Naming Convention and Laboratory Methods: PROVEDIt Database

Sec 1. Naming Convention and Laboratory Methods

Single source sample names will follow the format below and are best explained through example:

RD14-0003-21d1x-0.5IP-Q0.8_002.20sec or RD12-0002-21d1-0.5IP-002.20sec

RD14-0003 and RD12-0002 are the project numbers, 21 is the sample identifier within that project, and d_ is the dilution number which was used by laboratory personnel to distinguish between extracts. It is to be noted that each sample is designated by the combination of project number and sample identifier. For example, RD14-0003-21 will have a different known genotype than RD12-0002-21. Within the RD14-0003 project sample names, the 'x' designator indicates DNA condition (Supplementary Methods Table 1), 0.5 represents the template mass in nanograms (typically ranges from 0.007 to 1 ng), and IP is the amplification kit type (i.e., IP=Identifiler® Plus, GF=Globalfiler®, PP16=PowerPlex®16 HS). If the extracts were quantified with both a small and large autosomal fragment, the Quality Index is presented in the form of a Q value (Q0.8 in this example). In some instances, the Q designator is followed by "LAND," which stands for "large autosomal not detected." This term is used for samples in which the large autosomal fragment was not detected during qPCR and so a numerical Q value was not obtained. The designator 002 is the capillary number, where capillaries are numbered 001-004 on the 3130 electrophoresis platform and 01-08 on the 3500 platform, and 20 sec is the injection time. Injection times of 5, 10, and 20 sec were utilized for samples run on the 3130, and 5, 15, and 25 sec injections were utilized for samples run on the 3500 platform.

Mixtures are named similarly, with the addition of a mixture ratio. For example:

RD14-0003-31_32-1;2-M1x-0.062IP-Q14.4_003.10sec.

This is a two-person mixture of contributors RD14-0003-31 and RD14-0003-32. The ratio follows the sample names (i.e., 1;2); thus, person RD14-0003-31 is contributing one part to the mixture, and person RD14-0003-32 is contributing two parts. M1 is the mixture dilution number used to distinguish between extracts. The rest of the sample name follows the convention outlined above for single-source samples.

Methods Table 1. A summary of the protocols used to generate untreated or compromised WBM samples for the RD14-0003 sample-set. To demonstrate the naming convention utilized for each protocol, a corresponding example is provided using single-source samples.

Protocol	Single-Source Sample Name	x	Description
Untreated	RD14-0003-19d2**a**-0.5IP-Q0.8_002.5sec	a	Letter a indicates that the sample was not treated with any protocol intended to induce PCR inefficiencies.
DNase I Degradation	RD14-0003-21d2**b**-0.5IP-Q0.8_002.20sec	b to e	Letters b to e indicate units of DNase I in degradation reaction. (b) 3, (c) 6, (d) 12, and (e) 24 mU enzyme.
Fragmentase® Degradation	RD14-0003-36d1**-15**-0.5IP-Q1.4_003.10sec	-15 to -45	-15 indicates enzyme digestion/incubation time in minutes. 15, 30, and 45 minute digestion times were utilized.
UV Damage	RD14-0003-03d2**U60**-0.5IP-Q8.8_003.10sec	U15 to U105	U60 indicates 60 minutes of UV exposure. Times ranged from 15-105 minutes.
Sonication	RD14-0003-12d3**S30**-0.0078IP-Q17.1_002.20sec	S2 to S30	S30 indicates DNA was damaged with 30 cycles of sonication. 2, 10, and 30 cycles were utilized.
Humic Acid Inhibition	RD14-0003-49d2**I22**-0.5IP-Q2.4_002.10sec	I15 to I35	I22 indicates volume of 2 mg/mL humic acid (in µL) added to whole blood lysate. Three volumes were utilized (15, 22, and 35 µL).

RD12-0002: DNA Extract Mixtures (DEM)

Extraction

DNA extracts were obtained by isolating DNA from 69 donors using phenol/chloroform purification and alcohol precipitation. The DNA was dissolved in 50 mL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) at 56°C.

Quantification

The extracts were quantified with the Quantifiler® Duo DNA Quantification Kit (Life Technologies) on the 7500 Real-Time PCR System using the manufacturer's recommended protocol and an external calibrator [1, 2]. The reactions consisted of 12.5 µL PCR reaction mix, 10.5 µL primer set, and 2 µL sample. Negative controls, which used TE buffer in place of DNA extract, were run concurrently and showed expected results. The Quantifiler® Duo kit co-amplifies three targets: a human target, a human male target, and an internal PCR control (IPC). The concentration of each target was calculated by determining the cycle number at which the emitted fluorescence of the given target reached a defined threshold and comparing this cycle number to a calibration curve.

