QIAGEN Validation Report

Developmental validation of the Investigator® IDplex GO! Kit

The QIAGEN Investigator IDplex GO! Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. It is used for direct amplification of single source blood or buccal cells on Whatman® FTA® paper, and crude buccal swab lysates.

The performance of the Investigator IDplex GO! Kit was evaluated with regard to various sample types and conditions commonly encountered in reference and database sample analysis. The validation study was based on the recommendations of the European Network of Forensic Science Institutes (ENFSI) (1) and the Revised Validation Guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM) (2). The assay makes use of well-established methodologies for forensic DNA analysis. It co-amplifies 15 polymorphic STR markers, the 13 CODIS (Combined DNA Index System) markers (TH01, D3S1358, vWA, D21S11, TPOX, D7S820, D5S818, D16S539, CSF1PO, D13S317, FGA, D18S51, D8S1179), plus D2S1338, D19S433, and the gender-specific Amelogenin. These genetic loci have been characterized in numerous studies by other laboratories (see 3 for review). Optimal reaction conditions were established, and the effects of variations in those conditions were assessed. The kit was tested in house and at independent, external forensic laboratories.

The effect of variations in buffer component concentrations is described using the example of $MgCl_2$ concentration (pages 2–3). The effects of using different PCR cycle numbers were analyzed (pages 5–6). Variations in PCR cycling conditions and a range of thermal cyclers and genetic analyzers were used to demonstrate the robustness of the assay (pages 7–9). Sensitivity was addressed by amplifying a dilution series of blood on FTA paper, or buccal swab lysates (pages 9–11).Cross-reactivity with non-human DNA was also assessed (pages 15–16). The reproducibility of the results was verified (pages 17–27). This validation study covers a fully automated workflow for blood or buccal cells on FTA paper using the Hamilton easyPunch STARlet system (page 28).

Results of developmental validation

The validation study has been performed by QIAGEN research and development. All of the electropherograms shown were generated on an Applied Biosystems® 3500™ Genetic Analyzer. The standard conditions specified in the Investigator IDplex GO! Handbook were used for electrophoresis. Unless stated otherwise, a GeneAmp® PCR System 9700 with Gold-plated Silver 96-Well Block was used for amplification. Data were analyzed using the QIAGEN Investigator IDproof Software, v2.0 or Applied Biosystems GeneMapper ID-X software, v1.2. Whatman EasiCollect™ indicator cards were used to transfer buccal cells to FTA paper, and Whatman FTA cards were used for blood samples. Puritan® polyester or cotton tipped swabs, or Sarstedt® cotton tipped swabs were used to collect buccal swab samples. Investigator STR GO! Lysis Buffer was used to create a crude lysate from buccal swab samples.

Reaction conditions

Reaction conditions were established for optimal performance in terms of robustness, specificity, and reproducibility. The influence of critical buffer components on the amplification efficiency was also assessed. For example, various concentrations of $MgCl_2$ were evaluated with blood on FTA paper (Figure 1) and buccal swab samples (Figure 2). The assay yielded robust results within a $MgCl_2$ concentration range of ±20% from the optimum, and full profiles within an $MgCl_2$ concentration range of ±30% (data not shown).

