RapidINTEL™ Sample Cartridge for blood and saliva samples

RapidHIT™ ID System v1.1.3

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Overview

This user bulletin is for use by customers who are running RapidINTEL™ Sample Cartridges on a RapidHIT™ ID System v1.1.3.

The first version of the system, RapidHIT™ ID System v1.0 with RapidHIT™ ID ACE GlobalFiler™ Express Sample Cartridges (ACE GFE cartridges), is designed for analysis of single-source, buccal reference samples. The system uses single-use sample cartridges for sample introduction, mixing, and PCR.

The RapidINTEL™ Sample Cartridge expands the capability of the system to allow analysis of blood and saliva samples. The RapidINTEL™ Sample Cartridge uses the same GlobalFiler™ Express chemistry as the buccal sample cartridges, with the following enhancements to increase sensitivity:

- PCR cycle number is increased from 28 to 32 PCR cycles
- Lysis buffer volume is decreased from 500 µL to 300 µL

Note: Because of the increased sensitivity, DNA profiles from the RapidINTEL™ Sample Cartridges may require more manual review than DNA profiles from the ACE GFE cartridges.

IMPORTANT! The RapidINTEL™ Sample Cartridge is recommended for use with blood and saliva samples only. Epithelial touch samples were also tested, but successful results were not obtained.

The RapidINTEL™ Sample Cartridge runs on the RapidHIT™ ID System v1.1.3.

Threshold optimization and validation

The studies described in this user bulletin were performed in 2 phases:

- Optimize system threshold settings to address the increased sensitivity of the RapidINTEL™ Sample Cartridge (see "Development of RapidINTEL™ system thresholds" on page 6).
- Perform full validation studies using the optimized threshold settings (see "Objective of the validation" on page 8).

Instrument and system background information

Traditional vs. RapidHIT™ ID forensic workflows

In a traditional Applied Biosystems™ forensic workflow, Thermo Fisher Scientific validates an STR kit on a variety of samples. The validation data is provided to customers for reference when they optimize system thresholds for peak detection, genotyping, and quality value flagging.

In the RapidHIT™ ID workflow, Thermo Fisher Scientific optimizes and validates system thresholds on a specific verification sample set, then performs complete validation studies on the verification sample set and a variety of other samples. The customer does not have access to system threshold settings.

Primary analysis with RapidHIT™ ID

Primary analysis is automatically performed on the RapidHIT™ ID System v1.1.3 by an internal version of the GeneMarker™ HID STR Human Identity Software on the instrument. The software uses system threshold settings to perform the analysis, then the instrument displays a result (Table 1).

System thresholds define criteria such as minimum peak height ratio and stutter filter percentages (for more information, see "Development of RapidINTEL™ system thresholds" on page 6). If one or more system thresholds are not met:

- One or more internal quality score flags are triggered (see "Quality score flags" on page 4). These quality score flags are not viewable on the instrument.
- A ✓ result is generated on the instrument. The sample requires manual *secondary analysis* in the RapidLINK™ Software/GeneMarker™ HID software.

Table 1 Primary analysis results on the RapidHIT™ ID system

Status	DNA profile is generated	Action
Green ✓	Yes	Green: All peaks and markers in the DNA profile met system threshold criteria.
Yellow ✓	Yes	Samples only, not positive or negative controls. Yellow: One or more system threshold criteria was not met. An internal quality score flag is triggered (for more information, see "Quality score flags" on page 4).
Red ✗	No	Red: The DNA profile was not generated.

Note: The Y-markers (Y indel and DYS391) are not considered during primary analysis. They are considered during secondary analysis. Yindel and DYS391 markers are not included in the auto-generated CMF file from the GeneMarker™ HID software. If needed, you can manually generate a CMF file that includes the markers.

Secondary analysis with RapidLINK™/GeneMarker™ HID software

When primary analysis on the instrument yields a ✓, it indicates that quality score flags are present and the sample requires manual *secondary analysis* in the RapidLINK™ Software.

A ✓ primary analysis result from the instrument is marked with → (requires review) in the RapidLINK™ Software.

When a sample is selected for review in the RapidLINK™ Software, a version of the GeneMarker™ HID STR Human Identity Software opens on the RapidLINK™ Software computer. The DNA profile can be reviewed and edited in the GeneMarker™ HID software. If a sample is determined to be acceptable after review (for example, a single-source sample that was flagged because of a heterozygote peak height imbalance), a CMF file can be manually exported for database searching.

For more information, see *RapidLINK™ Software v1.0 User Guide* (Pub. No. MAN0018038), "Edit allele calls and save changes to the Match database".

Quality score flags

The RapidHIT™ ID system and the RapidLINK™ Software run an internal version of the GeneMarker™ HID STR Human Identity Software that evaluates DNA profiles. Quality score flags are triggered if peaks do not meet system thresholds. See Table 2.

Table 2 GeneMarker™ quality score flags

Flag	Name	Description
OL	Off Ladder	**Marker** indicator: Allele is outside a marker range.
OB	Out of Bin	**Bin** indicator: Allele is not within a bin in the marker range.
BC	Bin Conflict	**Bin** indicator: More than 1 allele is within a bin in the marker range.
PL	Ploidy	**Mixture** indicator: More than the maximum number of expected peaks is detected in a single-source DNA profile or within a marker . The lowest peak at the marker displays the PL flag. **Note:** The PL flag does not differentiate between haploid and diploid markers.
IHE	Inconclusive Heterozygous	**Heterozygous minimum peak height** indicator: Peak intensity is below the heterozygous inconclusive range set for this locus.
IMB	Heterozygote Imbalance	**Minimum peak height ratio** indicator: Peak intensity does not exceed the minimum percentage of the major peak within the marker.
IHO	Inconclusive Homozygous	**Stochastic threshold indicator**: Peak intensity is below the homozygous inconclusive range set for this locus.

Considerations for secondary analysis in the RapidLINK™ Software/ GeneMarker™ HID software

- The increased sensitivity provided by the RapidINTEL™ Sample Cartridge can exacerbate stochastic effects in low-level samples. Although system thresholds are set to minimize stochastic effects, secondary analysis will be required for more samples than are required for ACE GFE cartridges.
- Evaluate all markers in a sample, do not independently evaluate allele calls for individual markers. A ✔ result from the instrument is generated if *any one* of the system thresholds is not met.
- Because a single marker can prevent a sample from passing primary analysis, a sample could theoretically generate a full genotype and still not pass primary analysis (for example, a marker might contain a slightly elevated stutter peak). To obtain a true evaluation of a system's efficiency, it is important to measure not only the primary analysis pass rate, but also the number of correctly identified peaks/markers that do not trigger quality score flags.
- Because of the GlobalFiler™ Express kit design (more primers at higher molecular weight to compensate for the loss of high-molecular weight fragments in the direct workflow), you may observe a reverse ski-slope pattern across markers in the electropherogram.
- The Y indel and DYS391 markers are not considered during primary analysis on the instrument.

  Note: The Y indel and DYS391 markers are not included in the CMF file that is auto-generated by the GeneMarker™ HID software and do not affect database searches.
- A locus-specific filter is not applied to the Y indel and DYS391 markers in female samples. Therefore, pull-up artifact peaks may be called. These alleles can be deleted during secondary analysis.

The following interpretation guidelines for difficult samples were defined during validation and were used for secondary analysis of validation data. Use these guidelines as a starting point for your laboratory standard operating procedure.

Table 3 Guidelines for allele interpretation and database searching that were developed and used during validation

Observation	Action
Inconclusive Heterozygous (IHE) flag is triggered because: • One or both peaks in a heterozygous marker are below the minimum heterozygous peak height threshold. • The peak height ratio is less than the minimum peak height ratio threshold.	Confirm that neither peak is due to a mixture by assessing the profile for presence of a second contributor.
	Confirm that 2 peaks are present at the marker.
	Check for marker drop-out anywhere in the DNA profile.
	Confirm that the average peak height across the profile is approximately ≥250 RFU. **Note:** During validation, stochastic effects were consistently observed at ≤250 RFU in disparate mixture and in some single-source samples.
	Ensure that samples in the stochastic range (<1,600 RFU) have a peak height ratio >30%.
	If all condtions listed above are verified, confirm heterozygous pair.

Observation	Action
A single allele in a marker is below the 1,600 RFU stochastic threshold	A single peak that is below the stochastic threshold may not be a true homozygote. It is more likely a heterozygous allele. Ignore the peak, do not add the allele to the profile.[1]
Peaks are below the 50 RFU analytical threshold	Ignore the peaks. Data below the 50 RFU analytical threshold can be baseline noise.
Peaks are below the locus-specific filter (21% for samples)	Ignore the peak, do not add the allele to the profile.[1]
Stutter peak exceeds the locus-specific stutter threshold	Delete the peak after confirming it is a stutter peak.

[1] Guideline was followed during secondary analysis. Your laboratory standard operating system may differ.

Development of RapidINTEL™ system thresholds

Specific system thresholds are required to address the increased sensitivity of the RapidINTEL™ Sample Cartridge (which can introduce artifacts such as stutter and allele drop-in, or stochastic effects that occur with amplification of limited DNA amounts).

IMPORTANT! System thresholds were set so that all questionable peaks/markers are flagged during primary analysis and require secondary analysis by a forensically trained analyst.

The system threshold development process included the following steps:

1. Prepare a verification sample set: Process different volumes of blood and saliva from 97 individuals to generate 398 single-source samples (see Table 6 on page 11).
2. Run all samples on 6 RapidHIT™ ID System v1.1.3 (“Instruments and software“ on page 9).
3. Establish system thresholds for primary analysis. For more information, see “Development of RapidINTEL™ system thresholds“ on page 6 and Table 4.
4. Confirm system thresholds with secondary analysis by reviewing all sample data in the RapidLINK™ Software to:

   Note: Two forensically trained analysts performed a blind review on the verification sample set. The results for the blind reviews were compared and results were reported only for results upon which both analysts agreed.

   - Confirm that all samples that passed primary analysis (result) generated full, high quality DNA profiles
   - Review all samples that did not pass primary analysis (result) and optimize system thresholds as needed (see Table 4).
   - Ensure that mixtures triggered quality score flags (described in “Quality score flags“ on page 4).
   - Confirm that negative controls fail primary analysis (result) if signal >50 RFU analytical threshold is present.

Table 4 RapidINTEL™ Sample Cartridge on RapidHIT™ ID System v1.1.3 system thresholds

Thresholds and filters	Setting	Quality score flag that is triggered if the threshold is not met (see "Quality score flags" on page 4
Analytical threshold	50 RFU	—
Stochastic threshold	1,600 RFU	IHO Inconclusive homozygous
	50 RFU for Y indel and DYS391	
Minimum peak height ratio threshold	40%	IMB Heterozygous imbalance
	99% for Y indel and DYS391[1]	
Minimum heterozygous peak intensity threshold	640 RFU	IHE Inconclusive Heterozygous
Stutter filters	Locus-specific	—
Locus-specific filter	21%	—
	30% for the positive control	
Maximum number of expected peaks in a marker	2	PL Ploidy
Global filter (between loci)	21%	—
	30% for the positive control	
Minimum off-ladder (OL) intensity	30 RFU	OL Off-ladder

[1] Minimum peak height ratio for Y markers was set to 99% to flag mixture samples at these hemizygous markers.

For more information on system thresholds, see "Appendix A: RapidINTEL™ system threshold development data" on page 50.

Objective of the validation

The objective of the validation was to assess the performance of the RapidINTEL™ Sample Cartridges on the RapidHIT™ ID System v1.1.3 when analyzing high quality, single-source blood and saliva samples. The validation was performed according to guidelines from the *Scientific Working Group for DNA Analysis Methods* (SWGDAM, December, 2016). The following studies were performed:

- Sizing precision and accuracy
- Genotyping concordance
- Sensitivity
- Contamination
- Mixture
- Mock casework
- Non-allelic peak assessment
- Comparison of primary analysis results for the ACE GFE cartridge and the RapidINTEL™ Sample Cartridge, and evaluation of secondary analysis allele recovery for the RapidINTEL™ Sample Cartridge.

The validation studies assessed:

- Overall performance using the RapidINTEL™ system thresholds.
- Accurate detection and flagging of peaks that do not comply with the RapidINTEL™ system thresholds.
- Ability to detect mixtures and the presence of contamination.
- Primary analysis pass rate (the percentage of samples that meet all system thresholds and generate full, single-source profiles without manual review).
- For samples with ✓ or ✓ results, the number of correctly called peaks/markers that are identified during primary analysis.
- Secondary analysis allele recovery rate (the percentage of markers that can be correctly called with manual review).

In addition, sample input amounts were assessed to determine optimal sample type and amounts that provide accurate results.

Materials and methods

Instruments and software

Six RapidHIT™ ID System v1.1.3 instruments were used for validation studies. All instruments were not used in every study; however, each instrument was used in at least one study.

Before validation studies were run, an instrument ladder cartridge and a positive control cartridge were run on all instruments.

Component	Cat. No. or version number
RapidHIT™ ID system	v1.1.3
RapidINTEL™ Sample Cartridge	A43942
RapidLINK™ Software	v1.1.5
GeneMarker™ HID STR Human Identity Software	v2.9.5 (embedded in the RapidLINK™ Software)

Thermal cycler and injection parameters

Thermal cycler parameters were optimized using a Design of Experiments (DOE) approach that identifies the combination of temperatures and hold times that produce the best assay performance. Optimal assay performance was determined through evaluation of assay sensitivity and peak height balance.

Table 5 RapidINTEL™ Sample Cartridge thermal cycler conditions (controlled internally by the RapidHIT™ ID System v1.1.3)

Step	Temp (°C)	Time (second)
Activation	95°C	60 seconds
Denaturation	94°C	3 seconds
Annealing	61°C	30 seconds
Extension	61.5°C	30 seconds
Final Extension	60°C	480 seconds
Cycle number	32	

The RapidHIT™ ID System v1.1.3 uses the same injection parameters as RapidHIT™ ID System v1.0 (5 kV 8 s injection).

Samples and sample collection

Swabbed samples were collected with Puritan™ 3" sterile standard cotton swabs with a semi-flexible polystyrene handle.

The samples were prepared as follows:

- **Verification sample set, sensitivity, and mixture samples**—The referenced volume of blood or saliva (Table 6) was pipetted directly onto the swab and allowed to dry before processing (Figure 3).
- **Mock-casework samples**—
  - Blood and saliva samples were deposited on various substrates, allowed to dry, and then swabbed (Figure 3). Punches were obtained from denim and some cotton samples.
  - Cigarette butt and gum samples were swabbed directly, or were cleaned, handled by a single individual, then swabbed.
  - Hair sample was pulled from the head, inspected for the presence of root, then placed in a sample cartridge.
  - Touch samples were cleaned, handled by a single individual, then swabbed.

A dual wet-swab technique was used for all swabs (Figure 1 A) unless otherwise indicated.

One swab from each collection was added to a RapidINTEL™ Sample Cartridge, then run on a RapidHIT™ ID System v1.1.3. The other swab was used for the cartridge comparison study (see "Cartridge comparison: ACE GFE cartridge to RapidINTEL™ Sample Cartridge" on page 43).

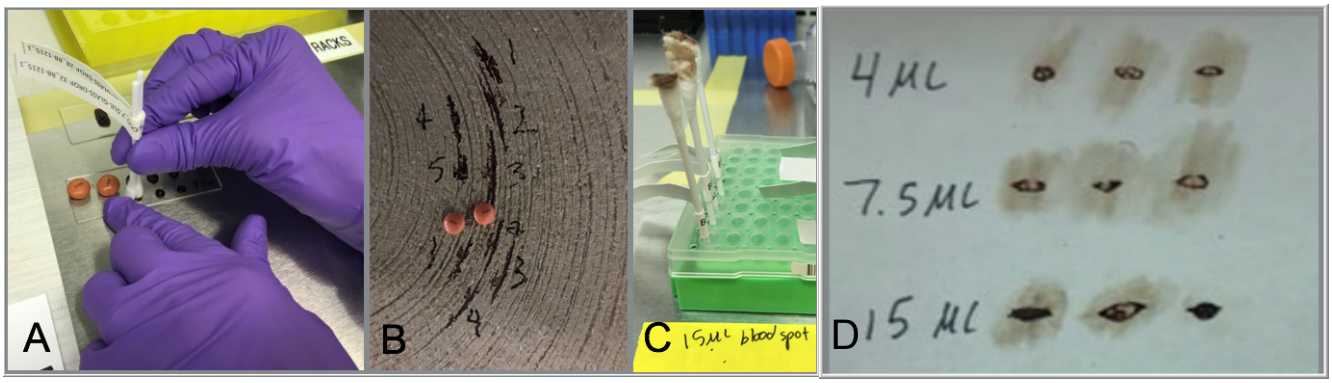


Figure 1 Sample collection examples

- (A) Dual wet-swab technique for collecting blood on glass samples: 2 swabs are held together with swab heads touching, wetted with sterilized de-ionized water, pressed firmly against the sample, then moved in a circular motion for 10–15 seconds to collect the sample.
- (B) 15 µL of blood on a tarred surface (parking lot or road).
- (C) Collection of the blood from the tarred surface caused the cotton to pull away from the tightly wound swab head. When adding a swab with a disrupted head to a sample cartridge, make sure to push the swab into the cartridge until the head is at the bottom of the swab receptacle (Figure 2).
- (D) Blood on drywall after dual wet-swab collection.

Figure 2 Sample cartridge

① Ensure the swab head is at the bottom of the swab receptacle

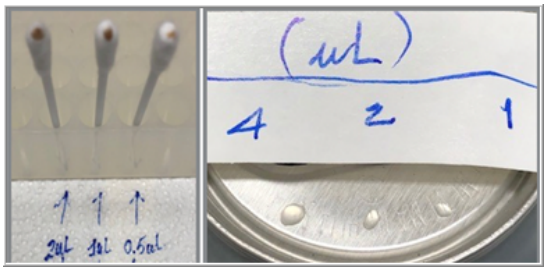


Figure 3 Blood on swabs at 2, 1, and 0.5 µL (left) and saliva on the top of a soda can at 4, 2, and 1 µL (right)

Verification sample set

The verification sample set included samples from 46 unique blood donors and 51 unique saliva donors with varying sample input. The total number of samples processed was 398.

