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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

the peak height and position data from an electropherogram to develop most likely explanations for the profile by use of Markov chain Monte Carlo (MCMC) sampling of the data. The TrueAllele® Casework (TA) mixture deconvolution process is performed in the absence of any reference profiles unless a reference is "assumed". No references were assumed for this study. There is no drop-in or drop-out factor calculated or needed for the TA analysis process. Instead, the allele data, in the form of peaks, is modeled *de novo* for each electropherogram. Every possible allele pair combination is tested and the probability assessed to explain that mixture profile. After the mixture deconvolution process is complete, then comparisons, in the form of likelihood ratios, are performed for all reference profiles of interest. Moreover, the TA process requires a minimum of two reproducible independent TA analyses of the STR data, thus if a value brackets zero, small positive log(LR) for one run and small negative log(LR) for the other, it will also be interpreted as inconclusive.

The hypothesis utilized in this study for all mixtures was as follows: the LR hypothesis (Hp) is that a person contributed their DNA to the mixture, along with N-1 unknown contributors. The alternative (Hd) is that the mixture contains N unknown contributors. Qualitative and quantitative assessments of blood samples were compared for concurrence of results.

3. Results and discussion

3.1. Blood mixture samples

Blood from five different contributors was used to prepare mixture samples derived from two, three, four or five of those donors in specified ratios (Table 1). White blood cells from each of these mixture samples were labeled with HLA-A*02 antibody and sorted by FACS to the P2 or P3 fractions corresponding to cell populations that bound to the antibody probe and cell populations that did not bind to the probe, i.e., A*02 positive and A*02 negative phenotypes, respectively. The fluorescence histograms and sorting gates for the two contributor mixtures are shown in Fig. 1, while the three, four, and five contributor fluorescence histograms and sorting gates are shown in Fig. 2.

STR profiles were generated from each sorted cell population and compared to the reference profiles of the contributors for that mixture sample. For three, four, and five contributor mixtures, alleles unique to each contributor are color-coded in the genotype table for ease of visualization. Each donor was assigned a color: donor 103 = gold, 104 = purple, 105 = red, 106 = green, 107 = blue. Within each subsample profile an allele unique to a donor was marked with that donor's color (Tables 4,6,8,10), otherwise uncolored boxes indicate that allele was shared by more than one donor in the mixture. All mixture samples and sorted cell populations were qualitatively analyzed in this manner.

3.2. Blood mixture samples – two contributor mixtures

Two separate, two contributor mixtures containing an A*02 positive and an A*02 negative contributor were created in 1:1 ratios. The fluorescence histogram of the first cell mixture (contributors 93 and 94) after antibody hybridization shows two distinct peaks consistent with the presence of an A*02 positive and an A*02 negative contributor (Fig. 1 left panel). DNA profiling of the unsorted mixture (Table 2) showed full STR profiles for both donors. After sorting, the P2 sorted fraction (A*02 positive) showed a complete, single source profile for donor 94. There were no alleles from 93 detected in this fraction (Table 2). The P3 sorted fraction (A*02 negative) showed a full profile for the negative donor, 94, with only five minor alleles consistent with 93 detected. The peak height ratio of major to minor contributor ranged between 8:1 to 10:1.

A second mixture composed of donors 95 and 96 showed similar results (Table 3). Although the fluorescence histogram showed two distinct peaks consistent with an A*02 positive and an A*02 negative contributor, cell populations exhibited more apparent overlap with less distinct differences in peak fluorescent intensity compared to the previous mixture histogram (Fig. 1, right). Complete STR profiles for both

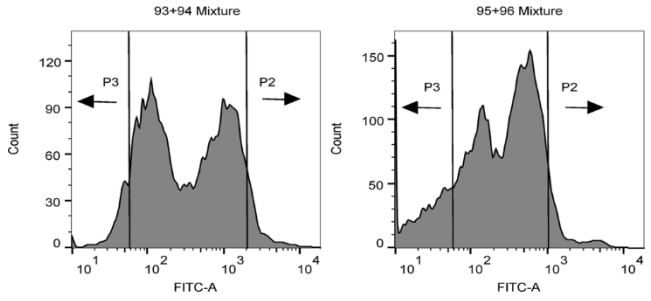

Fig. 1. Fluorescence histograms and sorting gates for 93 + 94 and 95:96 two contributor mixtures. HLA-A*02-labeled cells were sorted into the P2 fraction, and HLA-A*02-unlabeled cells were sorted into the P3 fraction.

