to STR profile analysis and both human and software interpretations using the TrueAllele® Casework System ('TA') for probabilistic modeling. Probabilistic interpretations were compared to traditional analyst assessments using standard caseworking protocols.

2. Materials and methods

2.1. Blood sample preparation

Human whole blood samples (n = 9) were obtained from the Tissue and Data and Acquisition and Analysis Core Facility at Virginia Commonwealth University pursuant to Institutional Review Board protocol #870. Blood samples were screened for the HLA-A*02 allele as previously described [3]; four were HLA-A*02 positive (sample IDs 93, 96, 103, 106) and five were HLA-A*02 negative (sample IDs 94, 95, 104, 105, 107). Multiple contributor blood mixture samples of two to five donors were prepared in the ratios (volume:volume) shown in Table 1. Next, 500 µl of each whole blood mixture was dried in a petri dish and incubated at room temperature for approximately 16 h. After the incubation, cells were eluted from the surface by pipetting 1 ml of 1x Phosphate Buffered Saline solution into the petri dish and transferring the cell solution into a 1.5 ml microcentrifuge tube. Samples were then subjected to red blood cell lysis using the Ammonium-Chloride-Potassium (ACK) lysis buffer (Thermofisher Scientific, Waltham, MA). A 50 µl aliquot of each lysed mixture was retained for the unsorted samples and the remainder of each mixture was labeled with FITC-conjugated anti-human HLA-A*02 antibody (BioLegend, San Diego, CA). As part of our initial optimization experiments, we tested three different concentrations of antibody probe: 5 µg, 2 µg, and 0.5 µg (per 30,000 cells). No appreciable differences in the proportion of hybridized cells were observed between 5 µg and 2 µg samples (Figure S1). Five micrograms was used for all hybridization experiments. Mixtures were then processed using FACS to produce the sorted samples as described in [3]. Untreated blood for each of the nine contributors was used for donor reference samples.

2.2. Fluorescence activated cell sorting (FACS)

Fluorescence activated cell sorting (FACS) was performed using a BD FACS Aria II (Becton Dickenson, Franklin Lakes, NJ) in the Flow Cytometry Core Laboratory on the Medical College of Virginia campus of VCU. FACS separation of antibody-labeled white blood cells was accomplished using a 488 nm laser and gating criteria for discrimination of HLA-A*02-labeled and HLA-A*02-unlabeled cells into the P2 and P3 fractions, respectively.

Table 1
Contributors and ratios for each mixture.

Number of Contributors	Mixture Ratios (vol:vol)	Contributors in Mixture [1]
2	1:1	93(+):94(-)
2	1:1	95(−):96(+)
3	1:1:1	105(−):106(+):107(−)
3	1:1:2	105(−):106(+):107(−)
4	1:2:2:3	103(+):104(−):106(+):107(−)
5	1:1:1:1:1	103(+):104(−):105(−):106(+):107(−)

[1] Contributors are listed in the same order as the mixture ratios. "+" or "-" indicates whether donor cell populations exhibited interactions with the HLA-A*02 antibody.

2.3. DNA extraction

DNA extraction was performed using the DNA IQ™ system which was previously validated for low level samples [12]. All DNA purification reagents were provided in the DNA IQ™ kit (Promega, Madison, WI). Briefly, samples were placed in 1.5 ml microcentrifuge tubes and cell lysis was performed in 160 µl of a Proteinase K buffer (TNE, 2.5% Sarkosyl), 20 µl of 0.39 M Dithiothreitol (DTT), and 20 µl of 20 mg/ml Proteinase K). Samples were incubated at 56 °C for 2 h, then substrate material was removed to a spin basket in the sample tube and centrifuged at 10,000 x g for 5 min to remove excess liquid. DNA preparations of the blood mixture and reference samples were also performed using the Biomek®NX^P Automation Workstation (Beckman Coulter, Inc., Indianapolis, IN) following the same process but automated. The purified DNA was stored at 4 °C.

2.4. DNA quantification

DNA was quantified by real-time PCR (qPCR) using the Plexor® HY System (Promega) in a MX3005P™ Quantitative PCR instrument (Stratagene, Santa Clara, CA) equipped with Plexor® HY Analysis software, as detailed in [13]. The Plexor® HY System (Promega, Madison WI) simultaneously quantifies human and male DNA and amplifies an internal positive control that may indicate sample inhibition.

2.5. STR amplification and analysis

STR amplification of extracted DNA was performed using the PowerPlex® Fusion System (Promega, Madison, WI) in a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, CA), as per manufacturer's protocol. The 25 µl reactions allowed for the addition of 15 µl template; the maximum amount used was 0.5 ng DNA in a STR amplification, though most samples had much less than this in the PCR. Separation of PCR products was accomplished by capillary electrophoresis (CE) in a 3500*xl* Genetic Analyzer followed by STR data analysis using the GeneMapper®ID-X v1.4 software program (Applied Biosystems, Carlsbad, CA) or data analysis using TrueAllele® Casework probabilistic modeling system (Cybergenetics, Pittsburgh, PA).

As part of our initial method development we also tested whether direct amplification and STR profiling of the sorted cell populations with the Powerplex Fusion system compared with results obtained from DNA IQ™ extraction. Direct amplification was performed according to the manufacturer's protocol with the following modification: 15 µl PunchSolution™ Reagent was added to a PCR tube containing the pelleted cell sample or reagent blank, mixed by pipetting, capped, and incubated at 70 °C for 30 min. The entire sample was then subjected to PCR amplification. Results indicated no clear differences in the number of alleles detected across either method (comparison tables shown in Table S1). All results reported in this study were obtained using DNA IQ™ method for extraction of DNA from unsorted mixture samples, contributor reference samples, and sorted cell fraction P2 and P3.

Qualitative (analyst) assessment of STR profiles followed Virginia Department of Forensic Science (VDFS) procedures for calling alleles, examination of controls and identification of artifacts in samples. For mixture samples, allele assignment to contributors was based on comparison to known donor reference profiles. Alleles were noted as either unique to a donor, shared with at least one other donor, or non-donor (not attributable to any of the contributors of the sample). In a casework setting, qualitative approaches alone would not utilize all of the data present within an STR profile, underscoring the need for quantitative interpretation protocols such as TA. Thus, we used both qualitative and quantitative analyses of mixtures for this study. Quantitative assessment of selected STR profiles was performed using TrueAllele® Casework software [5,8]. This probabilistic modeling system uses all of