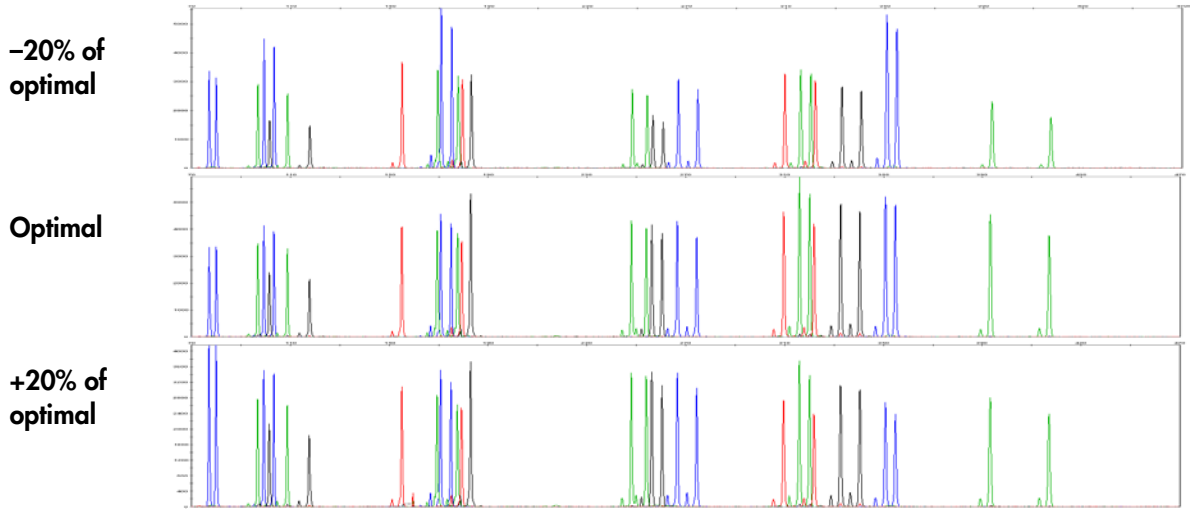


Figure 1. Influence of $MgCl_2$ concentration. Examples of blood on FTA paper analyzed in triplicate.

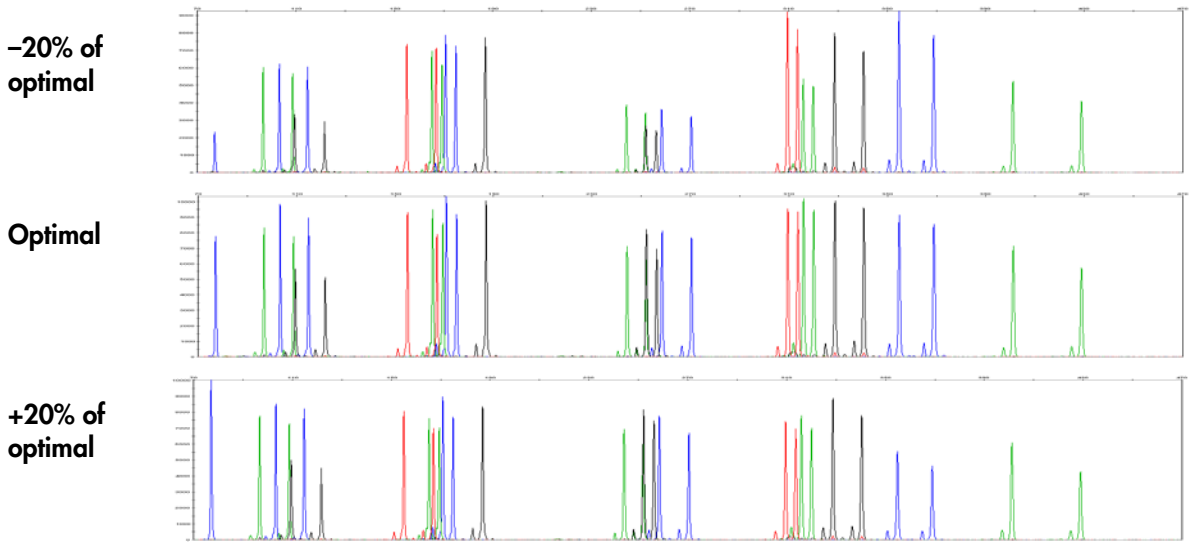


Figure 2. Influence of $MgCl_2$ concentration. Examples of a buccal swab sample analyzed in triplicate.