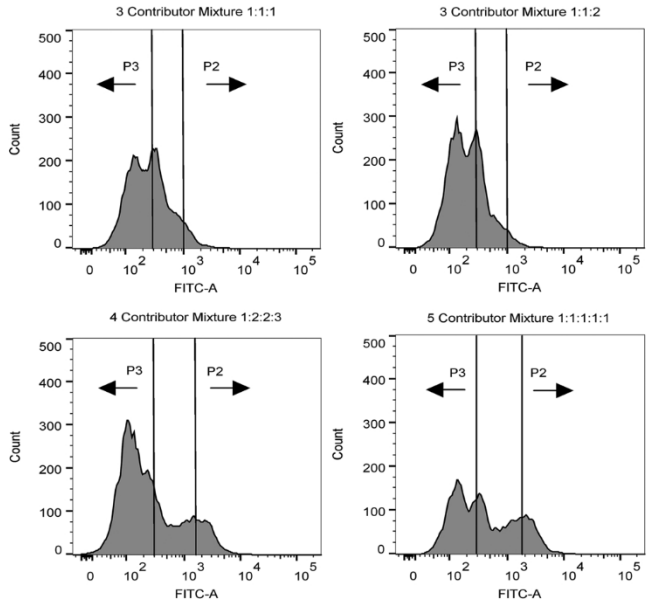

Fig. 2. Fluorescence histograms and sorting gates for the three, four, and five contributor mixtures. HLA-A*02-labeled cells were sorted into the P2 fraction, and HLA-A*02-unlabeled cells were sorted into the P3 fraction.

Table 2
STR profiles from two-person mixture (Donors 93, 94).

	94 Reference (−)	93 Reference (+)	93 + 94 UnSorted [1]	P2 Sorted [1]	P3 Sorted [1]
D8S1179	13,15	10,13	10,13,15	10,13	13,15
D21S11	28,32.2	28,31	28,31,32.2	28,31	28,32.2
D7S820	12	10,11	10,11,12	10,11	12
CSF1PO	10,11	10,12	10,11,12	10,12	10,11
D3S1358	16,17	16,17	16,17	16,17	16,17
TH01	6,7	7,8	6,7,8	7,8	6,7
D13S317	11,12	8,12	8,11,12	8,12	11,12
D16S539	11,12	9,13	9,11,12,13	9,13	(9),11,12
D2S1338	20,25	19,24	19,20,24,25	19,24	(19),20,25
D19S433	13,15	12.2,15.2	12.2,13,15,15.2	12.2,15.2	13,15
VWA	17	15,16	15,16,17	15,16	17
TPOX	8,10	8,9	8,9,10	8,9	8, (9),10
D18S51	15,17	12,17	12,15,17	12,17	15,17
AMEL	XY	XY	XY	XY	XY
D5S818	11,13	8,12	8,11,12,13	8,12	11, (12),13
FGA	23	21	21,23	21	(21),23

[1] Minor peaks are shown in parentheses.

Table 3
STR profiles from two-person mixture (Donors 95, 96).

	95 Reference (A*02−)	96 Reference (A*02+)	95 + 96 UnSorted [1]	P2 Sorted [1]	P3 Sorted [1]
D8S1179	14,16	14,15	14,15,16	14,15	14,16
D21S11	28	28,29	28,29	28,29	28
D7S820	11	8,10	8,10,11	8,10	11
CSF1PO	8,10	10,12	8,10,12	10,12	8,10
D3S1358	15,16	15,16	15,16	15,16	15,16
TH01	8,9	6,7	6,7,8,9	6,7,(8)	8,9
D13S317	10,13	11,12	10,11,12,13	11,12,(13)	10,13
D16S539	11	8,10	8,10,11	8,10,(11)	11
D2S1338	16,20	22,25	16,20,22,25	(16), (20),22,25	16,20
D19S433	12,14	13.2,18.2	12,13.2,14,18.2	13.2, (14),18.2	12,14
VWA	15,18	15,17	15,17,18	15,17	15,18
TPOX	8,11	8,11	8,11	8,11	8,11
D18S51	15,17	16,23	15,16,17,23	16,23	15,17
AMEL	XX	XY	XY	XY	XX
D5S818	11,12	11,12	11,12	11,12	11,12
FGA	22,24	22,23	22,23,24	22,23,(24)	22,24