Table 6 Verification sample set (n=398)

Blood			Saliva		
Sample	**Volume (µL)**	**# of replicates per volume**	**Sample**	**Volume (µL)**	**# of replicates per volume**
BB-1055	0.25, 0.5, 1, 2	1	DNR-1	1, 2, 4, 6	1
BB-1063	0.25, 0.5, 1, 2	1	DNR-2	1, 2, 4, 6	1
BB-1069	0.5, 1, 2	1	DNR-3	1, 2, 4, 6	1
BB-1076	0.5, 1, 2	1	DNR-4	1, 2, 4, 6	1
BB-1080	0.25, 0.5, 1, 2, 4	1	DNR-5	1, 2, 4, 6	1
BB-1117	0.25, 0.5, 1, 2, 4	1,2,2,2,1	DNR-6	1, 2, 4, 6	1
BB-1141	0.5, 1, 2, 4	1	DNR-7	1, 2, 4, 6	1

Blood			Saliva		
Sample	**Volume (µL)**	**# of replicates per volume**	**Sample**	**Volume (µL)**	**# of replicates per volume**
BB-1144	0.5, 1, 2, 4	1	DNR-8	1, 2, 4, 6	1
BB-1160	0.5, 1, 2, 4	1	DNR-9	1, 2, 4	1
BB-1169	0.5, 1, 2, 4	1	DNR-10	1, 2, 4, 6	1
BB-1180	0.5, 1, 2, 4	1	DNR-11	1, 2, 4, 6	1
BB-1193	0.5, 1, 2, 4	1	DNR-12	1, 2, 4, 6	1
BB-1195	0.5, 1, 2, 4	1	DNR-13	1, 2, 4, 6	1
BB-1196	0.5, 1, 2, 4	1	DNR-14	1, 2, 4, 6	1
BB-1206	0.5, 1, 2	1	DNR-15	1, 2, 4, 6	1
BB-1208	0.5, 1, 2, 4	1	DNR-16	1, 2, 4, 6	1
BB-1215	0.5, 1, 4	1,1,2	DNR-17	1, 2, 4, 6	1
BB-1220	0.5, 1, 2, 4	1	DNR-18	1, 2, 4, 6	1
BB-1222	0.5, 1, 2, 4	1	DNR-19	1, 2, 4	1
BB-1225	0.5, 1, 2, 4	1	DNR-20	1, 2, 4	1
BB-1226	0.5, 1, 2, 4	1	DNR-21	1, 2, 4, 6	1
BB-1230	0.5, 1, 2, 4	1	DNR-22	1, 2, 4, 6	1
BB-1233	0.5, 1, 2, 4	1	DNR-23	1, 2, 4, 6	1
BB-1240	0.5, 1, 2, 4	1	DNR-24	1, 2, 4, 6	1
BB-1247	0.5, 1, 2, 4	1	DNR-25	1, 2, 4, 6	1
HC-1005	0.5, 1, 2, 4	1	DNR-26	1, 2, 4, 6	1
HC-1017	0.5, 1, 2, 4	1	DNR-27	1, 2, 4, 8, 12	1
HC-1032	1, 2, 4	1	DNR-28	1, 2, 4	1
HC-1047	0.5, 1, 2, 4	1	DNR-29	1, 2, 4, 6	1
HC-1049	0.25, 0.5, 1, 2, 4	1,2,2,2,1	DNR-30	1, 2, 4, 6	1
IB-0079	0.25, 0.5, 1, 2, 4	1	DNR-31	1, 2, 4, 6	1
IB-0221	1, 2, 4	2,2,2	DNR-32	1, 2, 4, 6	1
IB-0336	1, 2, 4	2,2,2	DNR-33	1, 2, 4, 6	1
IB-0382	0.5, 1, 2, 4	1	DNR-34	1, 2, 4, 6	1
IB-0506	0.5, 1, 2, 4	1	DNR-35	1, 2, 4, 6	1
IB-0635	0.5, 1, 2, 4	1	DNR-36	1, 2, 4, 6	1

Blood			Saliva		
Sample	**Volume (µL)**	**# of replicates per volume**	**Sample**	**Volume (µL)**	**# of replicates per volume**
IB-0663	0.25, 0.5, 1, 2, 4	1	DNR-37	1, 2, 4, 6	1
IB-0666	0.5, 1, 2, 4	1	DNR-38	1, 2, 4, 6	1
IB-0694	0.5, 1, 2, 4	1	DNR-39	1, 2, 4, 6	1
IB-0819	1, 2, 4	1	DNR-40	1, 2, 4, 6	1
IB-0851	0.5, 1, 2, 4	1	DNR-41	2, 4, 6	1
IB-0902	0.25, 0.5, 1, 2, 4	1	DNR-42	1, 2, 4, 6	1
IB-0908	0.25, 0.5, 1, 2, 4	1	DNR-43	1, 2, 4, 6	1
IB-0921	0.25, 0.5, 1, 2, 4	1	DNR-44	1, 2, 4, 6	1
IB-0926	0.25, 0.5, 1, 2, 4	1	DNR-45	1, 2, 4, 6	1
IB-0981	0.5, 1, 2, 4	1	DNR-46	1, 2, 4, 6	1
—	—	—	DNR-47	1, 2, 4, 6	1
—	—	—	DNR-48	1, 2, 4, 6	1
—	—	—	DNR-49	1, 2, 4, 6	1
—	—	—	DNR-50	1, 2, 4, 6	1
—	—	—	DNR-51	1, 2, 4, 6	1

Test cases

Study	Samples	Total number of samples	Method and analysis
Sizing precision and accuracy	25 allelic ladder cartridges	5 allelic ladder cartridges were run on 5 instruments.	• Calculated the standard deviation of the size values for each allele in the allelic ladder across 5 instruments. • Evaluated the allelic ladder results.
Genotyping concordance	• 81 positive control cartridges • 1,216 samples from the population, casework, and mixture studies	• 81 positive control cartridges were run on 6 instruments. • See sensitivity, mixture, and mock casework studies for the number of samples.	• Compared sample and control genotypes to previously generated 3500xL results or expected positive control genotype. • Evaluated the peak height, peak height ratio, color balance, and signal variation for the positive control data.
Sensitivity population study 1	• 10 unique blood donors at 4, 2, 1, 0.5, 0.25, 0.125 µL, and 0.0625 volumes • 10 unique saliva donors at 8, 4, 2, 1, 0.5, and 0.25 µL volumes	409 samples were run on 6 instruments: • 201 blood samples • 208 saliva samples	Evaluated the primary analysis pass rate.
Sensitivity population study 2	Verification sample set	See Table 6 on page 11.	• Evaluated the primary analysis pass rate. • Evaluated peak height, peak height ratios, and allele drop-out for the verification sample set. • Evaluated secondary analysis marker recovery.
Contamination	42 negative control cartridges	42 negative control cartridges were run on 6 instruments.	• Ran negative controls after running high-concentration samples. • Evaluated peaks above the 50 RFU analytical threshold.

Study	Samples	Total number of samples	Method and analysis
Mixture	Saliva mixture of 2 donors: 1:0, 1:1, 1:3, 1:8, 8:1, 3:1, 0:1 at 4 µL total volume	44 samples were run on 6 instruments: • 2 replicates of 1:0 and 0:1 samples • 8 replicates of other sample ratios	• Evaluated the quality score flagging during primary analysis. • Evaluated the number of unique donor alleles identified during primary analysis.
	Blood mixture: 1:1 1:2, 1:4, 1:8, 1:16, 16:1, 8:1, 4:1, at 1.5–0.66 µL total volume	80 samples were run on 6 instruments.	
Mock casework	• Blood samples from 7 different substrates • Saliva samples from 4 different substrates • Epithelial cell touch samples from 4 different substrates • Pulled hair sample **Note:** Touch samples were used to test the limitations of the system. Touch samples are not a supported sample type.	121 samples were run on 6 instruments: • Blood—69 • Saliva—35 • Touch—15 • Hair—3	• Evaluated primary analysis pass rate by sample and substrate type. • Evaluated secondary analysis marker recovery by sample and substrate type.
Non-allelic peak assessment	1,216 samples from the population, casework, and mixture studies	See sensitivity, mixture, and mock casework studies for the number of samples.	Evaluated non-allelic peaks, including pull-up and stutter peaks.
Cartridge comparison	• Blood mixtures • Mock-casework samples: – Blood on glass, drywall, tarred surface (road or parking lot), denim, white cotton – Saliva on cigarette butts – Swabs from gum, coffee cup, and water bottle	See mock-casework study and mixture study for the number of samples.	• Ran each sample using RapidINTEL™ Sample Cartridges and ACE GFE cartridges. • Evaluated primary analysis pass rate for both cartridges. • Evaluated allele recovery for RapidINTEL™ Sample Cartridges.

Results summary

Test case	Result
Sizing precision and accuracy	• Sizing precision <0.14 bp. • All allelic ladders passed.
Genotyping concordance	• 79 of 81 positive controls passed primary analysis (✓ result), which means they generated the expected 007 control DNA genotype. • 2 controls failed primary analysis (✗ result). • 99% of sample results (n=1,216) were concordant with data from the 3500xL Genetic Analyzer.
Sensitivity	• Peak heights and peak height ratios generally increased at higher concentrations. • ≥20 correct markers were identified during primary analysis with ≥2 µL of blood per sample. • ≥17 correct markers were identified during primary analysis with ≥6 µL. • 17–18% of markers in the verification sample set that generated quality score flags were recovered during secondary analysis.
Contamination	2 out of 42 negative controls yielded in-bin peaks above the analytical threshold: • 77 RFU (1) peak at Y indel, which was present in the previous sample run. • 65 RFU (13) peak at D8S1179 at a pull-up position of the size standard peak. Did not match the genotype of the previous sample run.
Mixture	The minor contributor was detected and flagged in: • 39 of 40 saliva mixtures • 78 of 80 blood mixtures
Pull-up	• 16 instances of pull-up were detected, 15 of the observed peaks were in the Y indel and DYS391 markers. • The average observed pull-up peak was 4.5% of the parent peak. When the outliers were removed, the average pull-up peak was 1.5%.
Stutter	37 elevated stutter peaks were observed above the locus-specific stutter thresholds, all of which triggered quality score flags.
Artifact	An n –5 nt artifact peak was intermittently observed at the TH01 locus.
Mock casework	• Marker and allele recovery for blood on non-porous substrates was generally better than blood on porous substrates. • 28% of blood, 67% of hair root, 14% of saliva, and 0 touch mock-casework samples generated a ✓ primary analysis result. • Secondary analysis increased marker recovery by 7% for blood, 8% for saliva, and 3% for touch samples.
Cartridge comparison	• **Blood and saliva mock casework samples**—RapidINTEL™ Sample Cartridges with secondary analysis generate an average of 25% more markers than ACE GFE cartridges. • **Blood mixture samples**—RapidINTEL™ Sample Cartridges with secondary analysis generate an average of 57% more markers than ACE GFE cartridges.

Precision and accuracy results

Sizing precision allows accurate and reliable genotyping. Precision was measured by calculating and plotting the standard deviation of the size values for each allele.

The standard deviations of the size values for 25 allelic ladders run on 5 instruments were all <0.14 bp. This observation confirms the ability of the system to accurately size allele differences of 1 bp across theGlobalFiler™ Express kit sizing range.

All allelic ladders yielded passing results, indicating that each allele was within the specified bin definition of the system.

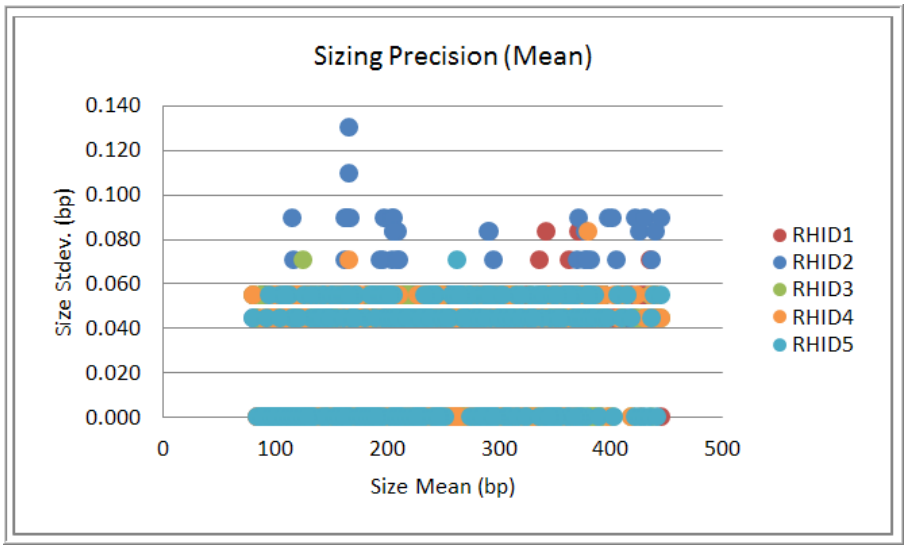


Figure 4 Sizing precision assessed by standard deviation of each allele in the allelic ladder run on 5 RapidHIT ID instruments.

Genotyping concordance: Concordance, peak height ratio, peak height, color balance, and variation comparisons

Results for positive controls

The expected genotype for 007 DNA control was generated for 79 positive control samples tested on 6 instruments. Two positive controls failed primary analysis (result on the intrument).

Positive control peak height ratio, peak height, color balance and signal variation were also evaluated for the 79 controls.

Table 7 Average peak height ratio (PHR) for 79 positive control samples calculated per heterozygote marker

Marker	Avg PHR	SD PHR
D3S1358	0.9083	0.071
vWA	0.7485	0.1174
D16S539	0.8689	0.0805
CSF1PO	0.8522	0.0992
AMEL	0.8259	0.1183
D8S1179	0.8548	0.0963
D21S11	0.7906	0.1187
D18S51	0.8761	0.0823
D2S441	0.8728	0.0759
D19S433	0.8416	0.0878
TH01	0.8547	0.099
FGA	0.8891	0.0721
D22S1045	0.7315	0.1086
D7S820	0.785	0.1083
SE33	0.7905	0.1115
D10S1248	0.855	0.0846
D1S1656	0.8307	0.0963
D12S391	0.8363	0.0909
D2S1338	0.8556	0.0923

The average peak height was calculated for the 79 positive controls for each dye channel and used to calculate intercolor balance (ratio of the average peak heights between dyes expressed as a percentage) and intracolor balance (ratio of the average lowest peak height for any allele by the average of the highest peak height for any

allele within a dye channel expressed as a percentage). All autosomal STR and amelogenin peak heights were normalized: homozygous alleles were divided by 2 and heterozygote peak heights were averaged; native peak heights were used for all Y-markers.

Signal variability was measured using %CV (the average SD for size standard peaks across injections divided by the mean of all size standard peaks) and is reported as a percentage.

Table 8 Average peak height, intracolor balance, intercolor balance, and variation as measured by %CV calculated from 79 positive control cartridges run on 6 instruments

Dye	Average Peak Height (RFU)	Intra-color Balance	Inter-color Balance	%CV
Blue	5,109	42%	23%	—
Green	3,476	35%	16%	
Yellow	5,460	40%	25%	
Red	4,707	36%	22%	
Purple	3,130	32%	14%	
Orange	—	—	—	10%

Results for samples from the sensitivity, mock casework, and mixture studies

1,199 of the 1,216 samples generated expected genotyping results based on the 3500xL data.

18 peaks were observed in 17 samples across 6 instruments that were not concordant with the 3500xL data.

- Samples 1 and 2 (run on different instruments) were flagged as out of bin (OB) instead of the expected allele calls. The migration difference of these two alleles was determined to be sample- and run-specific rather than an indication of a systemic issue.
- Sample 3 had 3 peaks at SE33: 27.2 (365 RFU), 28.2 (244 RFU), and 29.2 (205 RFU) respectively, which triggered the ploidy flag (PL). During secondary analysis, the 3 peaks were not edited because of the peak heights. The 3500xL-generated genotype was heterozygous 28.2, 29.2 at SE33 for this sample. It was hypothesized that this third peak could be elevated stutter or allele drop-in.

  Mulitple other markers in this sample triggered:
  - IHE flag for peaks >640 RFU minimum heterozygous peak intenstity threshold.
  - IHO flag for single peaks below the 1,600 RFU stochastic threshold.
- Sample 4 also triggered the IHO quality score flag because a peak at a different marker was below the stochastic threshold (1,600 RFU).

  The expected genotype at the TH01 locus was a 7 homozygote.

  Samples 4 and 5 generated a 5.3, 7 apparent heterozygous genotype. During secondary analysis, the heterozygous genotype was confirmed based on the a) peak height ratio of the two alleles (41% and 54%, both greater than the minimum peak height ratio) and b) the peak height of both alleles was >640 RFU minimum heterozygous peak intenstity threshold. Because these values exceed system

thresholds, no quality score flags were triggered and the peaks were not edited. However, when compared to the 3500xL data, the genotype was not concordant.

Of the 19 blood samples tested from this donor across sensitivity and casework studies, 37% showed the 5.3 stutter peak at TH01 above the 50 RFU analytical threshold. In two of the samples, the artifact did not trigger the IHE or IMB flags, therefore the peak was called as 5.3, 7. This DNA-dependant n –5 artifact has been observed in other samples and the positive control.

Figure 5 is an example where the TH01 artifact was not edited and was concluded to be part of the genotype. Figure 6 is an example where the TH01 artifact was edited and not considered to be part of the genotype.