Mixture Preparation

For mixture profiles, which consisted of 2 to 5 individuals, the concentrations of the human target obtained for the single-source extracts were utilized to calculate the appropriate volume of each single-source extract to combine based on the desired number of contributors to

the mixture and the contributor ratio (Supplementary Table 1); once combined, extract mixtures were quantified as described above, and the concentrations obtained were utilized to calculate the appropriate volume to amplify given the desired target mass. Where necessary, dilutions were prepared in TE buffer.

PCR Amplification

The concentration of the human target obtained during quantification was utilized to calculate the appropriate volume of extract to amplify given the desired target mass. The extracts were amplified with the AmpF*l*str® Identifiler® Plus PCR Amplification Kit (Life Technologies) (29 cycles) on the GeneAmp® PCR Amplification System 9700 using 9600 emulation mode following the manufacturer's recommended protocol at the following target masses: 0.5, 0.25, 0.125, 0.063, 0.047, 0.031, 0.016, 0.007 ng [3]. The total reaction volume was 25 µL which contained the appropriate volume of extract (up to 10 µL), 10 µL master mix, and 5 µL primer set.

A subset of the extracts were amplified with the PowerPlex® 16 HS System (Promega) (32 cycles), following the manufacturer's recommended protocol, using the aforementioned thermalcycler and target masses [4]. The reaction volume was 25 µL which contained the appropriate volume of extract, 5 µL master mix, 2.5 µL primer set, and amplification grade water to a final volume of 25 µL. Positive and negative amplification controls were processed in tandem for both amplification kits.

Electrophoresis

Amplicons were prepared for electrophoresis by combining the appropriate volumes of size standard, highly deionized (Hi-Di) formamide, and amplified product or ladder (Supplementary Methods Table 2) in the appropriate wells of a 96-well plate; samples were subsequently denatured at 95°C for 3 minutes, then snap-cooled at -20°C for 3 minutes. Identifiler® Plus amplicons were injected for 5, 15, and 25 s at 1.2 kV on the Applied Biosystems® 3500 Genetic Analyzer, and PowerPlex® 16 HS amplicons were injected for 5, 10, and 20 s at 3 kV on the Applied Biosystems® 3130 Genetic Analyzer. Both electrophoresis instruments utilized POP-4™ Polymer for separation. Electropherograms were analyzed with GeneMapper® *ID-X* using Local Southern sizing at an analytical threshold of one RFU. The genotype table for each sample was exported from GeneMapper® as a CSV file containing the allele, size, and height for all peaks.

Table 2. Electrophoresis parameters and reagents utilized for separation of DEM amplicons.

			Volume per Reaction (µL)		
PCR Kit	**CE Instrument**	**Size Standard Type**	**Size Standard**	**Hi-Di Formamide**	**Sample or Ladder**
Identifiler® Plus	3500	GeneScan™ 600 LIZ™ Size Standard v2.0	0.3	8.7	1
PowerPlex® 16 HS	3130	Internal Lane Standard 600	0.5	9.5	1

RD14-0003: Whole Blood Mixtures (WBM)

Extraction

Developing a process that included the dilution and mixing of whole bloods, rather than DNA extracts, allowed for the procurement of DNA profiles unaffected by dilution effects. In this dataset, single-source whole blood samples from a total of fifty donors were diluted to 1:10, 1:100, and 1:1000 in TE buffer and subjected to various protocols to generate untreated or compromised DNA, as described below. UV-damaged samples were extracted using the EZ1® DNA Investigator Kit (Qiagen) on the EZ1® Advanced following the manufacturer's recommended protocol for *Pretreatment for Various Casework and Reference Samples* [5]. Briefly, whole swabs were incubated at 56°C for one hour in 475 µL Buffer G2 and 25 µL Proteinase K; samples were occasionally vortexed during incubation. Following incubation, 400 µL Buffer MTL was added to each sample along with 1 µg carrier RNA. The samples were vortexed, and the swabs were transferred to spin baskets, then centrifuged for two minutes at maximum angular velocity. The sample lysates were then inserted in the EZ1® Advanced along with elution tubes, filter tips, and pre-filled reagent cartridges containing magnetic particles, lysis buffer, wash buffer, and elution buffer. Automated extraction was performed following the manufacturer's recommended protocol for *DNA Purification (Large-Volume Protocol)* [5], and extracts were eluted in 50 µL TE buffer.