[1] Minor peaks are shown in parentheses.

donors were detected in the unsorted mixture. The P2 sorted fraction contained a full profile for the positive donor, 96, with seven minor alleles detected from the negative donor, 95. The peak height ratio of major to minor contributor ranged in this mixture between 8:1 to 10:1. The P3 sorted fraction gave a complete, single source profile for the negative donor. No alleles from the positive donor were detected in this fraction (Table 3).

Results from the two-person mixtures indicate that antibody hybridization can be used to selectively label and sort contributor cell populations in a dried blood sample. Easily interpretable STR profiles consistent with each contributor were obtained from the two sorted cell fractions with only minor contributions from the non-target contributor. The decreased separation between A*02 positive and A*02 negative populations compared to earlier studies (i.e., (3)) may be due to increases in autofluorescence after drying as suggested previously [4] or due to increases in non-specific probe interactions due to degradation of cell targets after drying. Alternatively, differences in the efficiency of antibody hybridization may be donor-specific depending the presence of cross-reactive HLA alleles, i.e., non-A*02 antigens binding to A*02 antibody probe [14].

3.3. Blood mixture samples – three contributor mixtures

Next, three donor samples (105, 106, and 107) were used to create two separate, three contributor blood mixtures in ratios of 1:1:2 and 1:1:1. Donor 106 was HLA-A*02 positive whereas donors 105 and 107 were HLA-A*02 negative (Table 1). Therefore, in both mixtures the P2 fraction should have been enriched in donor 106 whereas the P3 fraction should have been enriched in cells from donors 105 and 107. For the 1:1:2 mixture, STR profiles from the unsorted mixture yielded full profiles for all three contributors and profiles from the P2 fraction primarily contained alleles consistent with donor 106 and with only two alleles from donor 107 and two alleles from donor 105 detected (Table 4). The STR profile from the P3 fraction was enriched for donors 105 and 107, with six alleles from donor 106 detected (Table 4).

Quantitative assessment by TA confirmed the qualitative results for the three contributor mixture sample (1:1:2). TA log(LR) values for the unsorted subsample were within 100-fold of each other, ranging from 9.4714 to 11.4591 (Table 5), which is equivalent to likelihood ratios of 2.9 billion and 287 billion, respectively. This indicates that it is 2.9 billion to 287 billion times more probable to observe the obtained DNA results if the person of interest contributed their DNA to the mixture,

Table 4
Genotype table for the three contributor (1:1:2) blood mixture.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	Unsorted Mixture 1:1:2						Sorted P2 (A*02+)				Sorted P3 (A*02-)				
Marker															
AMEL	X	Y					X	Y			X	Y			
D3S1358	14	15	17				15	17			14	15	17		
D1S1656	11	13	15.3	17	18.3		13	17.3	18.3		11	13	15.3	17.3	
D2S441	10	11	11.3				10				10	11	11.3		
D10S1248	13	14					13				13	14			
D13S317	11	12	13				12				11	13			
Penta E	5	7	11	12	13		11				5	7	11	12	13
D16S539	11	12	13				12	13			11	12	13		
D18S51	12	14	16	17			14	16	17		12	16	17		
D2S1338	17	19	20	23	24		17	20			19	23	24		
CSF1PO	10	11	12				11				10	11	12		
Penta D	2.2	9	10	13	14		9	13			2.2	10	13	14	
TH01	6	7	9				6	7			6	7	9		
vWA	15	16	18	19	20		15	20			15	16	18	19	
D21S11	28	30	31	33.2			28	31			28	30	33.2		
D7S820	10	11	12	13			11				10	11	12		
D5S818	11	12	13				11	13			11	12	13		
TPOX	8	9	11	12			9	11			8	11	12		
DYS391	11						11				11				
D8S1179	12	13	14	15			12	13	14	15	12	13	14	15	
D12S391	17	18	19	20	22	23	17	23			17	18	19	20	22
D19S433	12	13	14				12	13			12	13			
FGA	19	20	21	23	24	25	21	23			19	20	23	24	25
D22S1045	11	15	16				16				11	15	16		

Red = 105, Green = 106, Blue = 107.