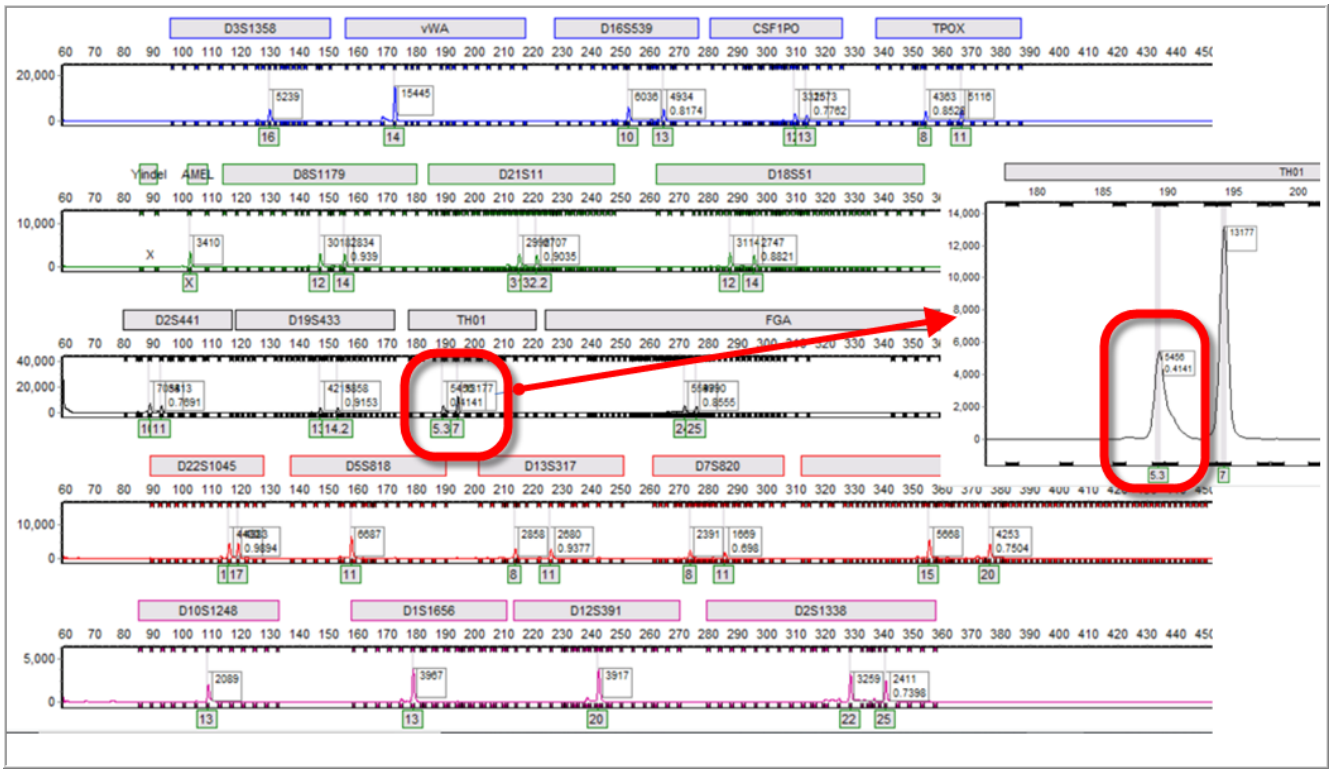


Figure 5 Electropherogram of the half-business-card-sized blood smear replicate 1, where the 5.3 was called at TH01. This peak is 5 nucleotides shorter than the 7 allele, and the peak height ratio between the two peaks is 41%. The inlay of TH01 shows the 5.3 peak called at 5,456 RFU and the main allele peak (7) at 13,177 RFU. The 5.3 peak was not deleted during secondary analysis.

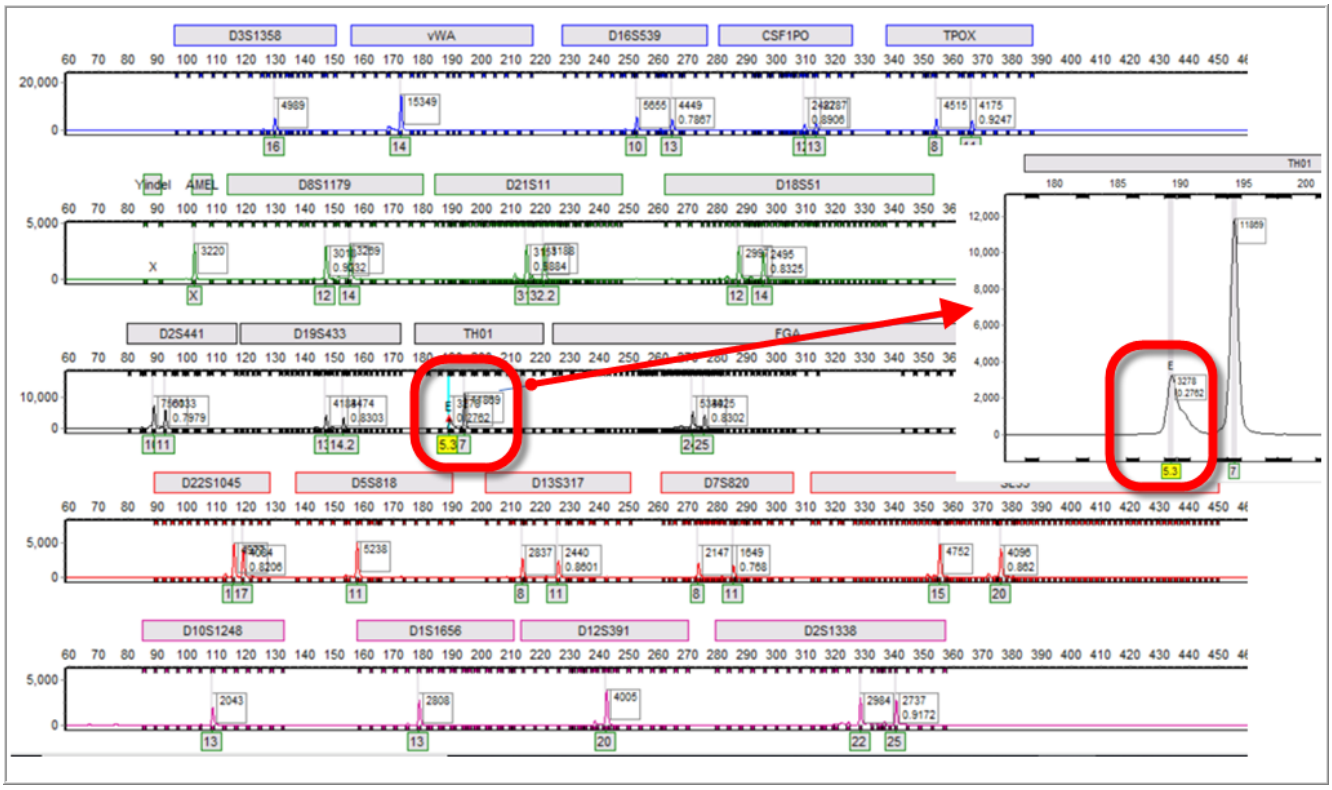


Figure 6 Electropherogram of the half-business-card-sized blood smear replicate 3, where the 5.3 was called and flagged at TH01 below the 40% peak height ratio flag (5.3 peak was 38% of the 7 allele) (marked as E=edited by user). This peak was deleted during secondary analysis.

- Samples 6–17 generated data with a false homozygote allele (a single peak at a marker above the 1,600 RFU stochastic threshold), when the true genotype at these markers was heterozygous. All of the samples had at least one other marker flagged during primary analysis; however, during secondary analysis the homozygote call was confirmed based on the peak height of the homozygote allele and drop-out of the heterozygote allele.

  Out of 10,437 markers evaluated across all samples, there were 13 observations in 12 samples of a single allele of a heterozygous pair above the stochastic threshold. This equates to a 0.1% false homozygote rate. This data confirms that the stochastic threshold of 1,600 RFU applied for the RapidINTEL™ Sample Cartridges is applicable across various DNA inputs for multiple sample and substrate combinations.

Table 9 Instances of false homozygotes detected above the stochastic threshold

Sample type	Volume	Marker	Peak height (RFU) of false homozygote
Blood	0.25 µL	D22S1045	2,031
	0.5 µL	TPOX	1,600
	1 µL	D2S1338	1,612
	1 µL	D21S11	1,706
		D5S818	1,738

Sample type	Volume	Marker	Peak height (RFU) of false homozygote
Blood	15 µL	vWA	1,902
	1 µL	SE33	3,109
	1 µL	D22S1045	2,474
	7.5 µL	D21S11	1,972
Saliva	2 µL	vWA	2,107
	4 µL	TPOX	1,890
	6 µL	D22S1045	2,345
	2 µL	FGA	1,944

Sensitivity results

Population study 1 used blood (at 7 different volumes) and buccal (at 6 different volumes) samples from 10 unique donors (Figure 7).

Population study 2 used blood samples (at 5 different volumes) from 46 unique donors and saliva samples from 51 unique donors (at 6 different volumes) (Figure 8 and Figure 9 and Table 10).

Both studies evaluated primary analysis pass rate (the number of ✅ results on the instrument) and the average number of correct, unflagged markers per sample after primary analysis.

In addition, population study 2 evaluated:

- Peak heights and peak height ratios for the blood and saliva samples (Figure 10 through Figure 13). Due to the sample size n=1 for the 8 and 12 µL volume saliva data, the two samples were excluded from peak height and peak height ratio calculations.
- Marker recovery after secondary analysis (Table 10 on page 24). Y indel and DYS391 are not included in the marker recovery evaluation; therefore, the total possible number of markers is 22.

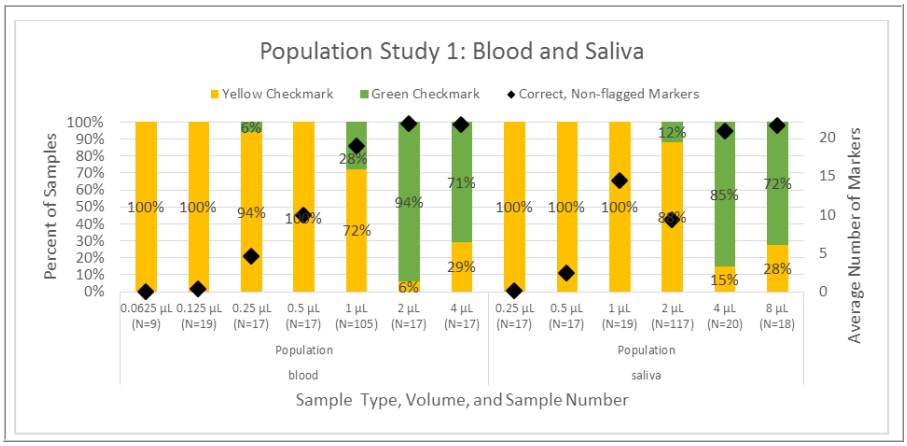


Figure 7 Population study 1: Primary analysis: Pass rate and average number of correct, unflagged markers for varying inputs of blood and saliva samples run on 6 instruments

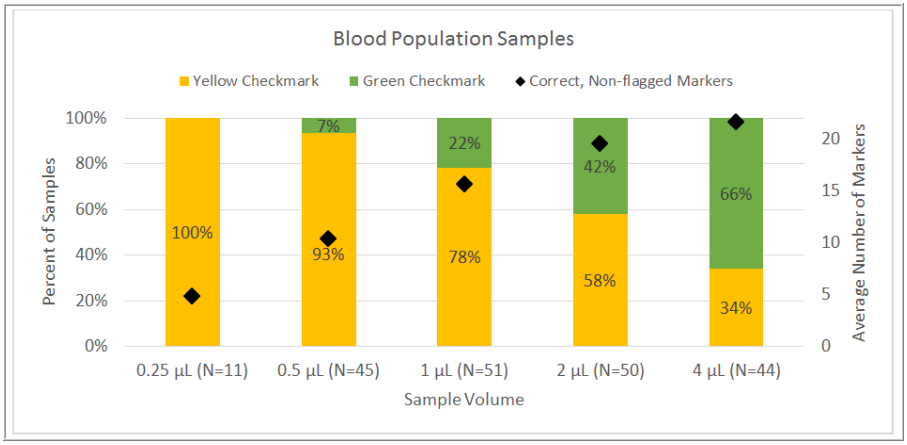


Figure 8 Population study 2: Primary analysis: Pass rate and average number of correct, unflagged markers for varying inputs of blood samples run on 6 instruments

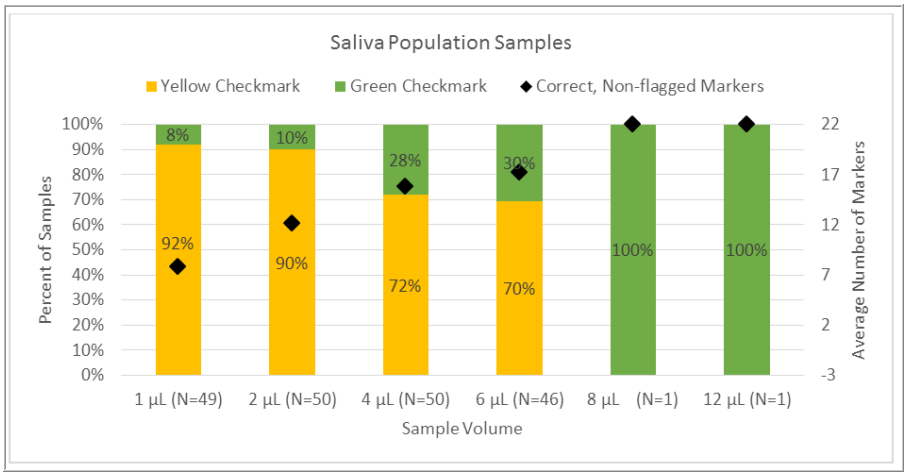


Figure 9 Population study 2: Primary pass rate and average number of correct, unflagged markers recovered for varying inputs of saliva samples run on 6 instruments

Overall, Table 10 demonstrates marker recovery increased 18% for blood samples and 17% for saliva samples after secondary analysis. The Y indel and DYS391 markers were not included in the calculations.

Table 10 Population study 2: Percent of correct, unflagged markers after primary analysis and markers recovered/ confirmed after secondary analysis for the 398 blood and buccal samples. The Y indel and DYS391 markers were not included; the 8 and 12 µL saliva samples were removed due to the limited sample size.

Sample type	Volume	Percent of correct, unflagged markers after primary analysis	Percent of correct, unflagged markers plus confirmed markers during secondary analysis
Blood	0.25 µL	16%	29%
	0.5 µL	34%	48%
	1 µL	49%	72%
	2 µL	58%	81%
	4 µL	77%	89%
Saliva	1 µL	19%	34%
	2 µL	35%	53%
	4 µL	44%	61%
	6 µL	55%	75%

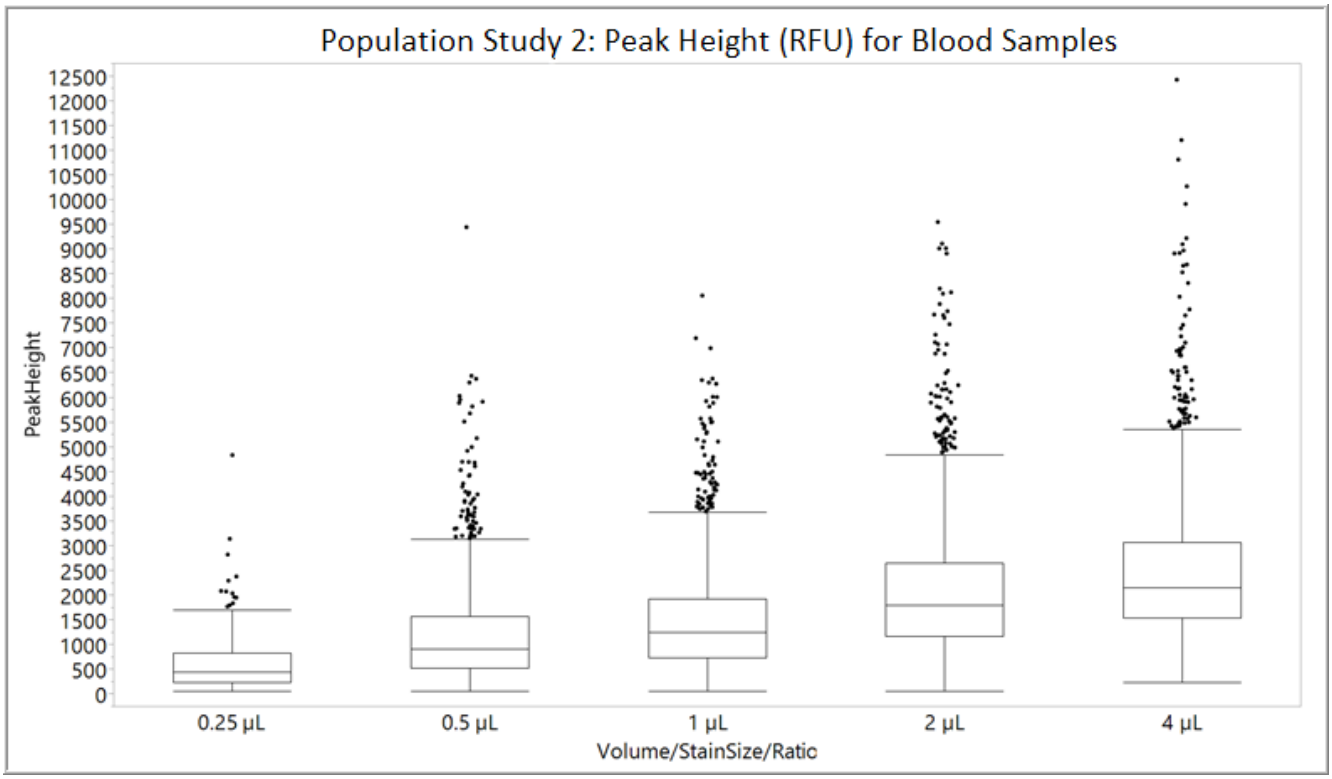


Figure 10 Peak height (RFU) for the population study 2 blood samples (n=201)