Effect of PCR annealing temperature variations

Specificity, sensitivity, and robustness are affected by the annealing temperature (Tm). Since the actual Tm may vary depending on cycler conditions, the assay was validated in a range surrounding the optimal Tm of the Investigator IDplex GO! reaction (61°C). Annealing temperatures between 57°C and 65°C were used for the amplification of blood on FTA paper and buccal swab samples. PCR was performed on an Eppendorf® Mastercycler® ep instrument (Figure 3). Reactions using annealing temperatures between 57°C and 64°C resulted in full profiles at a threshold of 100 RFU (see Figure 4 for example electropherograms). At 65°C heterozygous alleles of marker D13S317 dropped below 100 RFU. Overall signal heights were at a comparable level at 59°C–62°C. No non-specific PCR products were observed at any tested annealing temperature. In order to ensure optimal performance of the assay, we strongly recommend regular calibration of thermal cyclers.

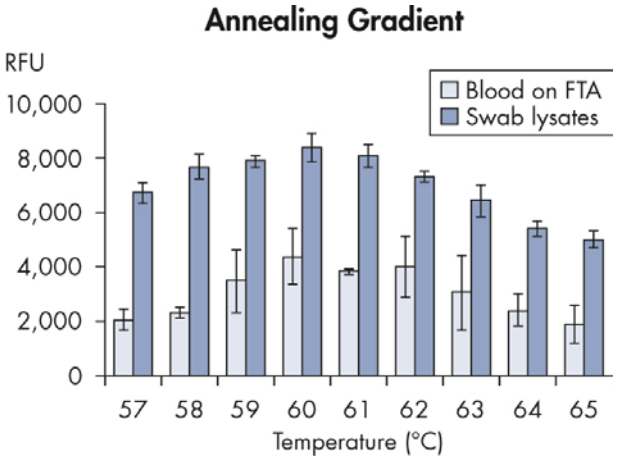


Figure 3. Effect of variations in the PCR annealing temperature. Blood on FTA paper and buccal swab lysates from a single donor were analyzed in triplicate using 25 PCR cycles for blood on FTA paper and 28 cycles for swab lysates.