Table 5
TrueAllele® Casework analysis for the three contributor (1:1:2) blood mixture sample.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	log Likelihood Ratio		
Contributor[1]	**Unsorted**	**Sorted P2 (A*02+)**	**Sorted P3 (A*02-)**
105 (HLA-A02-)	9.7462	-16.5399	11.5894
106 (HLA-A02+)	11.4591	13.0249	-15.7665
107 (HLA-A02-)	9.4714	-11.9631	20.5656

[1]Donor colors correspond to genotype charts in Table 4.

Table 6
Genotype table for the three contributor (1:1:1) blood mixture.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	Unsorted Mixture 1:1:1						Sorted P2 (A*02+)		
Marker									
AMEL	X	Y					X	Y	
D3S1358	14	15	17				15		
D1S1656	11	13	15.3	17.3	18.3		13	17.3	18.3
D2S441	10	11	11.3				10		
D10S1248	13	14							
D13S317	11	12	13				12		
Penta E	5	7	11	12	13		12		
D16S539	11	12	13				11	13	
D18S51	12	14	16	17			14	17	
D2S1338	17	19	20	23	24		17	20	
CSF1PO	10	11	12				12		
Penta D	2.2	9	10	13	14		9		
TH01	6	7	9						
vWA	15	16	18	19	20		20		
D21S11	28	30	31	33.2					
D7S820	10	11	12	13					
D5S818	11	12	13				11	13	
TPOX	8	9	11	12			11		
DYS391	11						11		
D8S1179	12	13	14	15			14	15	
D12S391	17	18	19	20	22	23	23		
D19S433	12	13	14				13		
FGA	19	20	21	23	24	25	23		
D22S1045	11	15	16						

Red = 105, Green = 106, Blue = 107.

Table 7
TrueAllele® Casework analysis for the three contributor (1:1:1) blood mixture sample.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	log Likelihood Ratio	
Contributor[1]	**Unsorted**	**Sorted P2 (A*02+)[2]**
105 (HLA-A02-)	13.1799	-15.3381
106 (HLA-A02+)	14.5489	5.1996
107 (HLA-A02-)	17.8216	-1.7661

[1]Donor colors correspond to genotype charts in Table 6.
[2]DNA was not detected in the P3 fraction for this mixture.

along with N-1 unknown contributors, than if the mixture contains N unknown contributors. TA analysis of the sorted cell populations provided quantitative support that donor 106 was enriched in the sorted P2 fraction and donors 105 and 107 were enriched in the sorted P3 fraction. Specifically, the sorted P2 fraction subsample yielded a log(LR) of 13.0249 for contributor 106, an enrichment of almost 100-fold greater from the unsorted subsample. Concurrently the log(LR) values for contributors 105 and 107 in the sorted P2 subsample were -16.5399 and -11.9631, suggesting that they were excluded from the P2 cell population (Table 5). TA analysis of the sorted P3 subsample yielded a negative log(LR) value for donor 106, which indicated that this contributor was excluded from the P3 subsample. Donor 105 had a log(LR) value of 11.5894, an almost 100-fold increase from the unsorted log (LR) of 9.7462. Donor 107 displayed a log(LR) value of 20.5656 in the sorted P3 subsample, an increase of more than 11 orders of magnitude from the unsorted subsample (Table 5).