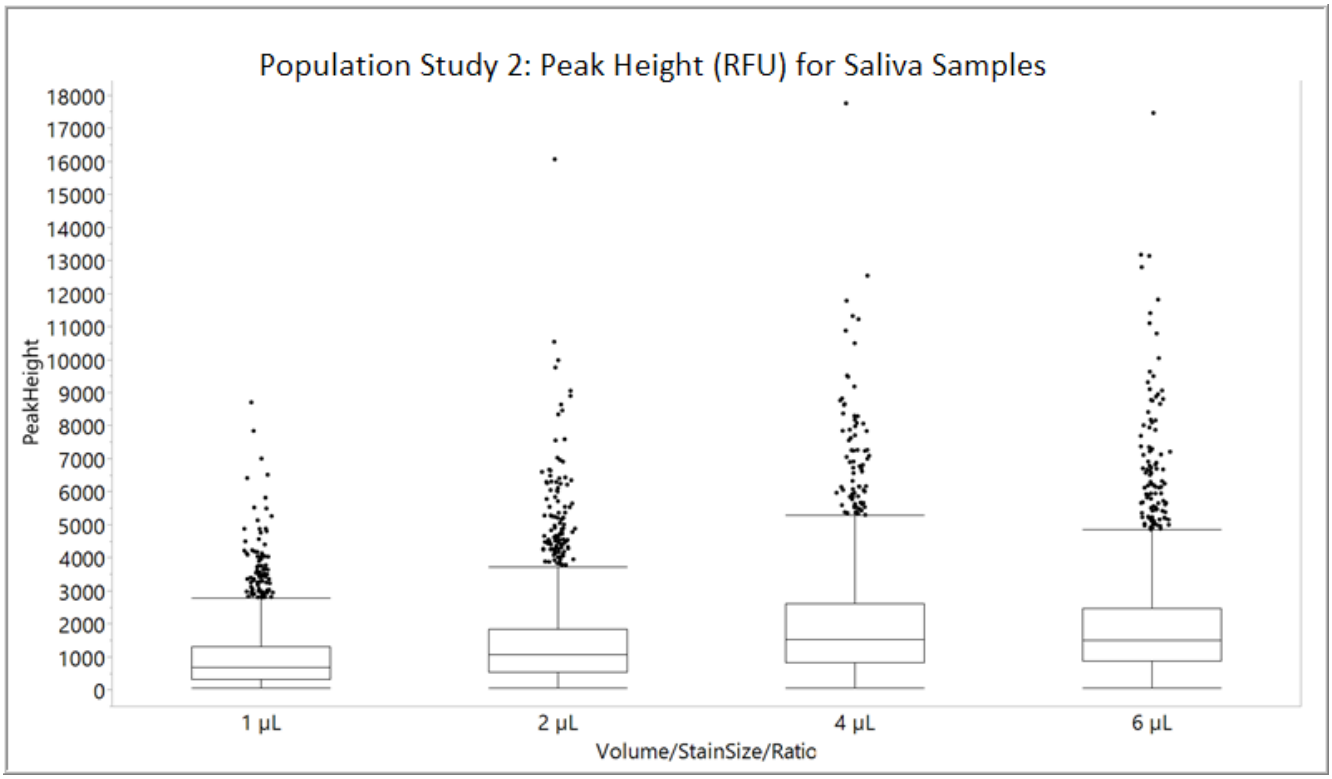


Figure 11 Peak height (RFU) for the population study 2 saliva samples (n=195, the 8 and 12 µL samples were excluded from analysis)

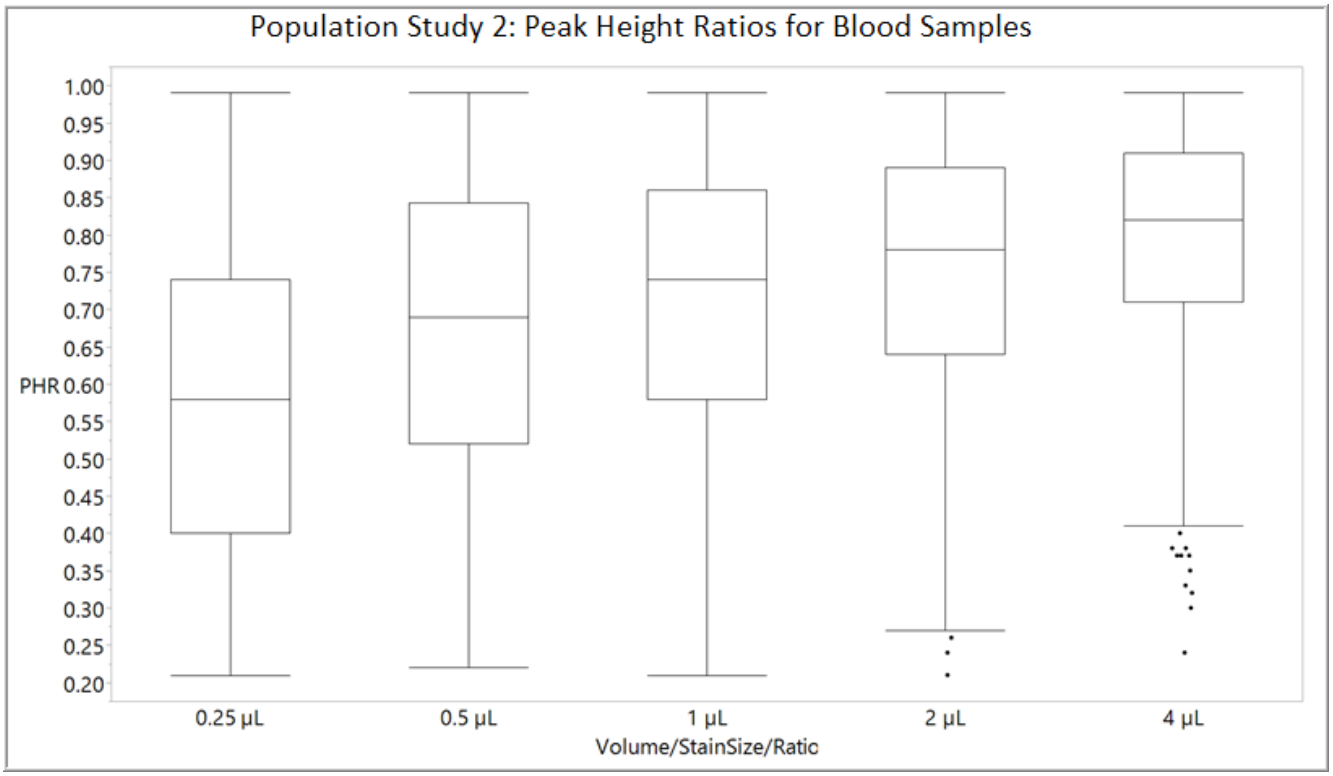


Figure 12 Peak height ratios for the population study 2 blood samples (n=201)

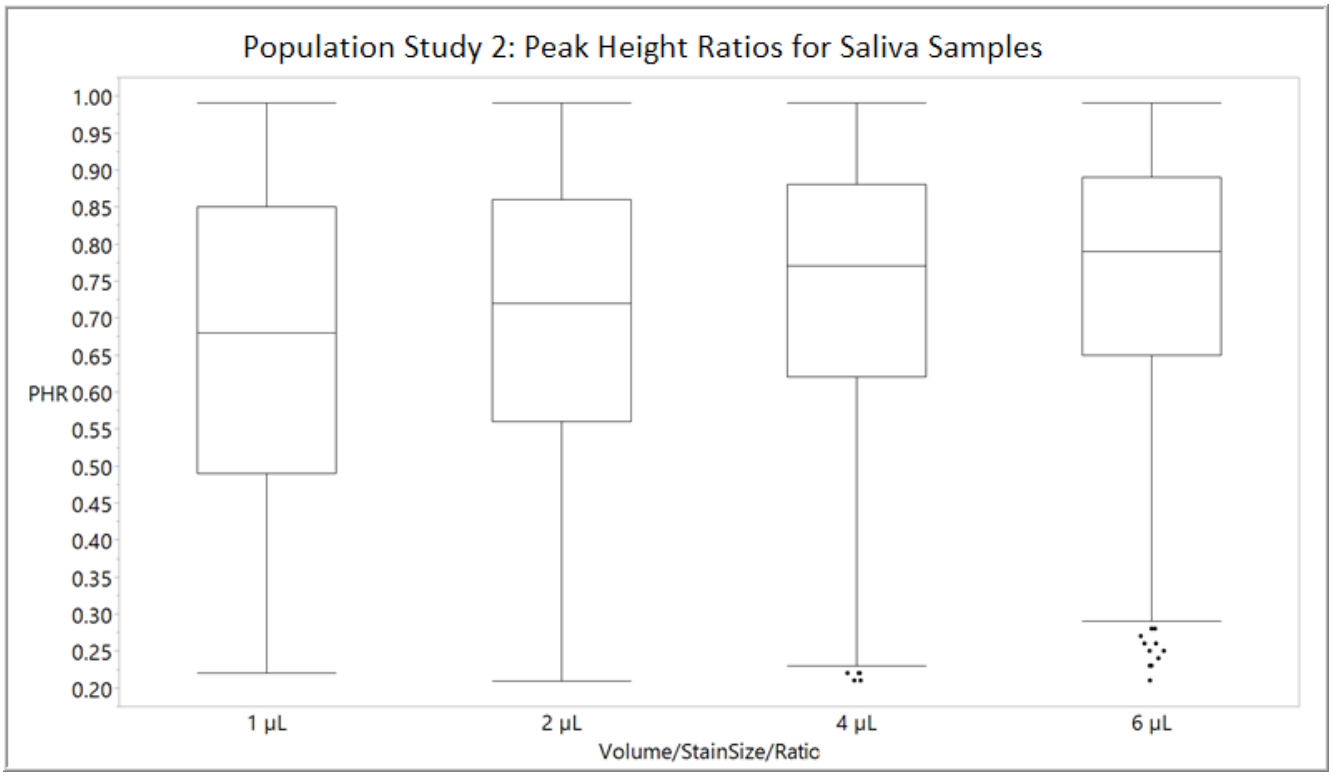


Figure 13 Peak height ratios for the population study 2 saliva samples (n=195, the 8 and 12 µL samples were excluded from analysis)

Mixture results

Three studies evaluated the performance of RapidINTEL™ Sample Cartridges with samples containing more than one contributor. Mixtures should trigger the ploidy flag (PL) in primary analysis if the peak height of the minor contributor peak in a mixture exceeds the analytical threshold and the locus-specific filter.

Mixture study 1

Mixture study 1 used a total of 4 µL of saliva from two donors run on 6 instruments at mixture ratios of donor A: donor B of 1:0, 1:1, 1:3, 1:8, 8:1, 3:1, 0:1. Primary analysis generated ✓ results (PL flag) for mixtures down to a 1:8 and 8:1 ratio. One of the eight 1:8 samples generated a ✓ result, which indicates that the mixture was not detected (Table 11 and Figure 15). All of the alleles in the ✓ 1:8 sample were from the major contributor. This data was analyzed with a 21% locus-specific filter, which can mask minor contributor alleles in low mixed-source ratio samples.

Table 11 Mixture study 1: Saliva mixture samples showing that 1 of the 1:8 mixture samples did not trigger the PL flag. As expected, the 0:1 and 1:0 single-source samples did not trigger the PL flag.

Mixture ratio	Donor A (µL)	Donor B (µL)	Yellow ✓	Green ✓	Replicates
0:1	0	1	1	1	2
1:0	1	0	1	1	2
1:1	2	2	18	—	8
1:3	1	3	8	—	8
1:8	0.45	3.55	7	1	8
3:1	3	1	9	—	8
8:1	3.55	0.45	8	—	8

The unique alleles that are not shared between donors for mixture study 1 are represented in Figure 14. There are 25 possible unique alleles for donor A and 26 possible unique alleles for Donor B.

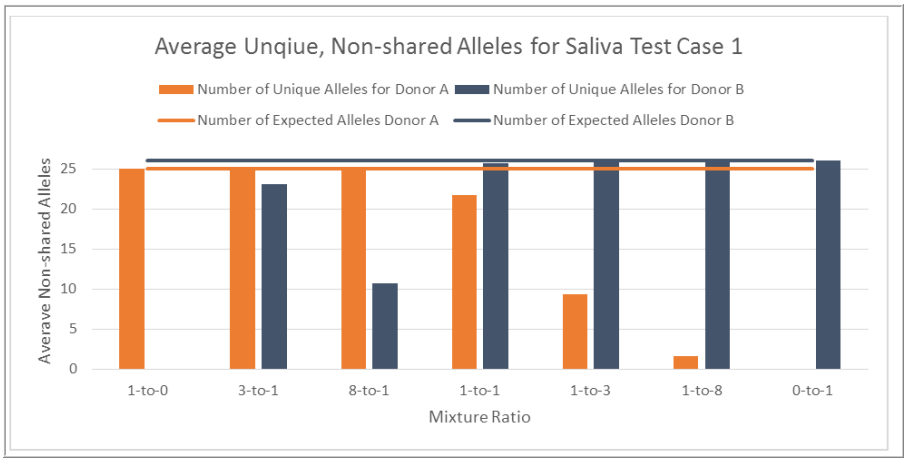


Figure 14 Mixture study 1: Average number of unique alleles detected for donor A and donor B in the saliva mixture samples. Two replicates were processed for the 1:0 and 0:1 and 8 replicates for all other mixture ratios.

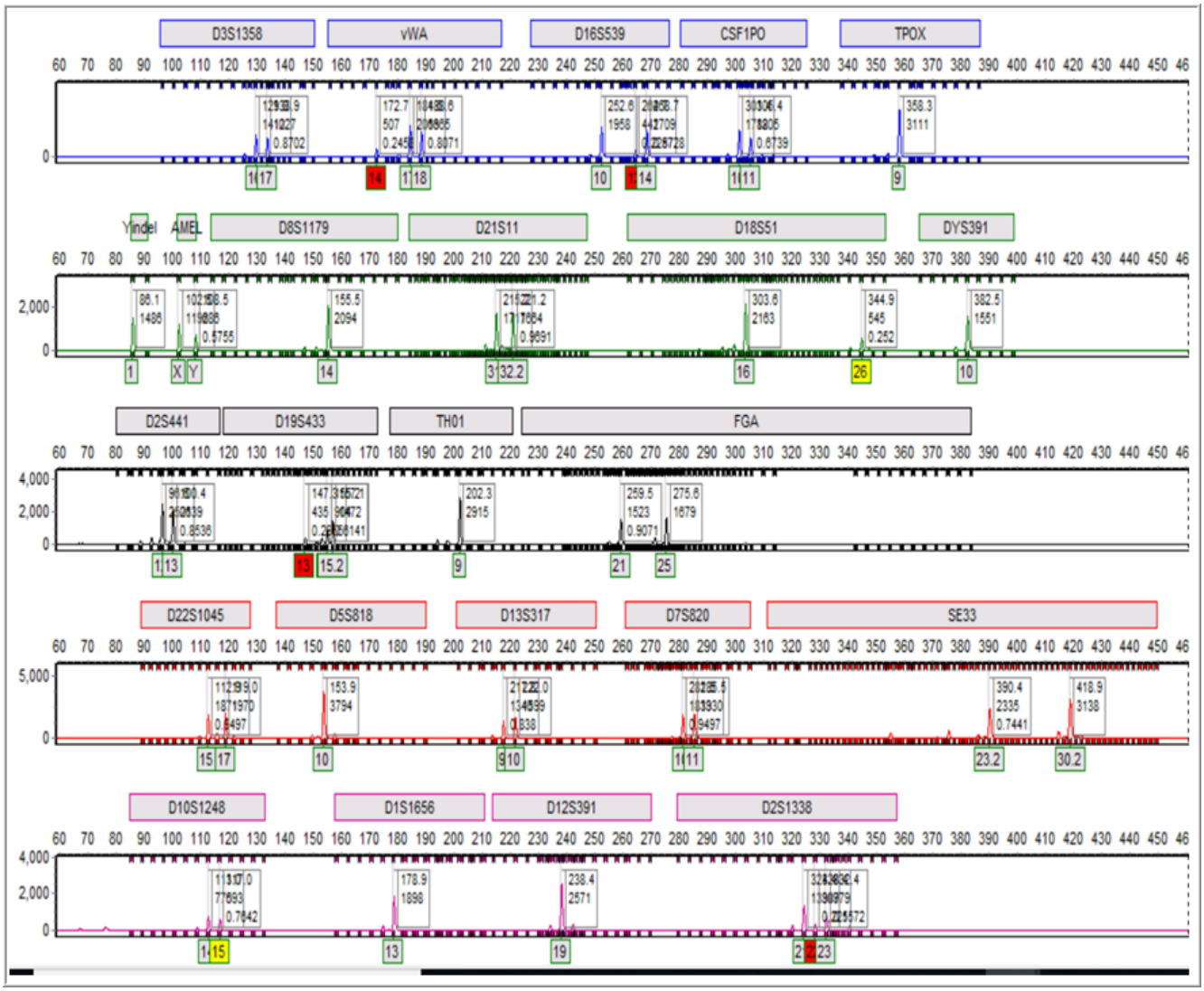


Figure 15 Mixture study 1: Electropherogram the ✔ 8:1 blood mixture sample in GeneMarker™ HID software, with 1 µL of Donor 1 and 0.125 µL of Donor 2. Ploidy flags (red PL allele labels) were triggered at 4 loci after primary analysis, which indicate a mixture. The D19S433 marker has an additional 15 major allele that is not visible in this image.

Mixture studies 2 and 3

Mixture studies 2 and 3 used varying inputs of blood from 2 donors on 6 instruments. Primary analysis generated ✓ results (PL flag) for mixtures down to 1:16 and 16:1 ratios. Two of the 5 (16:1) samples generated a ✓ result, which indicates that the mixture was not detected (Table 12 and Figure 18). All of the alleles in the ✓ 16:1 samples were from the major contributor. This data was analyzed with a 21% locus-specific filter which can mask minor contributor alleles in low mixed-source ratio samples.

Table 12 Mixture studies 2 and 3: Blood mixture samples showing that only two of the 16:1 mixture samples did not trigger the PL flag

Mixture ratio	Donor A (µL)	Donor B (µL)	Yellow ✓	Green ✓	Replicates
Test case 2: Higher input blood samples					
1:1	0.5	0.5	5	0	5
1:2	0.5	1	5	0	5
1:4	0.25	1	5	0	5
1:8	0.125	1	5	0	5
1:16	0.062	1	5	0	5
16:1	1	0.062	3	2	5
8:1	1	0.125	5	0	5
4:1	1	0.25	5	0	5
Test case 3: Lower input blood samples					
1:1	0.33	0.33	5	0	5
1:2	0.33	0.67	5	0	5
1:4	0.165	0.67	5	0	5
1:8	0.083	0.67	5	0	5
1:16	0.042	0.67	5	0	5
16:1	0.67	0.042	5	0	5
8:1	0.67	0.083	5	0	5
4:1	0.67	0.165	5	0	5

The unique alleles that were not shared between donors in mixture study 2 are represented in Figure 16. There are 30 possible unique alleles for donor A and 26 possible unique alleles for Donor B.