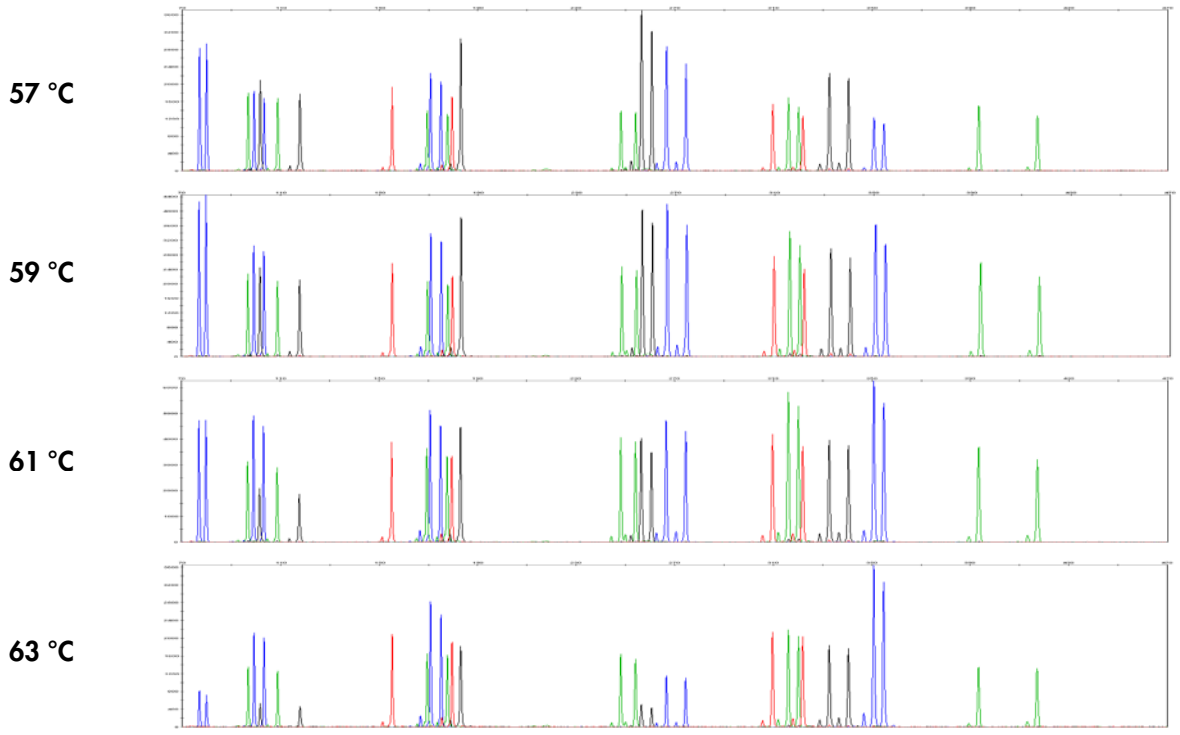


Figure 4. Variations in the PCR annealing temperature. Representative electropherograms for reactions using annealing temperatures of 57–63°C for blood on FTA paper.

Effect of different cycle numbers

The amount of sample material present for direct amplification varies between different reference sample types, donors, collection procedures, and storage conditions. Therefore, for optimal results it is important to evaluate a representative batch of samples and to adapt the reaction conditions if necessary. To adapt the reaction conditions to varying DNA template amounts we recommend adjusting the number of PCR cycles. Cycle numbers can either be increased to enhance amplification signals, or decreased when DNA is abundant.

Blood, or buccal cells on FTA paper and buccal swab lysates were initially amplified using the cycle number recommended for evaluation, ± 2 PCR cycles (Figure 5). Samples from 3 donors were analyzed in 5 replicates each. As expected, average signal heights increase by approximately a factor of 2 with each cycle added. Buccal cells on FTA paper showed a higher variation between individual punches, which is reflected in a weaker correlation between cycle number and profile peak height.

We recommend reviewing routinely used data periodically to ensure conditions chosen during evaluation still provide the best possible pass rates.