Although DNA was not recovered from the P3 fraction of the 1:1:1 mixture, STR profiling results from the unsorted mixture and the P2 fraction suggested efficient separation of the A*02 positive contributor from the mixture. Specifically, full STR profiles for each of the three contributors were detected in the unsorted fraction whereas a full profile for only donor 106 was detected in the P2 fraction (Table 6). Only one allele from a non-target contributor (107) was detected in this fraction. TA analysis indicated that there was only statistical support for

Table 8
Genotype table for the four contributor (1:2:2:3) blood mixture.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

Unsorted Mixture 1:2:2:3							Sorted P2 (A*02+)					Sorted P3 (A*02-)					
Marker						-					-						-
AMEL	X	Y					X	Y				X	Y				
D3S1358	14	15	16	17			14	15	16	17		14	15	16	17		
D1S1656	11	12	13	15.3	17	18.3	11	12	13	15.3	17.3	11	12	13	17.3	18.3	
D2S441	10	11	11.3				10	11	11.3			10	11	11.3			
D10S1248	13	14	15				13	14	15			13	14	15			
D13S317	11	12	13				11	12	13			11	12	13			
Penta E	5	11	12	14	17	19	5	11	14	19		5	11	12	14	17	19
D16S539	9	11	12	13			9	11	12	13		9	11	12	13		
D18S51	12	14	15	16	17		12	14	15	16	17	12	14	16	17		
D2S1338	17	19	20				17	19	20			17	19				
CSF1PO	10	11	12	13			10	11	12			10	11	12	13		
Penta D	2.2	9	10	12	13		2.2	9	10	13		2.2	9	10	12	13	
TH01	6	7	9	9.3			6	7	9	9.3		6	7	9			
vWA	15	16	17	18	19	20	15	16	19	20		15	16	17	18	20	
D21S11	28	29	30	31	32.2		29	30	31			28	29	30	31	32.2	
D7S820	9	10	11	12	13		10	11	13			9	10	11	12	13	
D5S818	11	12	13				11	12	13			11	12	13			
TPOX	8	9	11				9	11				8	9	11			
DYS391	10	11					10					10	11				
D8S1179	13	14	15				13	14	15			13	14	15			
D12S391	17	18	20	22	23		17	18	20	22	23	17	18	20	22	23	
D19S433	12	13	14	15			12	13	14	15		12	13	14	15		
FGA	21	22.2	23	24	25		21	22.2	23	24		21	22.2	23	24	25	
D22S1045	11	15	16	17			15	16	17			11	15	16			

Yellow = 103; Purple = 104; Green = 106; Blue = 107.

Table 9
TrueAllele® Casework analysis for four contributor (1:2:2:3) blood mixture sample.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	log Likelihood Ratio		
Contributor[1]	**Unsorted**	**Sorted P2 (A*02+)**	**Sorted P3 (A*02-)**
103 (HLA-A02+)	5.6952	25.8097	-0.4071
104 (HLA-A02-)	9.3661	1.9489	13.8653
106 (HLA-A02+)	10.2259	3.902	4.5862
107 (HLA-A02-)	6.3227	4.6193	10.6459

[1]Donor colors correspond to genotype charts in Table 8.

donor 106 in the P2 fraction (5.1996 106 compared to -1.7661 for 107 and -15.338 for 105, Table 7).

3.4. Blood mixture samples – four contributor mixture

The 1:2:2:3 four contributor blood mixture sample was prepared with donors 103, 104, 106, and 107. STR profiles from the unsorted subsample yielded full profiles for all four contributors (Table 8). Based on respective HLA phenotypes, P2 should have been enriched in donors 103 and 106 and P3 should have been enriched in donors 104 and 107. The actual STR profile from the P2 fraction was enriched for donor 103, as seen by the frequency of gold colored alleles in the genotype chart (Table 8, center). The P2 fraction shows few alleles from donor 106, compared to what we would expect given the dominance of their alleles in the P2 fraction of the three contributor mixtures. The STR profile resulting from the P3 fraction shows alleles consistent with donors 104 and 107, representing all alleles for both of those contributors. All alleles consistent with 106, except for one allele at D2S1338 are also present, however a qualitative analysis does not utilize much if any allele peak height information and mixture weight assessments and thus does not always provide a complete picture of the data.