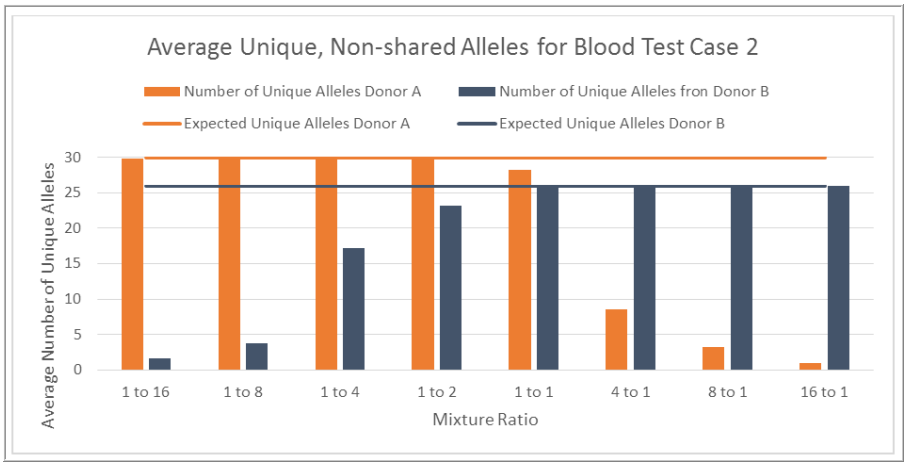


Figure 16 Mixture study 2: Average number of unique alleles detected for donor A and donor B in the higher input blood mixture samples. Five replicates processed for each mixture ratio.

The unique alleles that were not shared between donors for mixture study 3 are represented in Figure 17. There are 26 possible unique alleles for donor A and 30 possible unique alleles for Donor B.

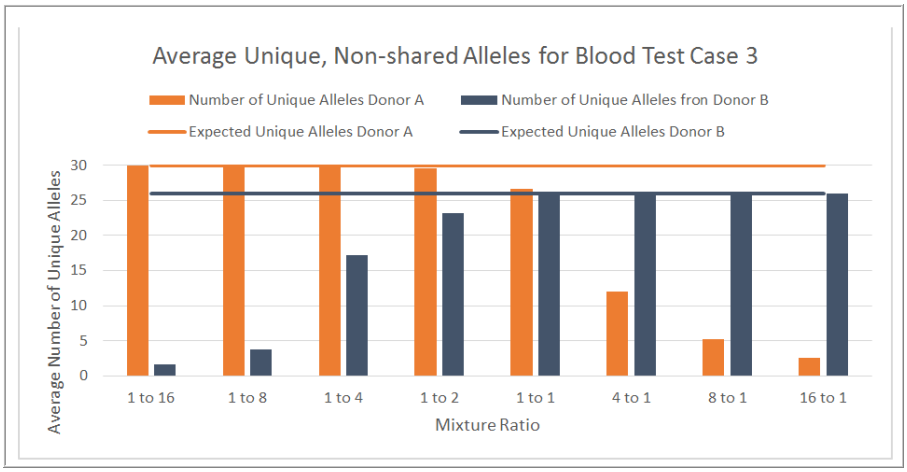


Figure 17 Mixture study 3: Average number of unique alleles detected for donor A and donor B in the lower input blood mixture samples. Five replicates were processed for each mixture ratio.

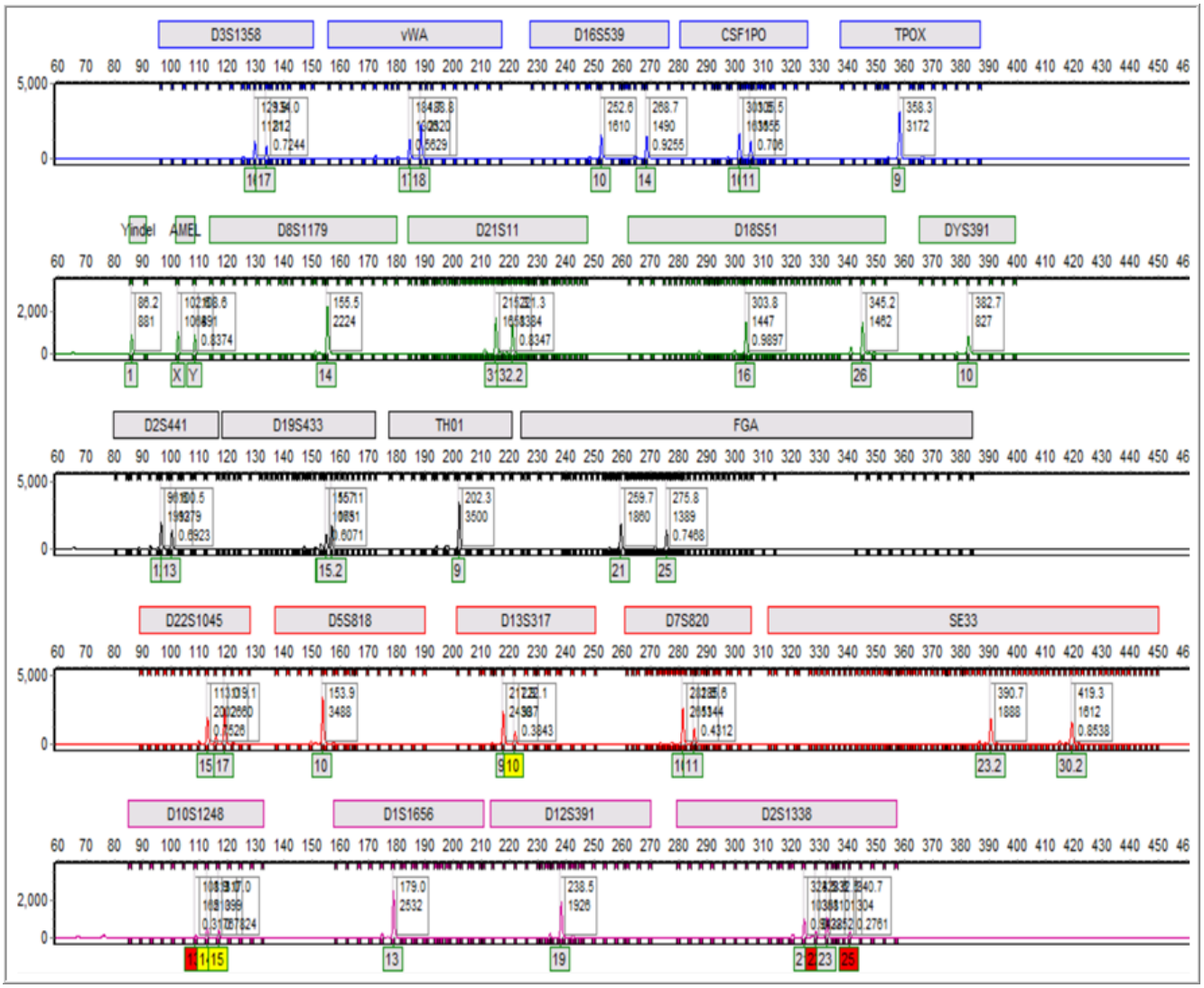


Figure 18 Mixture study 2: Electropherogram of the ✔ 16:1 blood mixture sample, with 0.67 µL of Donor 1 and 0.42 µL of Donor 2, where ploidy flags (red PL allele labels) were triggered at 2 loci after primary analysis, which indicate a mixture.

Mock casework results

Blood, saliva, hair, and touch samples were prepared at multiple sample volumes on different substrates to mimic casework-type samples.

Two mock-casework studies were performed to evaluate primary analysis pass rate, the average number of correct, unflagged markers after primary analysis, and secondary analysis marker recovery by sample and substrate type.

Table 13 Samples for mock-casework study 1

Sample type	Substrate	Volume or sample size[1]	Replicates
Blood	Cotton	2 µL drop, swab	n=4
	Denim: Blood was spotted and dried in 2013, then stored at room temperature.	1 µL drop, swab	n=5
Saliva	Coffee cup: Entire contents consumed, then top was swabbed	Swab	n=4
	Soda can: Entire contents consumed, then mouth area was swabbed	Swab	n=4
	Cigarette butts	Entire wrapper paper on the filter portion of the cigarette[2]	n=4
	Gum: Chewed for 30 minutes, rolled into slender shape, stored in refrigerator until processing.	Entire piece of gum[2]	n=4
Hair	Pulled hair	Hair root[2]	n=1
Touch	Firearm handled by a single donor	Swab	n=5
	Face mask worn by a single donor	Swab	n=4
	Hat worn by single donor	Swab	n=2
	Pen handled by a single donor	Swab	n=4

[1] Dual wet-swab technique was used for swab collections, one swab was processed. Therefore, the swab that was processed contained half the total amount of DNA collected.

[2] The item was placed directly into a sample cartridge.

Mock-casework study 1 processed a limited number of blood, saliva, epithelial/touch samples, and a hair sample. Primary pass rate and the average number of correct, unflagged markers per sample after primary analysis are shown in Figure 19 and Table 14. Yindel and DYS391 are not included in the markers recovered (therefore, the total possible number of markers is 22).

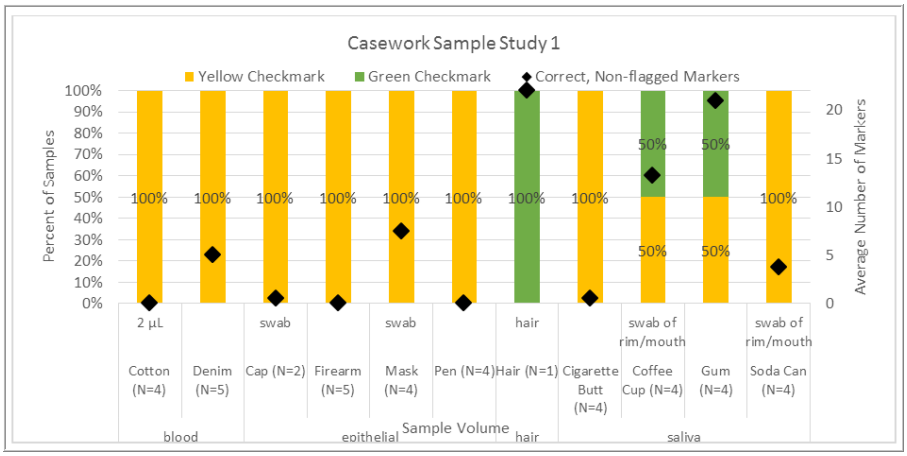


Figure 19 Mock-casework study 1: Primary analysis: Pass rate and average number of correct, unflagged markers for varying inputs of blood and saliva samples run on 6 instruments

Table 14 shows that secondary analysis generally improved marker recovery.

Table 14 Mock-casework study 1: Percent of correct, unflagged markers after primary analysis and markers recovered/ confirmed after secondary analysis

Sample type	Substrate type	Percent of correct, unflagged markers after primary analysis	Percent of correct, unflagged markers plus confirmed markers during secondary analysis
Blood	Cotton (n=4)	0%	0%
	Denim (n=5)	23%	33%
Epithelial	Cap (n=2)	2%	2%
	Firearm (n=5)	0%	0%
	Mask (n=4)	34%	47%
	Pen (n=4)	0%	0%
Saliva	Cigarette butt (n=4)	2%	2%
	Coffee cup (n=4)	20%	20%
	Gum (n=4)	91%	100%
	Soda can (n=4)	17%	44%
Hair Root	Hair root (n=1)	100%	100%

Mock-casework study 2 included differing inputs of blood and saliva on various substrates and hair roots. The data is shown in Figure 20 and Figure 21.

Table 15 Samples for mock-casework study 2

Sample type	Substrate	Volume or sample size[1]	Replicates
Blood	Glass (see Figure 1 on page 10)	• 7.5 µL drop (1/2 pill size) • 15 µL drop (1 pill size) • 30 µL drop (2 pill size)	3 swabs from each volume
	Tile (ceramic)	• 25 µL smear (quarter of business card) • 50 µL smear (half of business card) • 100 µL smear (business card)	3 or 6 swabs from each volume
	Tarred surface (see Figure 1 on page 10)	• 15 µL drop • 30 µL drop	4 or 5 swabs from each volume
	Drywall (see Figure 1 on page 10)	• 4 µL drop (1/2 pill size) • 7.5 µL drop (1 pill size) • 15 µL drop (2 pill size)	3 swabs from each volume
	Black underwear	• 3.75 µL drop (1/4 pill size) • 7.5 µL drop (1/2 pill size) • 15 µL drop (1 pill size) • 30 µL drop (2 pill size) • 60 µL drop (4 pill size)	1 to 5 swabs from each volume
	White cotton: Blood was spotted and dried in 2010, then stored at room temperature.	2 µL drop, 5 mm punch[2]	n=3
	Denim: Blood was spotted and dried in 2013, then stored at room temperature.	1 µL drop, 5 mm punch[2]	n=1
Saliva	Water bottle: Entire contents was consumed, then the top was swabbed.	Swab	n=3
	Coffee cup: Entire contents was consumed, then the top was swabbed.	Swab	n=4

Sample type	Substrate	Volume or sample size[1]	Replicates
Saliva	Cigarette butts	• Dual swab collection/one swab processed • Single swab collection/one swab processed • Half paper[2] • Whole paper [2]	• n=2 (dual swab collection) • n=3 (single swab collection) • n=3 (half paper) • n=1 (whole paper)
	Gum: Chewed for 30 minutes	Swab	n=3
Hair	Pulled hair	Root[2]	n=2

[1] Dual wet-swab technique was used for swab collections, one swab was processed (unless otherwise noted). Therefore, the swab that was processed contained half the total amount of DNA collected.

[2] The item was placed directly into a sample cartridge.

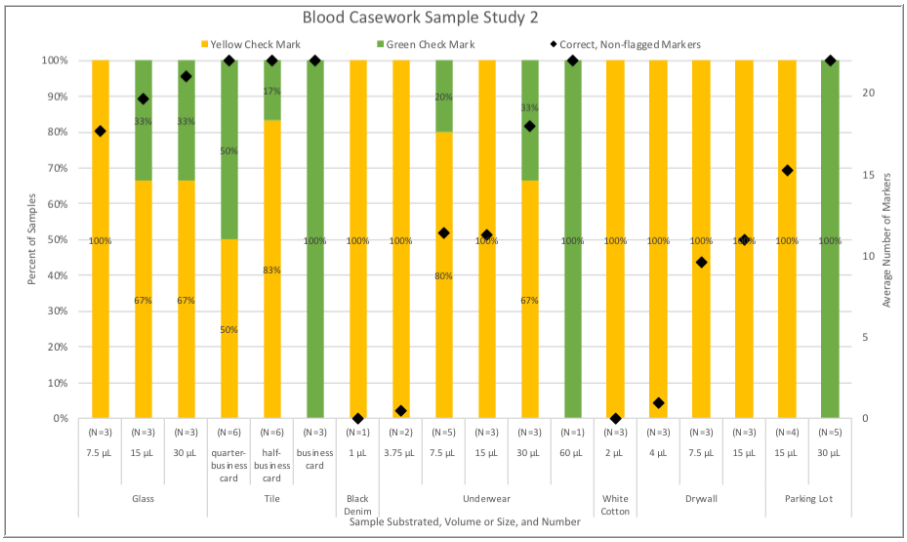


Figure 20 Mock-casework study 2: Primary analysis: Pass rate and average number of correct, unflagged markers recovered for varying inputs of blood mock casework samples run on 6 instruments.

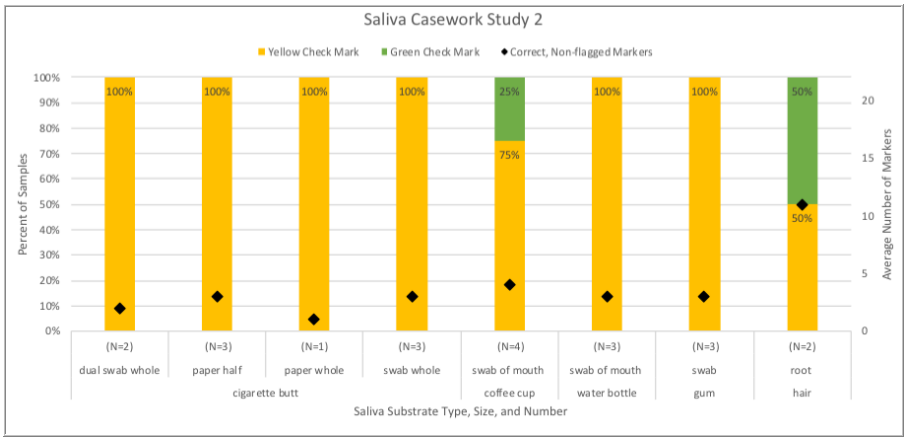


Figure 21 Mock-casework study 2: Primary analysis: Pass rate and average number of correct, unflagged markers for varying inputs of saliva and pulled hair mock casework samples run on 6 instruments.

Secondary analysis for casework test case 2 improved marker recovery for all samples where DNA was recovered with the exception of the blood on white cotton samples which remained the same, as seen in Table 16.

Multiple samples did not meet system thresholds during primary analysis (result on the instrument). However, secondary analysis showed a full profile with a single marker flagged. For example, the blood on glass, tarred surface, and tile recovered over 90% of markers after secondary analysis.

Table 16 Mock-casework study 2: Percent of correct, unflagged markers after primary analysis and markers recovered/ confirmed after secondary analysis

Sample type	Substrate type	Percent of correct, unflagged markers after primary analysis	Percent of correct, unflagged markers plus confirmed markers during secondary analysis
Blood	Denim (n=1)	0%	0%
	Drywall (n=9)	33%	57%
	Glass (n=9)	88 %	91 %
	Tarred surface (n=9)	86 %	93%
	Tile (n=15)	97%	99%
	Underwear (n=14)	55 %	69%
	White cotton (n=3)	6%	6%
Saliva	Cigarette butt (n=9)	8%	11%
	Coffee cup (n=4)	49 %	72%
	Gum (n=3)	0%	44%

Sample type	Substrate type	Percent of correct, unflagged markers after primary analysis	Percent of correct, unflagged markers plus confirmed markers during secondary analysis
Saliva	Water bottle (n=3)	0%	0%
Hair	Hair root (n=2)	50%	50%

Overall, blood samples show higher primary analysis pass rates and higher numbers of correct, non-flagged markers than saliva and touch samples. Variation in results between the sensitivity study and the mock casework study may be due to sampling technique or interferences from inhibitors present on the substrate.