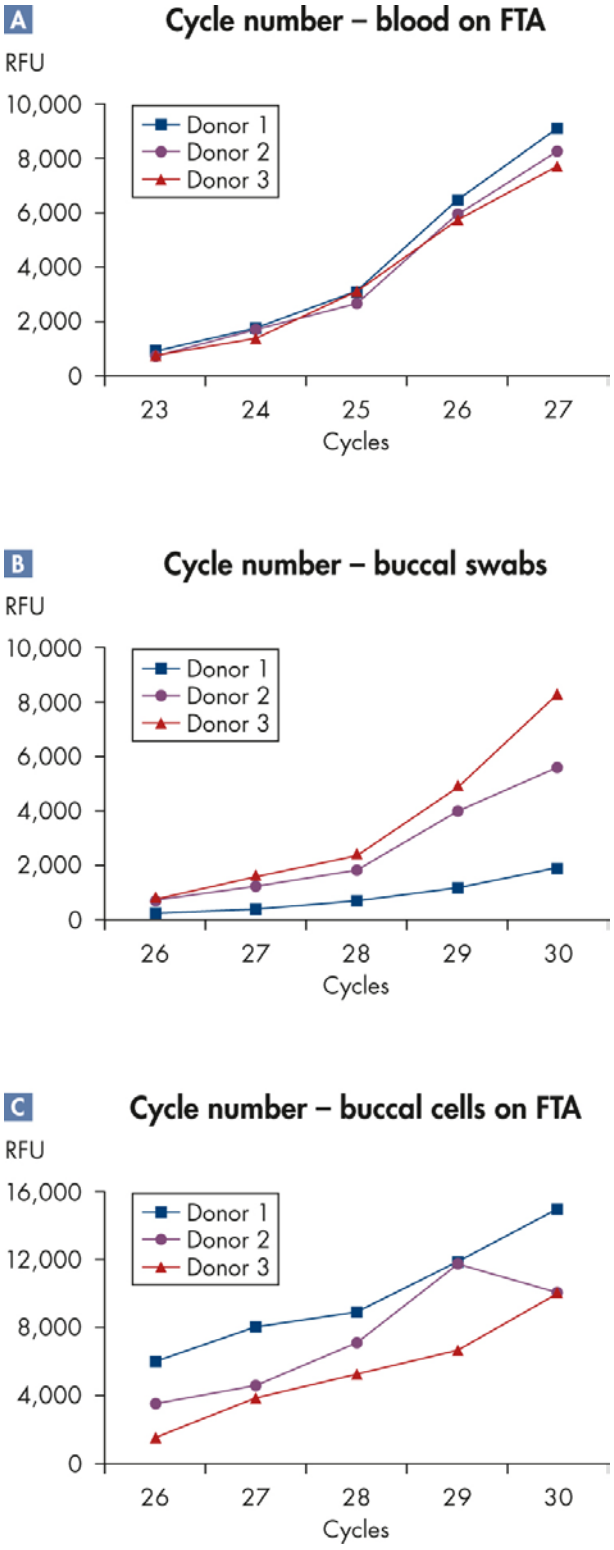


Figure 5. Effect of different cycle numbers on mean peak height. Samples were run in 5 replicates per donor. **[A]** Blood on FTA paper. **[B]** Buccal swabs. **[C]** Buccal cells on FTA paper.

Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator IDplex GO! Kit to demonstrate kit robustness independent of the instrument (Figure 6). Blood on FTA paper and buccal swab lysates from 5 different donors were run in 5 replicates each. For blood on FTA paper, 25 PCR cycles were used and for swab lysates, 28 PCR cycles were used. The following thermal cyclers were evaluated:

- GeneAmp PCR System 9700 with Aluminum 96-Well Block (Applied Biosystems Inc., Foster City, CA, USA)
- GeneAmp PCR System 9700 with Silver or Gold-plated Silver 96-Well Block (Applied Biosystems Inc., Foster City, CA, USA)
- Eppendorf Mastercycler ep
- MJ Research DNA Engine® PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany)
- UNO-Thermo Block (Biometra Biometra biomedizinische Analytik GmbH, Göttingen, Germany)

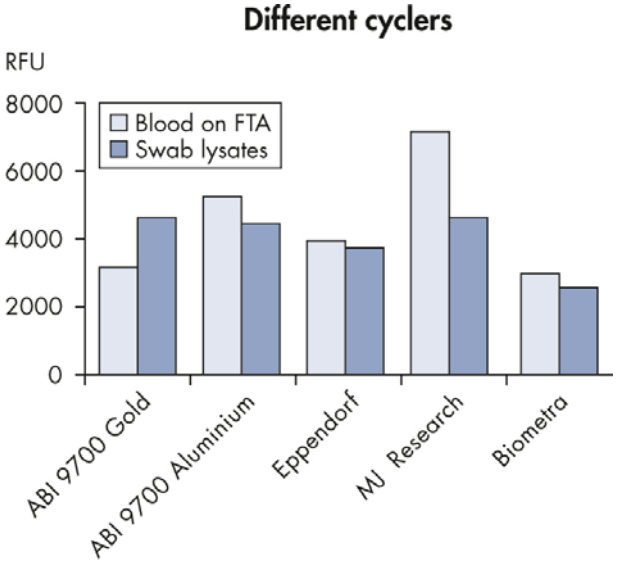


Figure 6. Performance on different PCR thermal cyclers. Average signal heights for blood on FTA paper and buccal swab lysates from 5 different donors, run in 5 replicates each.