Probabilistic modeling showed evidence of all four contributors in the unsorted mixture with LR values of ~5.7, 6.3, 9.3, and 10.2 for donors 103, 107, 104, and 106 respectively (Table 9). After sorting, the P2 fraction showed significant enrichment for donor 103 (25.8097) and the P3 fraction showed significant enrichment for donors 104 and 107 (13.8653, 10.6459 respectively). There was only limited statistical association of donor 106 in either sorted cell fractions (3.902 in P2 and 4.5862 in P3). Overall, TA analysis provided quantitative support for one of the A*02 positive contributors and both A*02 negative contributors in the corresponding sorted cell fractions. Although a few unique alleles for donor 106 were detected in the unsorted mixture profile as well as the sorted P2 and P3 fractions (Table 8), the lower statistical support for 106 from TA analysis suggests proportionally fewer cells were sorted into either P2 or P3 fractions. This may be due to incorrect partitioning of donor 106 cells into the P3 fraction from inefficient antibody hybridization. We note that poor detection of alleles from donor 106 was observed in multiple cell mixtures for this study (1:2:2:3 and 1:1:1:1:1 shown below). Direct comparison of hybridized cell populations from the donors 103 and 106 (both A*02

Table 10
Genotype table for the five contributor (1:1:1:1:1) blood mixture.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	Unsorted Mixture 1:1:1:1:1								Sorted P2 (A*02+)				Sorted P3 (A*02-)						
Marker								-					-						-
AMEL	X	Y							X	Y			X	Y					
D3S1358	14	15	16	17					16	17			14	15	17				
D1S1656	11	12	13	15.3	17.3	18.3			11	15.3			11	12	13	15.3	17.3		
D2S441	10	11	11.3						11	11.3			10	11	11.3				
D10S1248	13	14	15						13	15			13	14	15				
D13S317	11	12	13						12				11	12	13				
Penta E	5	7	11	12	13	14	17	19	14	19			5	7	11	12	13	17	19
D16S539	9	11	12	13					11	12	13		9	11	12				
D18S51	12	14	15	16	17				14	15	16	17	12	16	17				
D2S1338	17	19	20	23	24				17	19	23		17	19	23	24			
CSF1PO	10	11	12	13					10	11	12		10	11	12	13			
Penta D	2.2	9	10	12	13	14			9	13	14		2.2	9	10	12	13	14	
TH01	6	7	9	9.3					6	9.3			6	7	9				
vWA	15	16	17	18	19	20			15	19			15	16	17	18	19		
D21S11	28	29	30	31	32.2	33.2			29	31			28	30	31	32.2	33.2		
D7S820	9	10	11	12	13				10				9	10	11	12			
D5S818	11	12	13						11	12	13		11	12	13				
TPOX	8	9	11	12					9	11			8	11	12				
DYS391	10	11							10				11						
D8S1179	12	13	14	15					13	14	15		12	13	14	15			
D12S391	17	18	19	20	22	23			17	22			18	19	20	22			
D19S433	12	13	14	15					14	15			12	13					
FGA	19	20	21	22.2	23	24	25		19	22.2	23	24	19	21	24	25			
D22S1045	11	15	16	17					16	17			11	15	16				

Yellow = 103; Purple = 104; Red = 105; Green = 106; Blue = 107.

Table 11
TrueAllele® Casework analysis for five contributor (1:1:1:1:1) blood mixture sample.(For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

	log Likelihood Ratio		
Contributor[1]	**Unsorted**	**Sorted P2 (A*02+)**	**Sorted P3 (A*02-)**
103 (HLA-A02+)	7.7709	28.0754	-3.7186
104 (HLA-A02-)	7.7705	-10.3345	19.1138
105 (HLA-A02-)	8.4417	-7.6818	9.1347
106 (HLA-A02+)	7.9141	-7.8931	-12.6020
107 (HLA-A02-)	3.4128	-16.3560	9.4315

[1]Donor colors correspond to genotype charts in Table 10.

positive) after drying indicates that donor 103 cells have stronger interaction with the probe as evidenced by higher proportion of cells above 10 [3] RFU (Figure S2). Additionally, mixtures in which donor 106 is the only A*02 positive contributor exhibit lower fluorescence intensities in the P2 subpopulation compared to mixtures where donor 103 is present (e.g., P2 populations in top two histograms versus bottom two histograms in Fig. 2), further suggesting that this is a contributor-specific trend.

3.5. Blood mixture samples – five contributor mixture

The five contributor 1:1:1:1:1 blood mixture sample was composed of donors 103, 104, 105, 106, and 107. All alleles consistent with the five donors were observed in the unsorted subsample (Table 10). In a forensics laboratory this would be a very challenging mixture, not only due to the number of contributors but also because all were present in equal measure. In many, if not most forensic laboratories, the mixture would be deemed uninterpretable due to its complexity and potential information would be lost.