All other sample types were extracted in 50 µL aliquots using the QIAamp® DNA Investigator Kit (Qiagen) following the manufacturer's recommended protocol for *Isolation of Total DNA from Small Volumes of Blood or Saliva* [6]. Briefly, 50 µL aliquots of each whole blood dilution were incubated at 56°C for 10 minutes in a solution consisting of 50 µL Buffer ATL, 10 µL Proteinase K, 100 µL Buffer AL, and 1 µg carrier RNA. Following incubation, 50 µL 100% ethanol was added, and the samples were incubated at room temperature for 3 minutes. The samples were then transferred to a QIAamp® MinElute column and centrifuged at 8000 rpm for 1 minute. In succession, the following reagents were added: 500 µL Buffer AW1, 700 µL Buffer AW2, and 700 µL 100% ethanol. In between additions, the samples were centrifuged at 8,000 rpm for 1 minute, and the flow-through was discarded. The columns were dried by centrifuging at 14,000 rpm for 3 minutes, then incubating at room temperature for 10 minutes. The samples were eluted by adding 55 µL Buffer ATE, incubating at room temperature for 5 minutes, then centrifuging at 14,000 rpm for 1 minute.

Generation of Condition-Dependent Extracts

Untreated

Untreated samples were generated by extracting aliquots of each whole blood dilution as described above. These extracts were not subjected to any conditions intended to induce inefficiencies in amplification.

rDNase I-Degraded

rDNase I-degraded samples were produced using the DNA-*free*™ Kit (Life Technologies). The extracts were sonicated for 5 minutes, then 2 µL of 10X DNase I Reaction Buffer was added. Three levels of degradation were generated by digesting extracts with 6, 12, or 24 mU rDNase I for 10 minutes at 37°C. The reactions were

subsequently halted by adding 5 µL of DNase Inactivation Reagent and incubating at room temperature for 10 minutes. The samples were then centrifuged at 10,000 rcf for 1.5 minutes; the supernatant was transferred to a clean tube, and the remaining pellet was discarded [7].

Fragmentase®-Degraded

Fragmentase®-degraded samples were produced by extracting 50 µL aliquots of each whole blood dilution using the QIAamp® DNA Investigator Kit and a modified elution volume of 37 µL deionized water. Three levels of degradation were created using the NEBNext® dsDNA Fragmentase® Kit (New England Biolabs) by incubating the extracts with 4 µL Reaction Buffer v2 and 4 µL Fragmentase® enzyme cocktail for 15, 30, or 45 minutes. Following incubation, the reactions were halted by the addition of 10 µL 0.5 M EDTA [8]. To remove EDTA, all extracts subsequently underwent a second extraction using the QIAamp® DNA Investigator Kit, as outlined above.

Sonicated

Sonicated samples were generated by diluting extracts to a total volume of 200 µL with TE buffer. The extracts were sonicated using the Fisher Scientific™ Model 50 Sonic Dismembrator at 25% amplitude for 2, 10, or 30 sonication cycles, where one cycle was defined as 30 seconds sonication on followed by 30 seconds sonication off.

UV-Damaged

UV-damaged samples were created by spotting 100 µL aliquots of each whole blood dilution onto glass microscope slides and allowing the stains to air dry for 75 minutes. The stains were subsequently irradiated using the QIAgility® UV lamp for 15, 60, or 120 minutes. All stains were collected using the double swab method using cotton swabs moistened with deionized water [9]. Swabs were air dried overnight, then extracted as described above.

Humic Acid-Inhibited

Humic Acid-inhibited extracts were generated by combining 50 µL aliquots of each whole blood dilution with 50 µL Buffer ATL, 10 µL Proteinase K, 100 µL Buffer AL, and 1 µg carrier RNA [6]. These solutions were vortexed, incubated at 56°C for 10 minutes, then briefly centrifuged. A 2 mg/mL humic acid solution (Sigma-Aldrich) was prepared, and 15, 22, or 35 µL of this solution was added to each of the cell lysate solutions. The solution were subsequently incubated at room temperature for 2 hours, vortexing every 30 minutes to mix. After incubation, the extraction protocol described above was resumed to completion.

Quantification

All extracts were quantified using the Quantifiler® Trio DNA Quantification Kit (Life Technologies) on the 7500 Real-Time PCR System using the manufacturer's recommended thermalcycling protocol and an external calibrator[2, 10]. The reactions consisted of 10 µL PCR

reaction mix, 8 μL primer mix, and 2 μL sample. Negative controls, which used TE buffer in place of DNA extract, were run concurrently and showed expected results.