Effect of different genetic analyzers

Two different genetic analyzers were tested with the Investigator IDplex GO! Kit. Blood on FTA paper or buccal swab samples from 5 different donors were each amplified in 5 replicates. Samples from the PCR cycler comparison described in the previous section run on a GeneAmp PCR System 9700 with Gold-plated Silver 96-Well Block were analyzed on the following instruments:

- Applied Biosystems 3500 Genetic Analyzer (2 independent instruments)
- Applied Biosystems 3130™ Genetic Analyzer

Similar overall peak heights and a comparable balance of peaks between individual markers of the same color channel, as well as between different channels, were observed between the two evaluated Applied Biosystems 3500 Genetic Analyzers. Average signal heights were lower on the 3130 Genetic Analyzer. This was expected since many samples produced results at the upper end of the signal that can be reliably analyzed on this instrument, but well within the linear range of the 3500 Genetic Analyzers (Figure 7). An increased number of profiles showing pull-up peaks on the 3130 Genetic Analyzer also occurred as expected (Figure 8). Limitations of genetic analyzer sensitivity must be addressed by individual laboratories during an internal validation study using the instrumentation in their laboratory.

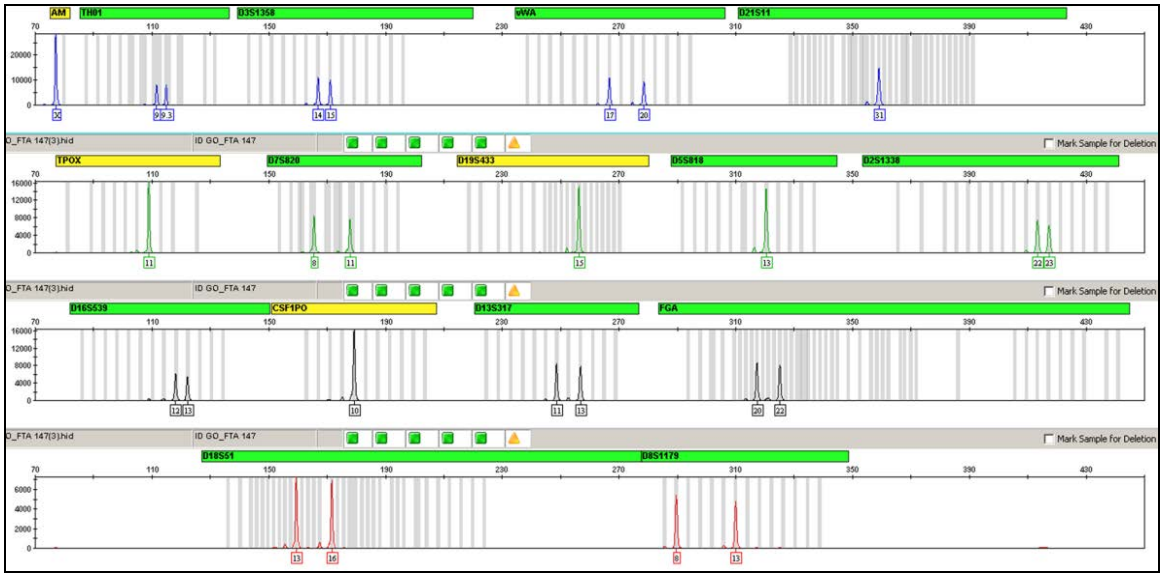


Figure 7. Analysis of PCR products on different genetic analyzers. DNA from blood on FTA paper was amplified on a GeneAmp PCR System 9700. Samples were run according to the *Investigator IDplex GO! Handbook*. Analysis was performed on an Applied Biosystems 3500 Genetic Analyzer.