Donors 103 and 106 were HLA-A*02 positive and are expected to sort into the P2 fraction whereas donors 104, 105 and 107 were HLA-A*02 negative and are expected to sort into the P3 fraction. Qualitatively, the STR profile generated from the sorted P2 fraction was enriched for donor 103, with 10 unique alleles detected from this contributor compared to three unique alleles detected from contributors 105 and 106 (Table 10). The STR profile generated from the sorted P3 fraction showed the highest number of unique alleles for donors 104 (= 8), 105 (= 10), and 107 (= 9), with only three alleles detected that were uniquely attributable to donors 103 or 106 (Table 10). Qualitative assessment determined that the sorted P2 subsample yielded all alleles for donor 103, and the sorted P3 subsample generated all alleles for donors 104 and 107 and nearly all for 105. The limited number of unique alleles from donor 106 detected in the P2 fraction are consistent with results from the 1:1:2 mixture and could indicate less efficient antibody hybridization to this contributor cell population and subsequent sorting into the A*02 negative fraction

(discussed further below). Additionally, physical adhesion or clumping of target cells to non-target cells has been observed in previous flow cytometry studies [3] and could have contributed to non-specific sorting, and allelic drop-out in this experiment.

Quantitative assessment of the five contributor sample was performed using TA (Table 11). The unsorted subsample included all five donors, with log(LR) values ranging between 3.4128-8.4417. TA analysis of the sorted P2 subsample yielded log(LR) of 28.0754 for donor 103, a value that is comparable to a single source sample [15], and negative values for the other four contributors. The sorted P3 subsample yielded log(LR) of 19.1138 for donor 104, an increase of nearly 12 orders of magnitude. While donor 105 produced equivalent values for unsorted and sorted fractions, the log(LR) for donor 107 increased by 6 (a million times more likely increase). Donors 103 and 106 showed LR values of -3.7186 and -12.6020 respectively suggesting that they were excluded from the sorted P3 subsample. The TA results provided quantitative confirmation that donor 103 was enriched in the sorted P2 fraction, donors 104 and 107 were enriched in the sorted P3 fraction while donor 105 stayed the same, and that donor 106 was not detected after the cell sorting process.

4. Conclusions

The data presented here suggests that antibody probes combined with FACS can be used for the front-end separation of contributor cell populations in two-person dried blood mixture samples to generate single source STR profiles. Further, for mixtures containing three, four, or five individuals, binary sorts based on the presence or absence of an HLA allele can be combined with probabilistic modeling procedures to enhance the interpretation of complex mixture samples. Results from mixtures containing three or more individuals may potentially be further improved by combining different antibody probes in the initial hybridization steps to enhance discrimination of cell populations during FACS and/or sorting cell populations into more than two fractions (i.e., non-binary sort) depending on the nature of the fluorescence histogram and the initial resolution of contributor cell populations with the mixture sample. Alternative antibody probes may be particularly useful for labelling contributor cell populations that exhibit decreased separation efficiency with a given probe (e.g., donor 106 with A*02 probe). With this, it may be necessary to systematically investigate binding efficiencies of specific antibody probes against dried cell populations containing a range of subtypes of the target allele (e.g., subtypes of A*02 described in ([16])) as well as cell populations with non-target antigens within the same cross-reactive group as the target allele [14,17].

Future studies can also make effort to collect and profile cells from the discarded fraction of the FACS instrument that result from sorting errors or events falling outside the initial gating parameters for target cells. For some complex mixtures retaining this fraction may help detect certain contributor cell populations that are incorrectly sorted and also would be a generally advantageous practice for degraded and/or low template samples. Although these results suggest that antibody based cell labelling and FACS separation may be used on dried/compromised samples, we acknowledge that as the extent of sample degradation increases (i.e., dried for > 24 h), decomposition of antigen targets and/or autofluorescence may present more significant obstacles. Applying this workflow to the full range of sample types and conditions encountered in forensic casework may require more robust probe targets or alternative autofluorescence-based signatures that can be detected in aged samples [18].

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Appendix A. Supplementary data

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