The Quantifiler® Trio kit co-amplifies four targets: a small autosomal human target (80 base pairs), a large autosomal human target (214 base pairs), a human male target, and an internal PCR control (IPC). The concentration of each target was calculated by determining the cycle number at which the emitted fluorescence of the given target reached a defined threshold and comparing this cycle number to an external calibration curve.

Mixture Preparation

To generate mixture samples, aliquots of the appropriate whole blood dilutions, selected based on the desired number of contributors (2 to 5) and contributor ratios (Supplementary Table 2), were extracted and quantified as described above. The concentrations of the small autosomal target obtained were used to approximate the volume of each whole blood dilution to combine. Once combined, the whole blood mixtures were extracted and treated with the various protocols described above to generate untreated or compromised DNA. Mixture extracts were subsequently quantified, and the concentrations obtained were utilized to calculate the appropriate volume of extract to amplify given the desired target mass.

PCR Amplification

The concentration of the small autosomal target was used to calculate the appropriate volume of extract to amplify given the desired template mass for all amplifications. Single-source extracts were amplified with the GlobalFiler® PCR Amplification Kit (Life Technologies) (29 cycles) on the GeneAmp® PCR Amplification System 9700 using 9600 emulation mode following the manufacturer's recommended protocol at the following target masses: 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, and 0.007 ng [11]. The reaction volume was 25 μL which included the appropriate volume of extract, 7.5 μL master mix, 2.5 μL primer set, and 5 μL TE buffer. Extracts were also amplified with the Identifiler® Plus PCR Amplification Kit (Life Technologies) (28 cycles) following the manufacturer's recommended protocol using the same thermalcycler and template masses specified above[3]. The total reaction volume was 25 μL which contained the appropriate volume of extract (up to 10 μL), 10 μL master mix, and 5 μL primer set.

Mixture extracts were amplified similarly; however, the target masses were calculated by setting the minor component equal to 0.015, 0.03, 0.06, and 0.125 ng. The total target masses for mixtures therefore varied depending upon the contributor ratio and number of contributors, but typically ranged from 0.03 to 1 ng. Positive and negative amplification controls were processed in tandem for all amplifications.

Electrophoresis

Amplicons were prepared for electrophoresis by combining the appropriate volumes of size standard, highly deionized (Hi-Di) formamide, and amplified product or ladder (Supplementary Methods Table 3) in the appropriate wells of a 96-well plate; samples were subsequently denatured at 95°C for three minutes, then snap-cooled at -20°C for three minutes. GlobalFiler® amplicons were injected for 5, 15, and 25 s at 1.2 kV on the Applied Biosystems® 3500 Genetic Analyzer, and Identifiler® Plus amplicons were injected for 5, 10, and 20 s at 3 kV on the Applied Biosystems® 3130 Genetic Analyzer. Electropherograms were analyzed with

GeneMapper® *ID-X* v1.4 using Local Southern sizing at an analytical threshold of 1 RFU. Both electrophoresis instruments utilized POP-4™ Polymer for separation. The genotype table for each sample was exported from GeneMapper® as a CSV file containing the allele, size, and height for all peaks.

Methods Table 3. Electrophoresis parameters and reagents utilized for separation of WBM amplicons.

			Volume per Reaction (µL)		
PCR Kit	**CE Instrument**	**Size Standard Type**	**Size Standard**	**Hi-Di Formamide**	**Sample or Ladder**
GlobalFiler®	3500	GeneScan™ 600 LIZ™ Size Standard v2.0	0.4	9.6	1
Identifiler® Plus	3130	GeneScan™ 600 LIZ™ Size Standard v2.0	0.3	8.7	1

Methods References

[1] Life Technologies Corporation. Quantifiler® Duo DNA Quantification Kit User's Manual 2012.

[2] Grgicak CM, Urban ZM, Cotton RW. Investigation of reproducibility and error associated with qPCR methods using Quantifiler® Duo DNA quantification kit. Journal of forensic sciences. 2010;55:1331-9.

[3] Life Technologies Corporation. AmpFlSTR® Identifiler® Plus PCR Amplification Kit User Guide 2015.

[4] Promega Corporation. PowerPlex® 16 HS System Technical Manual 2016.

[5] Qiagen Corporation. EZ1® DNA Investigator® Handbook 2014.

[6] Qiagen Corporation. QIAamp® DNA Investigator Handbook 2012.

[7] Life Technologies Corporation. DNA-free™ Kit DNase Treatment and Removal Reagents User Guide 2012.

[8] New England Biolabs. Digestion with NEBNext dsDNA Fragmentase 2015.

[9] Sweet D, Lorente M, Lorente JA, Valenzuela A, Villanueva E. An improved method to recover saliva from human skin: the double swab technique. Journal of forensic sciences. 1997;42:320-2.