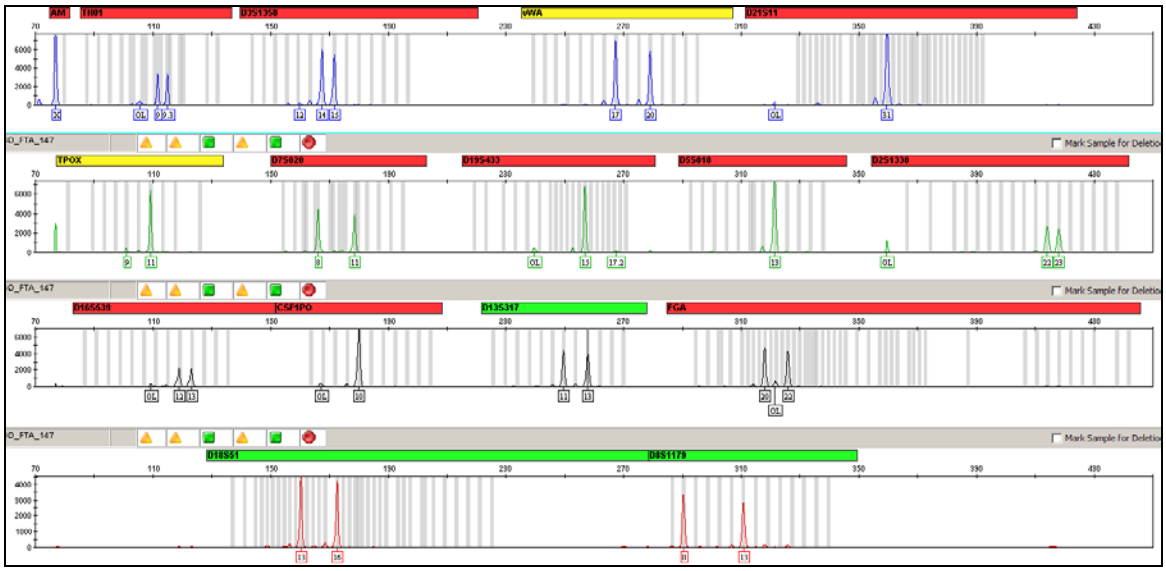


Figure 8. Analysis of PCR products on different genetic analyzers. Same sample as shown in Figure 7, analyzed on an Applied Biosystems 3130 Genetic Analyzer.

Sensitivity

The Investigator IDplex GO! Kit is designed to work robustly over a range of sample input amounts. A dilution series of blood was spotted on FTA paper and amplified using 25 PCR cycles. Swab lysates were diluted and amplified using 28 PCR cycles. For both sample types, 3-fold dilutions down to 1:81 were used.

For dilutions of swab lysates the obtained signal heights correlated well with the dilution factor of the sample material. For spotted blood dilutions, the signal was reduced by a factor of 2 for every 3-fold dilution step, which was consistently less than expected (Figure 9). This is most likely due to a concentration dependent build-up of white blood cells on the FTA paper during spotting.

Full profiles were achieved for all samples down to a 1:27 dilution. At a dilution of 1:81 individual alleles dropped below the 50 RFU thresholds for both blood on FTA paper and buccal cell lysates (Figure 10). However, the full profiles from these samples could be recovered by increasing the number PCR of cycles.

Issues with very high sample amounts

Fluorescence intensity depending on the instrumentation and settings used for capillary electrophoresis may go off the scale. "Off-scale" peaks are often accompanied by "pull-up" peaks. Furthermore, higher stutter-peak height ratios may be observed as the signal from the main peak gets saturated. Finally, "split-peaks" may occur as a result of incomplete +A nucleotide addition.

Poor STR profiles resulting from high sample input can be improved by either: re-amplifying using less amplification cycles, or re-injecting at a shorter injection time. Due to inherent sample-to-sample variation in direct amplification, the result of a re-injection has a higher predictability compared to re-amplification. This is especially true for buccal cells transferred to paper, which frequently show considerable punch-to-punch variability, even if indicator cards are used.

Issues with very low amounts of sample

Reference samples typically contain sufficient biological material to obtain full profiles with good heterozygote balance. Therefore, issues with stochastic allelic drop-out and drop-in and strong peak imbalance are not expected. However, individual samples that contain significantly less material may not be sufficiently amplified using the PCR cycle number chosen. Re-amplification at a higher cycle number will in most cases recover full profiles.