Non-allelic peak assessment

Non-allelic peaks were assessed for all samples in the sensitivity/ population, casework, and mixture studies (n=1,216).

Pull-up edited during secondary analysis

Sixteen total instances of spectral pull-up were observed. Two elevated pull-up peaks were observed in two 4 µL blood population study samples at 11% and 40%. Excluding the 2 elevated pull-up peaks, the average pull-up percentage observed was 1.5%.

Table 17 Pull-up observations in the verification sample set

Marker	Dye	Parent Color	Pull-up %
DYS391	Green	Red	0.7
DYS391	Green	Red	0.7
DYS391	Green	Blue	1
Y indel	Green	Yellow	1
DYS391	Green	Blue	1.3
DYS391	Green	Red	1.6
Y indel	Green	Yellow	1.3
Y indel	Green	Yellow	0.9
DYS391	Green	Blue	1.4
Y indel	Green	Yellow	3.8
DYS391	Green	Blue	1.6
DYS391	Green	Red	2
Y indel	Green	Yellow	11
D2S441	Yellow	Green	41

Marker	Dye	Parent Color	Pull-up %
Y indel	Green	Yellow	0.9
Y indel	Green	Yellow	2.9
Average	—	—	4.5
Average without outliers	—	—	1.5

Stutter

Stutter is a well-characterized PCR artifact that refers to the appearance of a minor peak 1 repeat-unit smaller than the target STR allele product (minus stutter), or less frequently, one repeat-unit larger (plus stutter) (Butler, 2005; Mulero et al., 2006). Sequence analysis of stutter products at tetranucleotide STR loci has revealed that the minus stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (Walsh et al., 1996).

Stutter was observed in data from all 6 instruments (Figure 22 through Figure 27). Some conclusions from these measurements and observations are:

- Smaller alleles generally display a lower level of stutter relative to the longer alleles within each locus. TH01 does not appear to follow this pattern and shows similar levels of stutter for all alleles.
- Each allele within a locus displays a consistent stutter percentage.
- Peaks in the stutter position that are above the locus-specific stutter threshold are assigned allele labels. Peaks in the stutter position that are not filtered out can be further evaluated during secondary analysis.

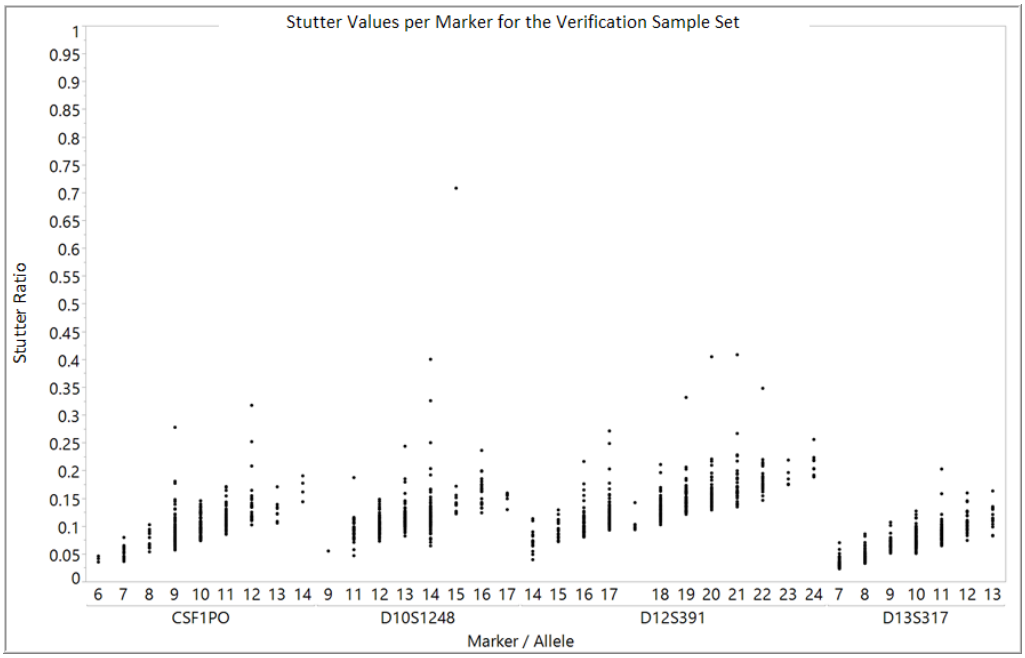


Figure 22 Stutter for CSF1PO, D10S1248, D12391, and D13S317 markers

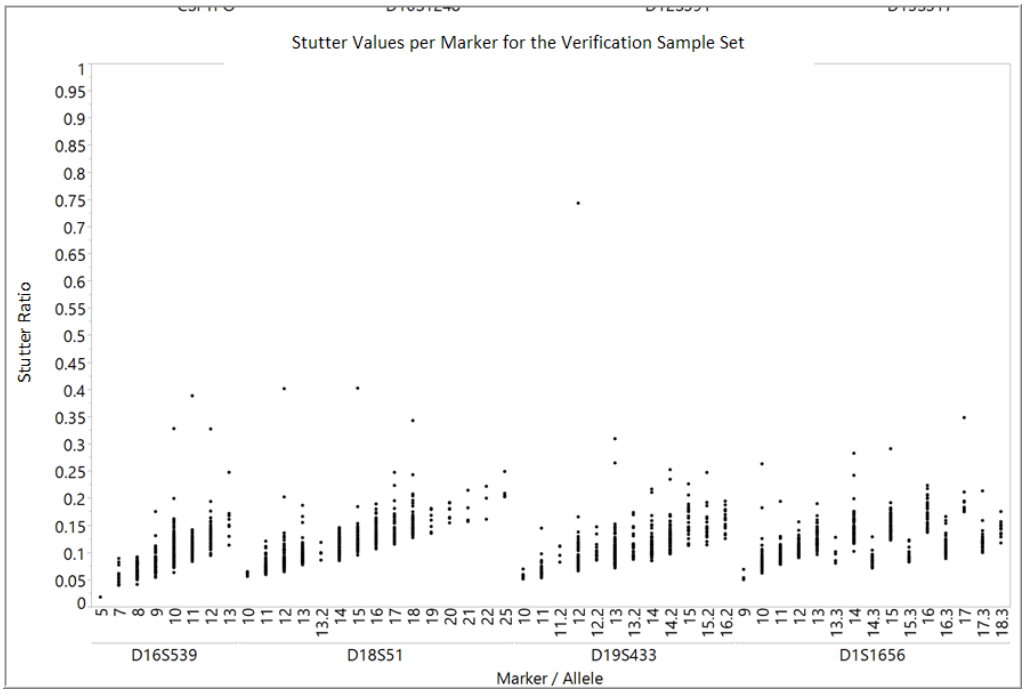


Figure 23 Stutter for D16S539, D18S51, D19S433, and D1S1656 markers

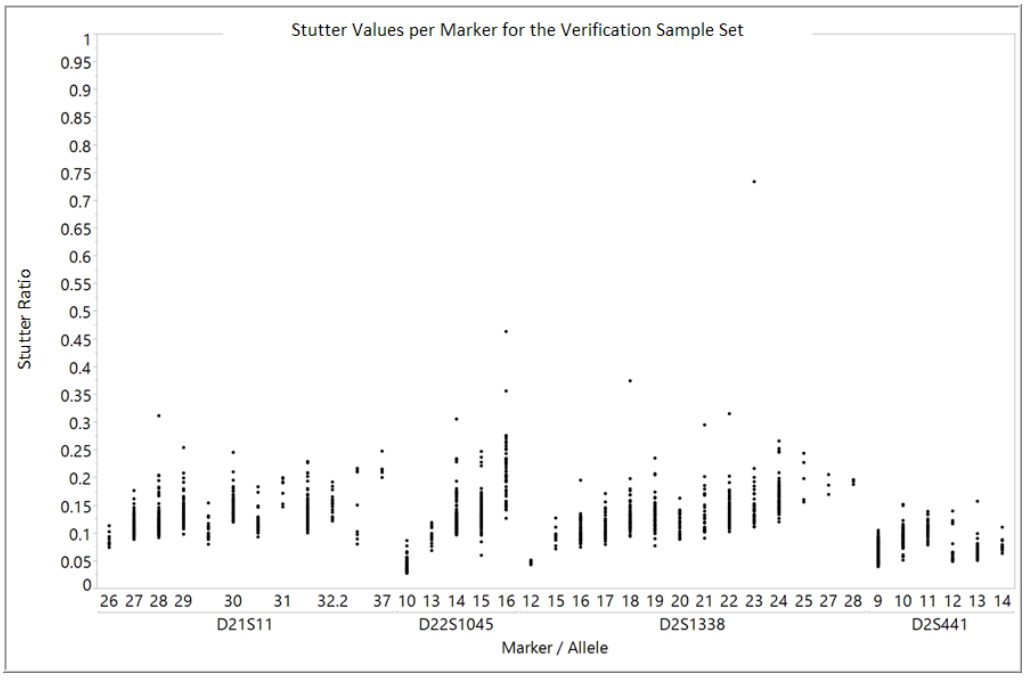


Figure 24 Stutter for D21S11, D22S1045, D2S1338, and D2S441 markers

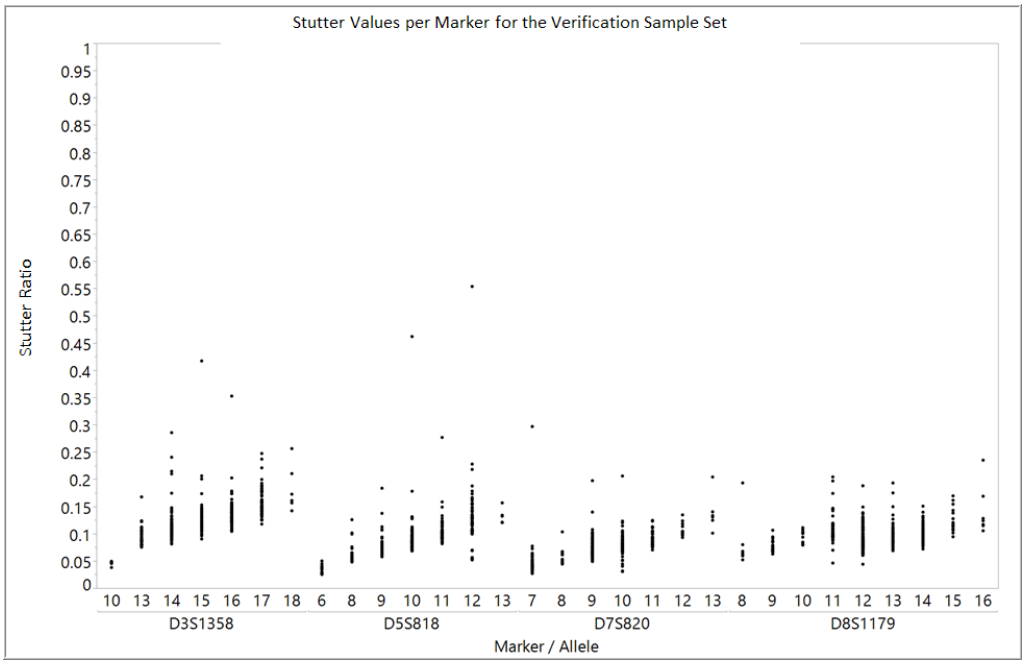


Figure 25 Stutter for D3S1358, D5S818, D7S820, and D8S1179 markers

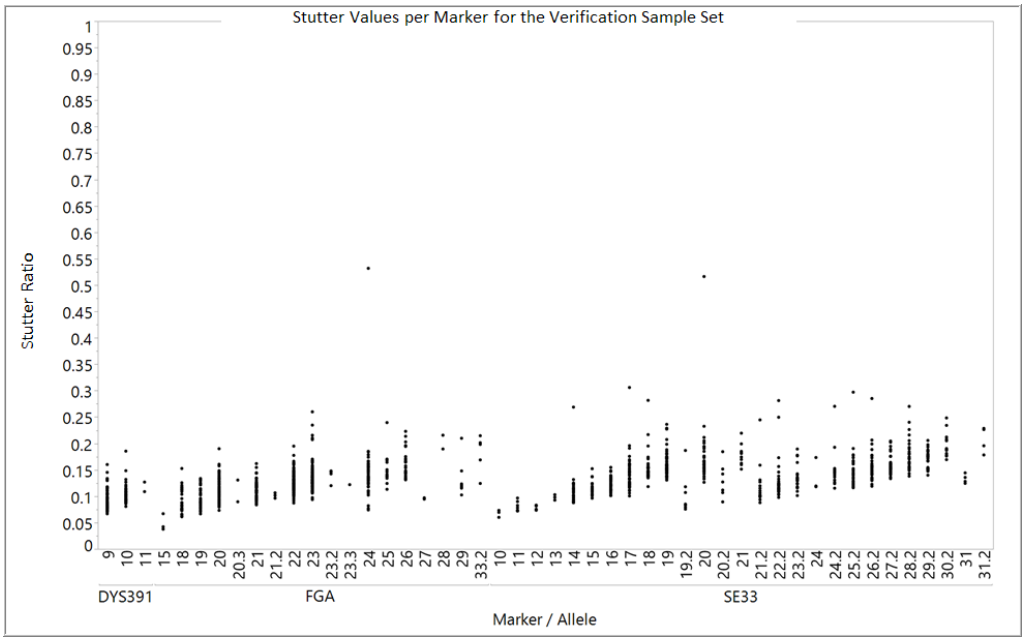


Figure 26 Stutter for DYS391, FGA, and SE33 markers

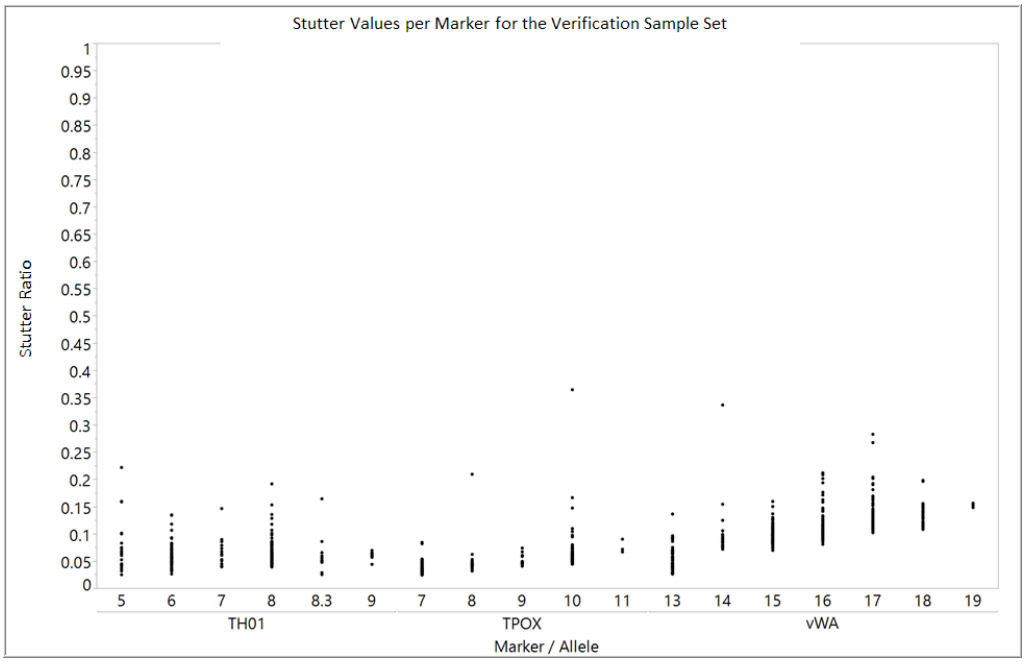


Figure 27 Stutter for TH01, TPOX, and vWA markers

Thirty-seven instances of elevated minus stutter were observed in the verification sample set and edited during secondary analysis. There was no observable pattern of sample type or locus that demonstrated elevated stutter.

Artifacts

Non-standard –5 nucleotide artifact peaks were observed for the TH01 marker in multiple samples across multiple donors, including the positive control. The artifact percentage for this peak was as high as 51% of the allele peak, and at times greater than the 640 RFU minimum heterozygous intensity inconclusive (IHE) flag threshold. This artifact is DNA template-dependent and is likely to be caused by the formation of a secondary structure in the target sequence. An example of the non-standard artifact peak at TH01 is shown in Figure 28.

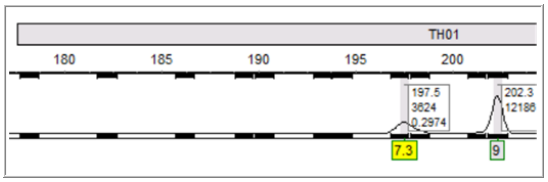


Figure 28 –5 nucleotide artifact peak at the TH01 marker. The stutter (7.3) peak height is 3,624 RFU and the allele peak (9) height is 12,180 RFU. The stutter peak is 30% of the allele peak and was edited during secondary analysis.

Cartridge comparison: ACE GFE cartridge to RapidINTEL™ Sample Cartridge

Primary analysis results from the RapidHIT™ ID ACE GlobalFiler™ Express Sample Cartridge were compared to the RapidINTEL™ Sample Cartridge using samples from the mock casework and blood mixture studies. Both cartridges use the GlobalFiler™ Express chemistry.

System thresholds for the RapidHIT™ ID System v1.1.3 and both cartridges are listed in Table 18.

Marker recovery for the RapidINTEL™ Sample Cartridge was also assessed after secondary analysis.

Table 18 RapidINTEL™ Sample Cartridge and ACE GFE cartridge on RapidHIT™ ID System v1.1.3 system thresholds

System threshold	RapidINTEL™ Sample Cartridge	ACE GFE cartridge
Analytical threshold	50 RFU	All loci 50 RFU, except: • TPOX = 84 RFU • Y indel = 74 RFU • D2S441 = 68 RFU
Stochastic threshold	1,600 RFU 50 RFU for Y indel and DYS391	All loci 100 RFU, except: • TPOX = 210 RFU • Y indel = 74 RFU • DYS391 = 50 RFU • D2S441 = 136 RFU • SE33 = 150 RFU • D2S1338 = 125 RFU
Minimum peak height ratio threshold(Heterozygote Imbalance (IMB flag) threshold)	40% 99% for Y indel and DYS391	50%
Minimum heterozygous peak intensity threshold (Inconclusive Heterozygous (IHE flag) threshold)	640 RFU	—
Stutter filters	Locus-specific	20%
Locus-specific filter	21% 30% for the positive control	20%
Ploidy (PL flag) threshold (maximum number of expected peaks)	2	2
Global filter (between loci)	21%	20%

System threshold	RapidINTEL™ Sample Cartridge	ACE GFE cartridge
Global filter (between loci)	30% for the positive control	20% for the positive control
Minimum off-ladder (OL) intensity	30 RFU	30 RFU

Table 19 Mock-casework study 2 for ACE GFE cartridge and RapidINTEL™ Sample Cartridge: Primary analysis: Percent of correct, unflagged markers; Secondary analysis: Markers confirmed samples from RapidINTEL™ Sample Cartridge.