[10] Life Technologies Corporation. Quantifiler™ HP and Trio DNA Quantification Kits User Guide 2017.

[11] Life Technologies Corporation. GlobalFiler™ PCR Amplification Kit User Guide 2016.

Sec 2. PROVEDIt Sample Summary

Sample Summary Table 1. The DNA extract mixtures (DEM) produced for the RD12-0002 sample set. The contributors and their respective ratios are listed in column 1. The kit(s) used to amplify the extracts is noted.

Mixture	Identifiler® Plus	PowerPlex® 16 HS
1,2-1:1	x	x
4,5-1:1	x	x
68,69-1:1	x	
1,2-1:2	x	x
4,5-1:2	x	
68,69-1:2	x	x
1,2-1:4	x	
4,5-1:4	x	
68,69-1:4	x	x
1,2-1:9	x	x
4,5-1:9	x	x
68,69-1:9	x	
1,2-1:19	x	
4,5-1:19	x	
68,69-1:19	x	x
68,69-1:49	x	
68,69-1:99	x	
1,2,5-1:1:1	x	x
6,44,66-1:1:1	x	
1,2,5-1:2:1	x	
6,44,66-1:2:1	x	x
1,2,5-1:4:1	x	x
6,44,66-1:4:1	x	x
1,2,5-1:9:1	x	
6,44,66-1:9:1	x	x
1,2,5-1:2:2	x	
6,44,66-1:2:2	x	x
1,2,5-1:4:4	x	
6,44,66-1:4:4	x	x
1,2,5-1:9:9	x	x
6,44,66-1:9:9	x	
4,5,1,2-1:1:1:1	x	x
4,5,1,2-1:1:2:1	x	x
4,5,1,2-1:1:4:1	x	x
4,5,1,2-1:1:9:1	x	x
4,5,1,2-1:2:2:1	x	x
4,5,1,2-1:4:4:1	x	x
4,5,1,2-1:9:9:1	x	x
1,2,4,5,6-1:1:1:1:1	x	x
1,2,4,5,6-1:1:2:1:1	x	x
1,2,4,5,6-1:1:4:1:1	x	x
1,2,4,5,6-1:1:9:1:1	x	x
1,2,4,5,6-1:1:2:2:1	x	x
1,2,4,5,6-1:1:4:4:1	x	x
1,2,4,5,6-1:1:9:9:1	x	x
1,2,4,5,6-1:2:2:2:1	x	x
1,2,4,5,6-1:4:4:4:1	x	x
1,2,4,5,6-1:9:9:9:1	x	x

Sample Summary Table 2. The whole blood mixtures (WBM) produced found within the RD14-0003 sample set. The contributors and their respective ratios are listed in column 1. The condition of DNA generated for each mixture is specified in the subsequent columns. All mixtures were amplified with Identifiler® Plus and GlobalFiler®.

Mixture	Pristine DNA	Degraded DNase I	Degraded Sonication	Damaged UV	Inhibited Humic Acid
31,32-1:1	x	x			
44,45-1:1	x	x	x	x	x
33,34-1:2	x	x			
39,40-1:2	x	x			
48,49-1:4	x	x			
40,41-1:4	x	x	x	x	x
35,50-1:9	x	x			
42,43-1:9	x	x	x	x	x
46,47,48-1:1:1	x	x	x	x	x
36,37,38-1:2:1	x	x			
49,50,29-1:4:1	x	x	x	x	x
41,42,43-1:9:1	x	x			
44,45,46-1:2:2	x	x			
30,31,32-1:4:4	x	x	x	x	x
47,48,49-1:9:9	x	x			
40,41,42,43-1:1:1:1	x	x	x	x	x
50,29,30,31-1:1:2:1	x	x			
44,45,46,47-1:1:4:1	x	x	x	x	x
32,33,34,35-1:1:9:1	x	x			
36,37,38,39-1:2:2:1	x	x	x		
33,34,35,36-1:4:4:1	x	x			
37,38,39,40-1:9:9:1	x	x			
48,49,50,29-1:4:4:4	x	x		x	x
30,31,32,33,34-1:1:1:1:1	x	x	x	x	x
43,44,45,46,47-1:1:2:1:1	x	x			
33,34,35,36,37-1:1:4:1:1	x	x	x		x
48,49,50,29,30-1:1:2:4:1	x	x			
31,32,33,34,35-1:1:2:9:1	x	x			
35,36,37,38,39-1:4:4:4:1	x	x	x	x	x
36,37,38,39,40-1:9:9:9:1	x	x			