Sample type	Substrate type	Percent of correct, unflagged markers for ACE GFE cartridge primary analysis	Percent of unflagged markers for RapidINTEL™ Sample Cartridge primary analysis	Percent of correct, unflagged markers plus confirmed markers during secondary analysis of RapidINTEL™ Sample Cartridge
Blood	Denim (n=1)	0%	0%	0%
	Drywall (n=9)	25%	33%	57%
	Glass (n=9)	81%	88%	91%
	Tarred surface (n=9)	77%	86%	93%
	White cotton (n=3)	2%	6%	6%
Saliva	Cigarette butt (n=9)	0%	8%	11%
	Coffee cup (n=4)	11%	49%	72%
	Gum (n=3)	0%	0%	0%
	Water bottle (n=3)	0%	0%	0%

Additional alleles were also observed for the minor contributor in the blood mixture samples processed with the RapidINTEL™ Sample Cartridge vs. the ACE GFE cartridge.

Representative electropherograms for the ACE GFE cartridge and RapidINTEL™ Sample Cartridge of dual-swabbed samples are shown in Figure 29. For each sample, both cartridges were run on the same instrument.

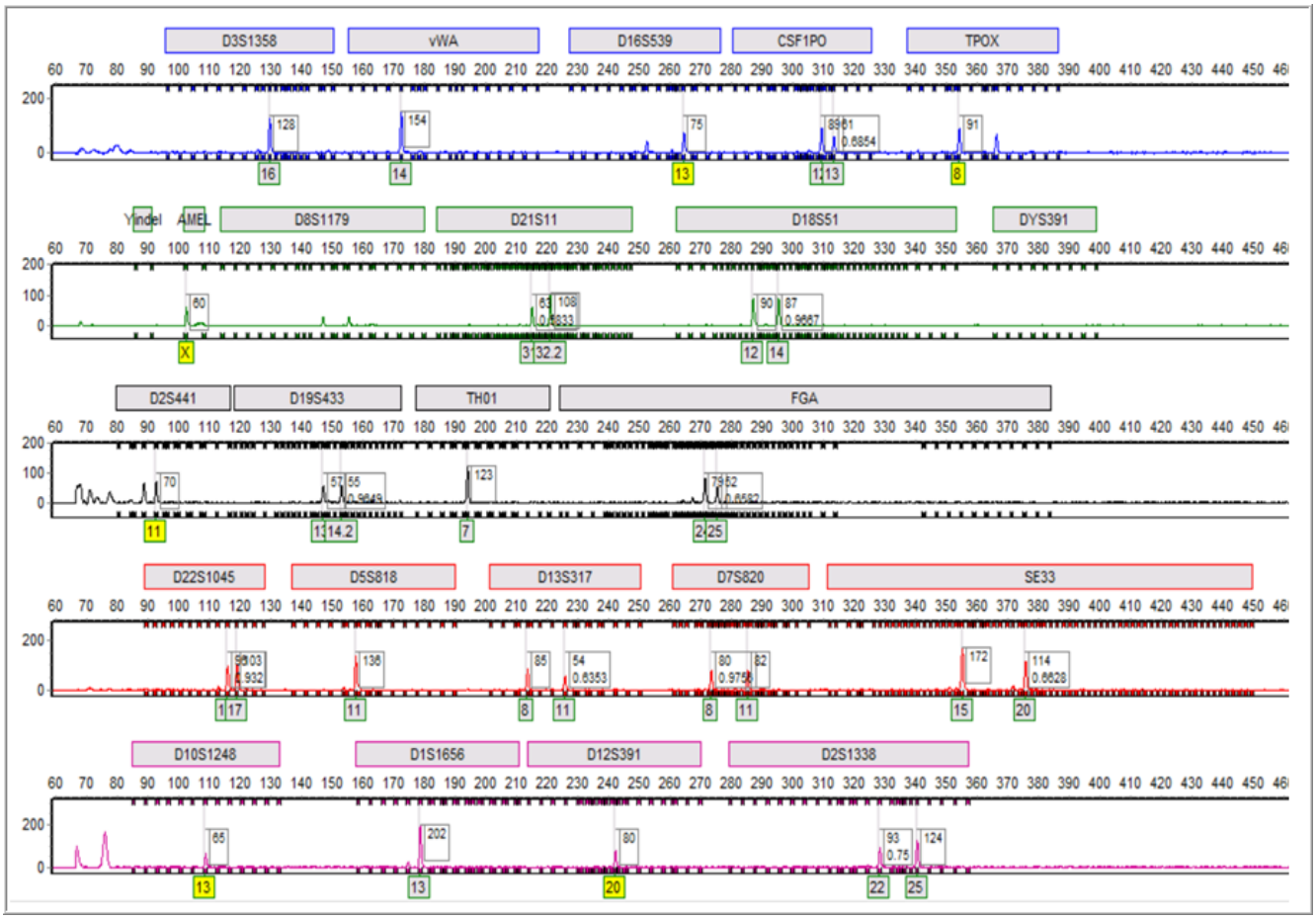


Figure 29 7.5 µL blood on glass sample run using the ACE GFE cartridge. Drop-out can be observed at the D8S1179 marker. Six flags are present (yellow peak labels) because a single peak is present below the ST.

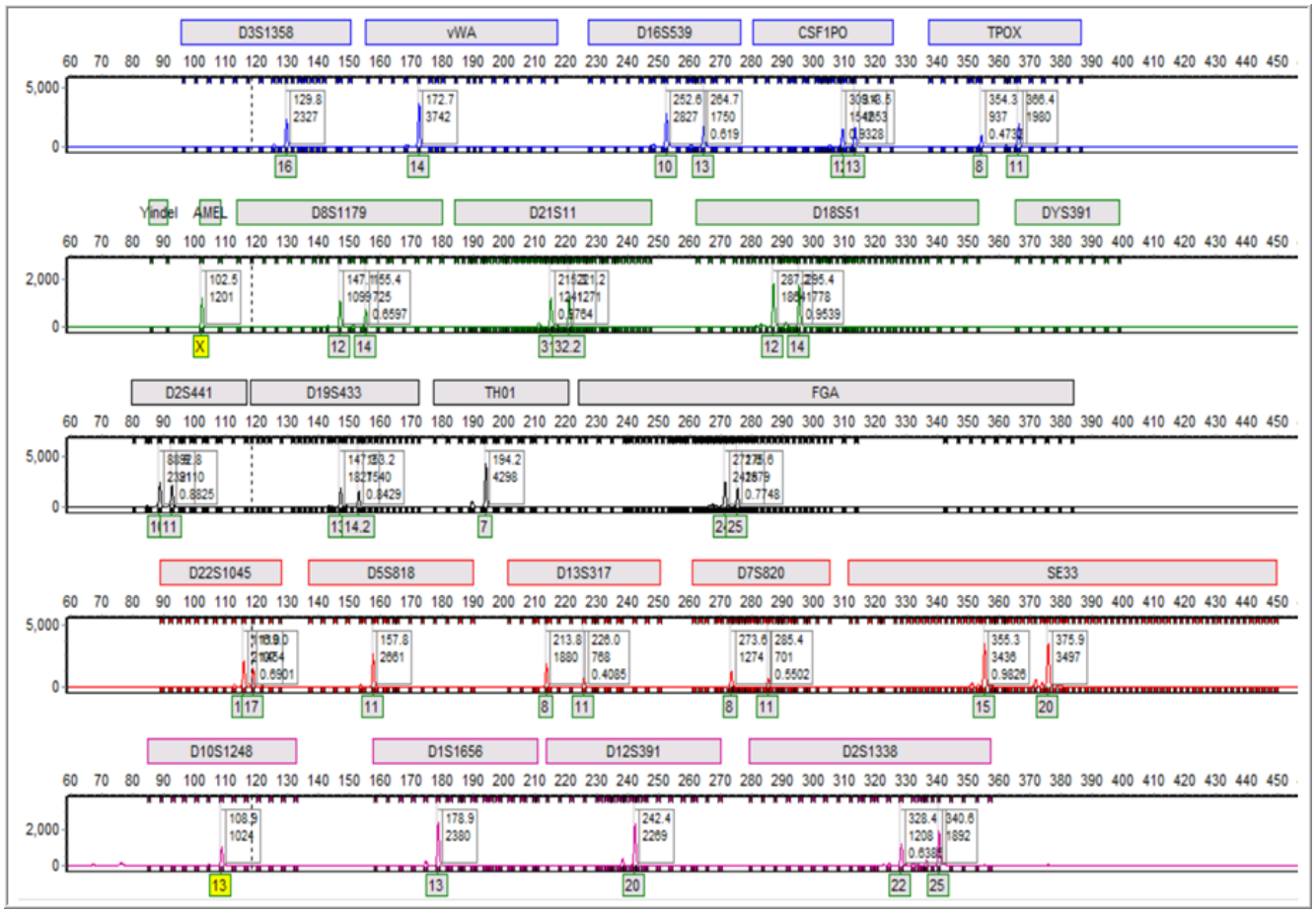


Figure 30 7.5 µL blood on glass sample run using the RapidINTEL™ Sample Cartridge. Two IHO flags are present (yellow peak labels) for single peaks below the stochastic threshold at Amelogenin and D10S1248 markers.

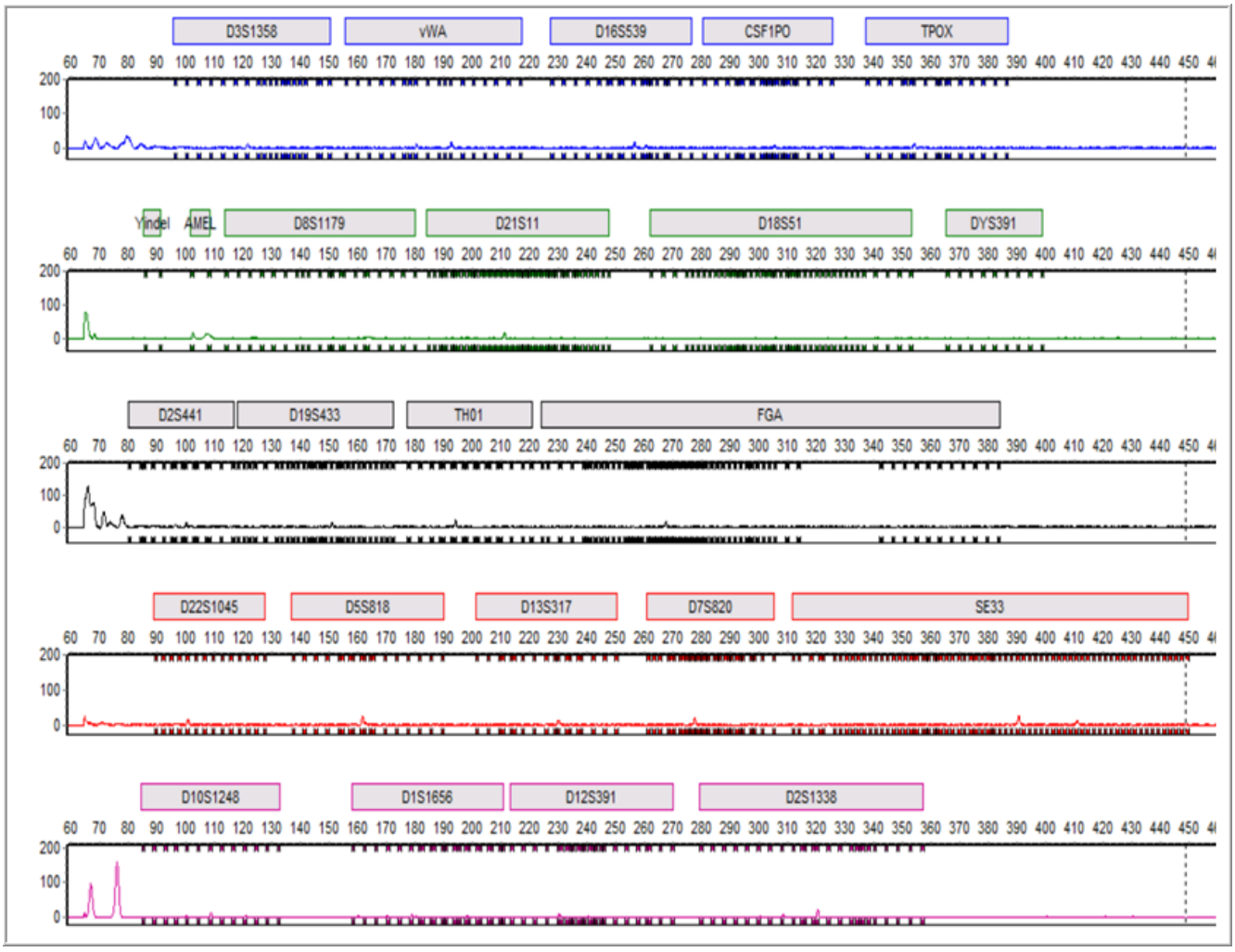


Figure 31 Water bottle swab run using the ACE GFE cartridge. No DNA was detected above the system thresholds.

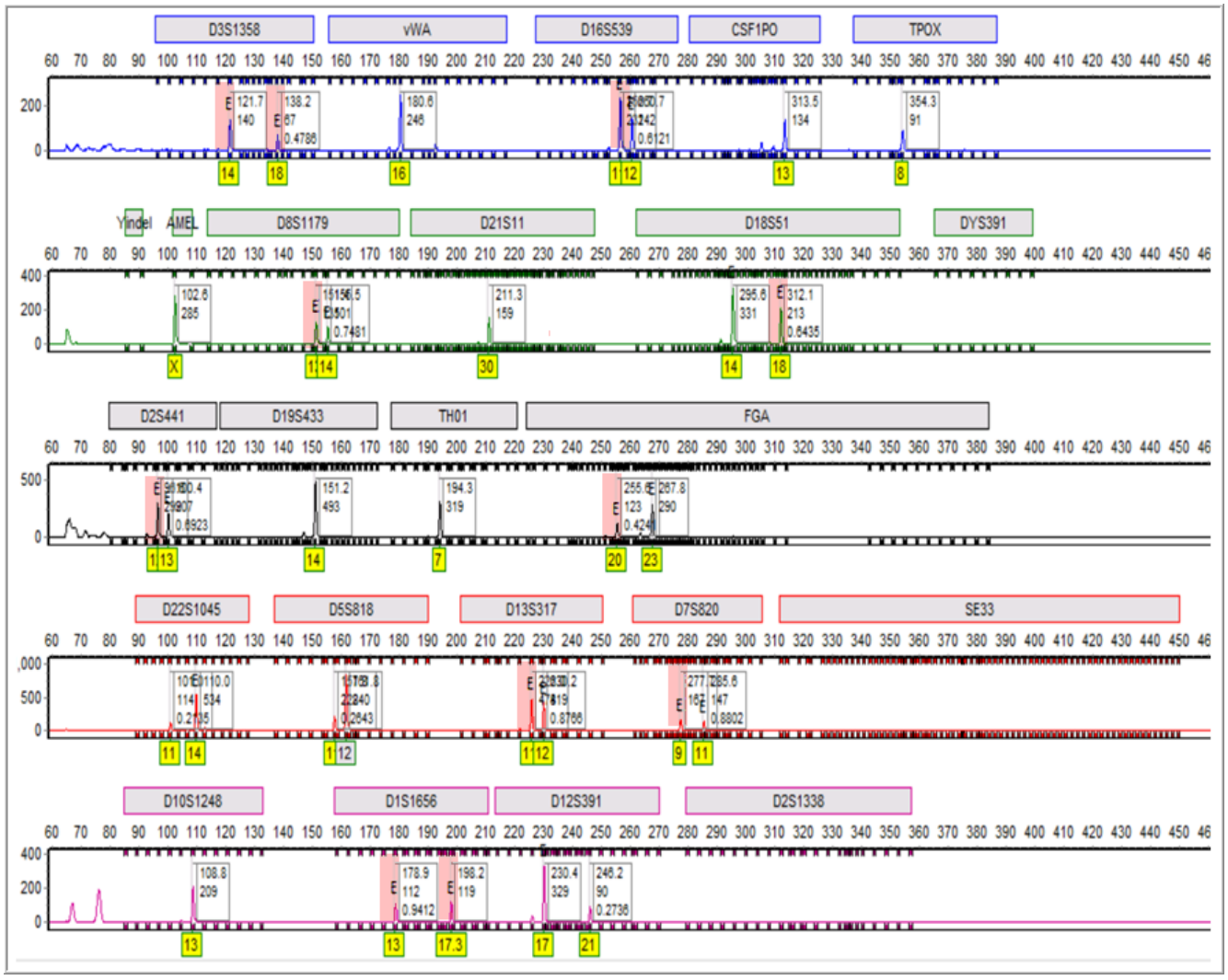


Figure 32 Water bottle swab run using the RapidINTEL™ Sample Cartridge. Multiple flags are present (yellow peak labels). "E" labels (highlighted in pink) are present in 9 markers where the minimum heterozygote intensity inconclusive flag (IHE) was overridden during secondary analysis.

Conclusions

RapidINTEL™ Sample Cartridges that are run on RapidHIT™ ID System v1.1.3 can accurately analyze moderate- to high-DNA content samples. This workflow expands the capability of the system to allow analysis of blood and saliva samples. Samples that do not meet the validated system thresholds are appropriately identified during primary analysis. Any samples that do not pass primary analysis require manual secondary analysis before results can be reported. This workflow supports the collaboration of decentralized locations for automatic primary analysis and forensic laboratories for required secondary analysis.

IMPORTANT! The RapidINTEL™ Sample Cartridge is recommended for use with blood and saliva samples only. Epithelial touch samples were also tested, but successful results were not obtained.

The validation studies confirmed:

- 99% genotype concordance between samples run on the RapidINTEL™ Sample Cartridge and 3500xL instruments.
- Samples that contain peaks/markers that do not comply with the RapidINTEL™ system thresholds generate a ☑ result (do not pass primary analysis).

  IMPORTANT! System thresholds were set so that all questionable peaks/markers are flagged during primary analysis and require secondary analysis by a forensically trained analyst.

- Mixtures were accurately detected.
- Negative controls that are run after high-concentration samples show no reproducible contamination.
- For mock-casework samples, the number of unflagged, correct alleles after primary analysis on RapidINTEL™ Sample Cartridges was generally higher than the number of unflagged, correct alleles after primary analysis on ACE GFE cartridges.
- The number of correctly called peaks/markers that were identified during primary analysis indicate high system efficiency.
- Because of the increased sensitivity, the RapidINTEL™ Sample Cartridges generate more data than ACE GFE cartridges.

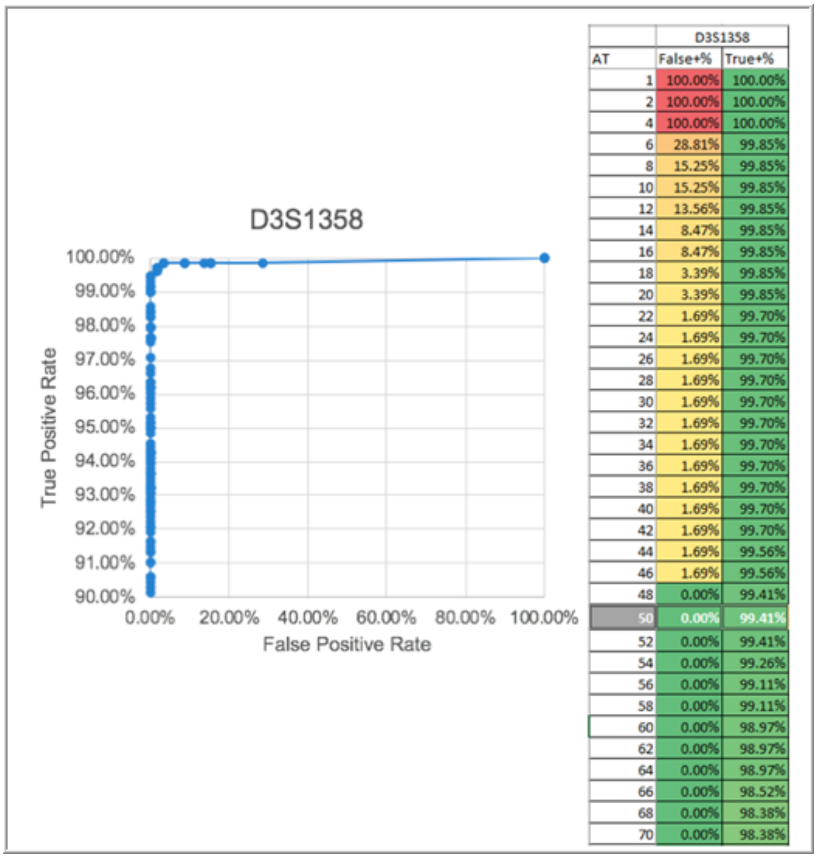
Appendix A: RapidINTEL™ system threshold development data

Analytical threshold

The analytical threshold is set to differentiate a true peak from baseline noise. There are multiple accepted approaches across the forensic DNA community to set analytical thresholds on Applied Biosystems™ 3130 and 3500 Series Genetic Analyzers (Bregu *et al.*, 2012, Butler 2015).

For this study, a receiver operating characteristic (ROC) curve was used to determine the RFU where false positive rate drops to 0.00% for peaks in bin (allele calls). The analytical and stochastic thresholds were set to 5 RFU and the stutter thresholds were set to 5%. Known artifact peaks were removed (Rakay *et al.*, 2012).

An analytical threshold of 50 RFU was determined to filter out ≥99% of noise peaks.



	D3S1358	
AT	False+%	True+%
1	100.00%	100.00%
2	100.00%	100.00%
4	100.00%	100.00%
6	28.81%	99.85%
8	15.25%	99.85%
10	15.25%	99.85%
12	13.56%	99.85%
14	8.47%	99.85%
16	8.47%	99.85%
18	3.39%	99.85%
20	3.39%	99.85%
22	1.69%	99.70%
24	1.69%	99.70%
26	1.69%	99.70%
28	1.69%	99.70%
30	1.69%	99.70%
32	1.69%	99.70%
34	1.69%	99.70%
36	1.69%	99.70%
38	1.69%	99.70%
40	1.69%	99.70%
42	1.69%	99.70%
44	1.69%	99.56%
46	1.69%	99.56%
48	0.00%	99.41%
50	0.00%	99.41%
52	0.00%	99.41%
54	0.00%	99.26%
56	0.00%	99.11%
58	0.00%	99.11%
60	0.00%	98.97%
62	0.00%	98.97%
64	0.00%	98.97%
66	0.00%	98.52%
68	0.00%	98.38%
70	0.00%	98.38%

Figure 33 ROC analysis of the D3S1358 marker where at an AT of 50 RFU, 99.41% of noise peaks are filtered.

Stochastic threshold

The stochastic threshold applies to low DNA input amounts where stochastic effects may be observed. Examples of stochastic effects are allele drop-out, allele drop-in, heterozygote allele imbalance, and elevated stutter. These effects occur more frequently in low-level DNA samples and may be exacerbated with increased PCR cycles.

If a peak exceeds the stochastic threshold, it is not typically affected by stochastic effects; therefore, heterozygote allele drop-out is unlikely. A single peak that is below the stochastic threshold may not be a true homozygote.

To determine the stochastic threshold, the verification sample set was analyzed at a 50 RFU analytical threshold. The RFU value at which 97% of false homozygotes were detected was selected for the stochastic threshold: 1,600 RFU for all non-Y markers; 50 RFU for the Y indel and DYS391 markers.

False homozygotes (where a one allele of a heterozygote pair is present above the AT at a marker) were evaluated in the verification sample set (Figure 34).

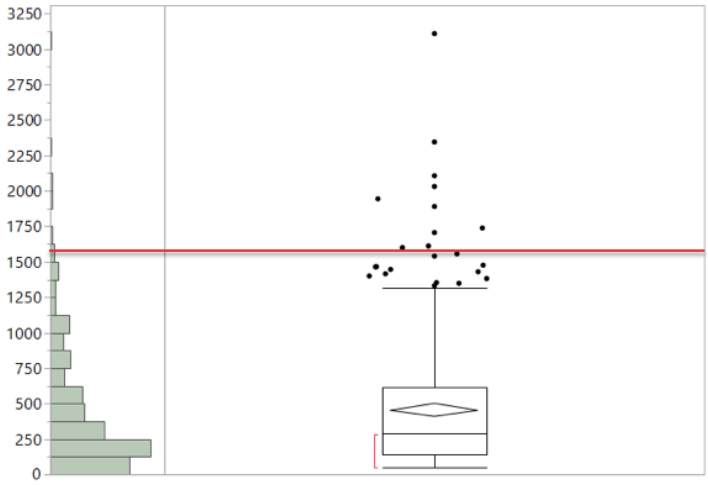


Figure 34 Evaluation of false homozygotes in the verification sample set with the analytical threshold set at 50 RFU

Minimum peak height ratio threshold (IMB flag)

Samples were analyzed with an analytical threshold of 50 RFU and a stochastic threshold of 1,600 RFU. Peak height ratio distribution was assessed (Figure 35).

The point at which 90% of heterozygote allele pairs would *not* be flagged was selected for minimum peak height ratio (40%).

As expected, when approaching stochastic (low DNA input) amplification levels, peak height ratios were lower.

Because the ploidy (PL) flag does not differentiate between haploid and diploid markers, the heterozygote imbalance flag threshold was set to 99% for Y indel and DYS391 to ensure flagging of two alleles at the markers.

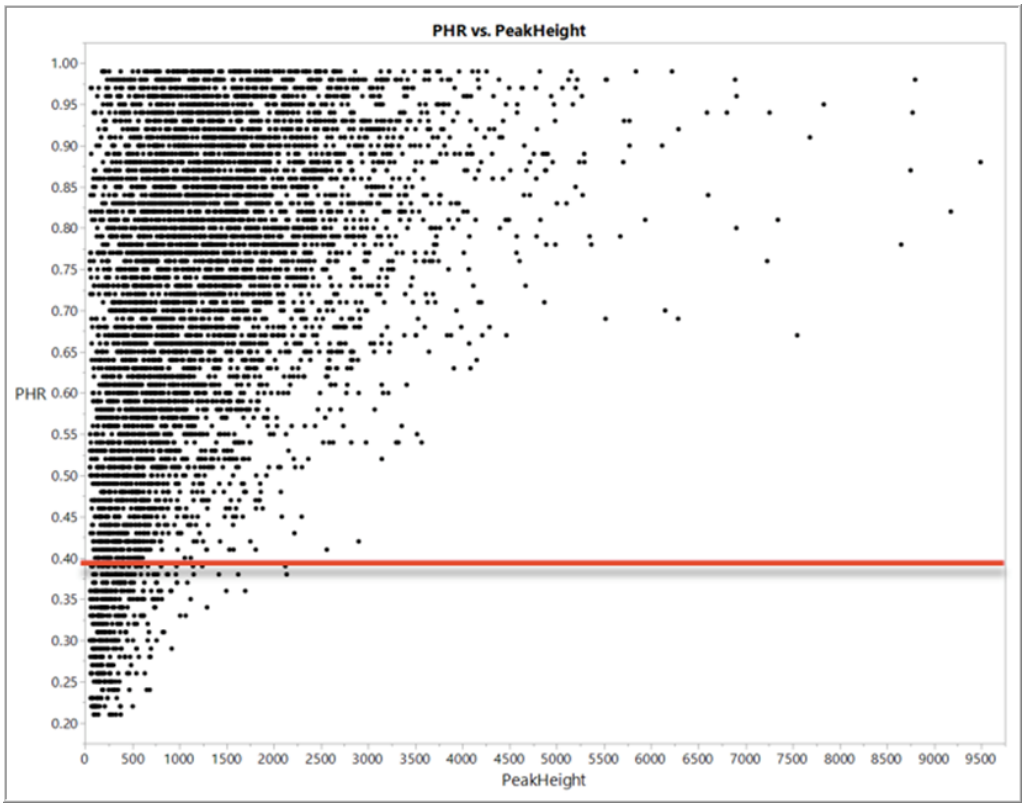


Figure 35 Peak height ratio distribution

Minimum heterozygous peak intensity threshold (IHE flag)

This threshold was calculated with the following formula to be 640 RFU for all markers:

IHE flag threshold = minimum peak height ratio × stochastic threshold

This threshold designates the minimum RFU for both peaks in a marker. If both peaks also exceed the minimum peak height ratio (no IMB flag is triggered), the peaks can be confidently identified as a heterozygous pair.

In conjunction with the analytical threshold (which is set to detect low-level mixtures) the Inconclusive Heterozygous (IHE) flag threshold provides an additional level of confidence for heterozygous allele calls.

Stutter filters

Stutter filters were based on the verification sample set that was analyzed with an analytical threshold of 50 RFU and without global or locus-specific filters. For each marker, the percentage of the stutter peaks to the allele peaks was averaged and the standard deviation (SD) was calculated. Stutter filters were set using the following calculation:

Stutter filter % = (average stutter peak height + 4 SD) × 100

The stutter filter values were rounded up to the nearest whole number as seen in Table 20.

Table 20 Stutter thresholds. When not indicated, the stutter threshold is –4 nucleotides from the allele peak.

Dye	Marker	Avg Stutter + 4SD (%)
6-FAM™	D3S1358	27
	vWA	25
	D16S539	25
	CSF1PO	22
	TPOX	16
VIC™	Y indel	21
	AMEL	21
	D8S1179	20
	D21S11	25
	D18S51	28
	DYS391	18
NED™	D2S441	16
	D19S433	29
	TH01	18
	FGA	27
TAZ™	D22S1045	34
	D5S818	26
	D13S317	18
	D7S820	18
	SE33	30
SID™	D10S1248	29
	D1S1656	26

Dye	Marker	Avg Stutter + 4SD (%)
SID™	D12S391	30
	D2S1338	31

Locus-specific filter

An analytical threshold of 50 RFU was used to evaluate artifact peaks at each locus. A threshold of 21% (artifact to allele peak) filtered out 99% of noise/artifact peaks observed at each marker (Figure 36).

For the positive control, a locus-specific filter of 30% allows for a high-pass rate of the single-source, high-quality, high-quantity control DNA. The positive control is used primarily for genotype concordance and troubleshooting purposes.

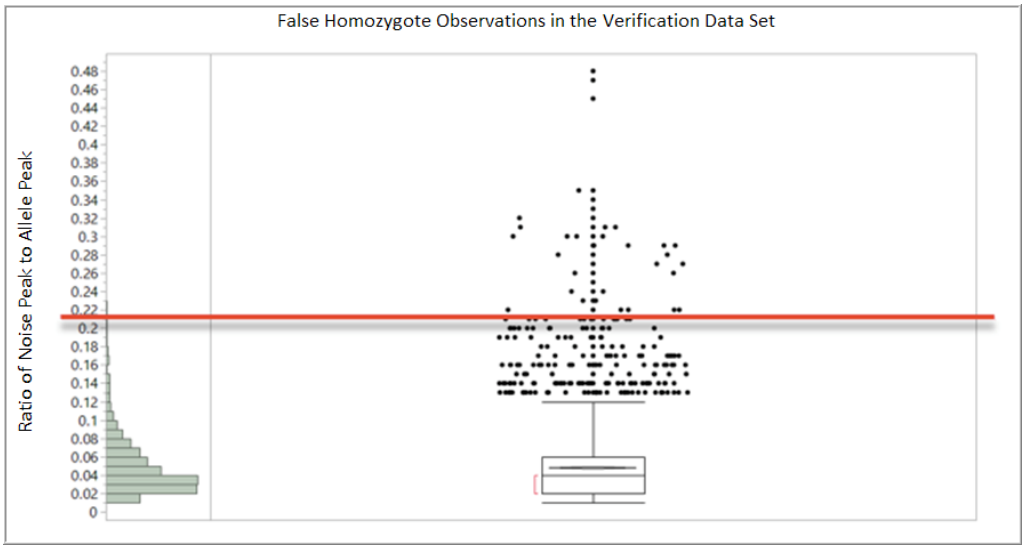


Figure 36 Locus-specific filters

Ploidy flag (PL)

The ploidy flag (PL) is triggered if the number of peaks identified within a marker exceeds the maximum number of expected peaks in a single-source DNA profile. It is used to identify a mixture.

All markers (including Y indel and DYS391) are evaluated for PL.

The PL flag setting was set to 2; therefore, any marker with three peaks detected above the analytical threshold will trigger the PL flag.

Appendix B: References

- *RapidHIT™ ID System v1.0 User Guide* Pub. No. MAN0018039 Rev A.0
- *RapidLINK™ Software v1.0 User Guide* Pub. No. MAN0018038 Rev A.0
- *GeneMarker HID User Guide* (**https://softgenetics.com/PDF/GeneMarkerHID_UserManual.pdf**).
- Rakay, et al., "Maximizing allele detection: Effects of analytical threshold and DNA levels on rates of allele and locus drop-out.", Forensic Sci Int Genet. 2012 Dec;6(6):723–8.
- Butler, J.M.. (2015). Advanced Topics in Forensic DNA Typing: Interpretation. 10.1016/B978-0-12-405213-0.
- Bregu, J. , Conklin, D. , Coronado, E. , Terrill, M. , Cotton, R. W. and Grgicak, C. M. (2013), Analytical Thresholds and Sensitivity: Establishing RFU Thresholds for Forensic DNA Analysis,. *J Forensic Sci*, 58: 120-129. doi:**10.1111/1556-4029.12008**.
- Mulero, J.J., Chang, C.W., and Hennessy, L.K. 2006. Characterization of N+3 stutter product in the trinucleotide repeat locus DYS392. *J. Forensic Sci.* 51:826–830.
- Scientific Working Group on DNA Analysis Methods (SWGDAM). 2012. Validation Guidelines for DNA Analysis Methods. Available at http://swgdam.org/SWGDAM_Validation_Guidelines_APPROVED_Dec_2012.pdf. Accessed 29 July 2013.
- Walsh, P.S., Fildes, N.J., Reynolds, R. 1996. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Res.* 24:2807– 2812.

Appendix C: Cartridge contents and storage

Contents	Cat. No.	Amount	Storage
RapidINTEL™ Sample Cartridge Kit	A43942	• 50 RapidINTEL™ Sample Cartridges • 2 positive-control RapidINTEL™ Sample Cartridges • 2 negative-control RapidINTEL™ Sample Cartridges	4–10°C Expires 6 months after the date of manufacture.
RapidINTEL™ Sample Cartridge Evaluation Kit	A439421	10 RapidINTEL™ Sample Cartridges	

Table 21 RapidHIT™ ID ACE GlobalFiler™ Express 150 Sample Kit (Cat. No. A41841)

Contents	Amount	Storage
Box 1		
Primary Cartridge GlobalFiler™ Express • Primary cartridge • 1 utility cartridge The utility cartridge is used for primary cartridge replacement.	1 cartridge for 150 runs[1]	Room temperature (15–25°C) Expires 6 months after the date of manufacture.
Box 2		
Primary Cartridge GlobalFiler™ Express • 1 gel cartridge The gel cartridge is used for primary cartridge replacement. • 1 GFE Control Cartridge (allelic ladder cartridge)	1 gel cartridge 1 GFE Control Cartridge (allelic ladder cartridge)	4–10°C Expires 6 months after the date of manufacture.
Positive control cartridge	1 positive control cartridge	

[1] Primary cartridges can be ordered separately (Cat. No. A41841). Includes gel, utility, and GFE Control Cartridge [allelic ladder] cartridges.

Life Technologies Ltd | 7 Kingsland Grange | Woolston, Warrington WA1 4SR | United Kingdom
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

Revision history: Pub. No. MAN0018979

Revision	Date	Description
A.0	10 December 2019	Validation user bulletin for RapidINTEL™ Sample Cartridges on the RapidHIT™ ID System v1.1.3.