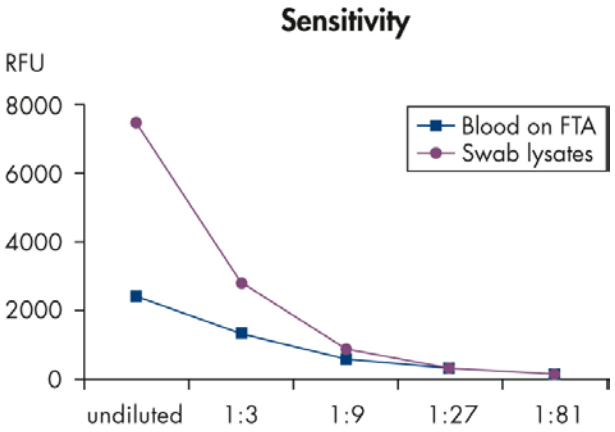


Figure 9. Sensitivity study. A serial dilution of a blood sample on FTA paper or a buccal swab lysate.

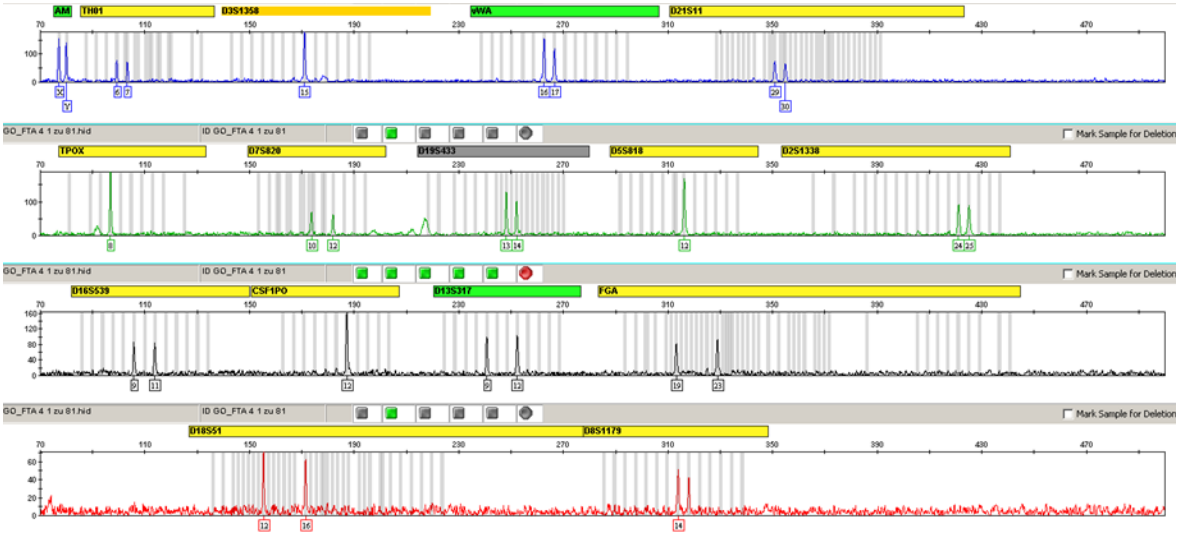


Figure 10. Sensitivity study. Example of blood on FTA paper diluted 1:81 prior to spotting. Allele 15 of D8S1179 dropped below 50 RFU.

Stability

Sample input

The robustness was tested using varying amounts of sample. For blood or buccal cells on FTA paper: 1, 2, or 3 punches of 1.2 mm diameter were used. For buccal swabs: 1, 2, or 4 µl of crude lysates were used (Figures 11–13 show example electropherograms). For all sample types, 3 replicate of 3 donors were run. Although increased sample amounts in general still provided full DNA profiles, occasionally the balance of markers was negatively affected. In particular, introduction of 3 FTA punches frequently results in reduced amplification of the longer STR markers and shoulder formation of markers: D18S51, CSF1PO, and D13S317. Both effects indicate inhibition of the PCR with increasing amounts of FTA paper. Using 4 µl buccal swab lysate in most cases increases the overall signal intensity, but Amelogenin typically shows lower signals compared to other markers. We recommend using sample amounts as indicated in the handbook and to increase PCR cycle numbers if signals are too low.

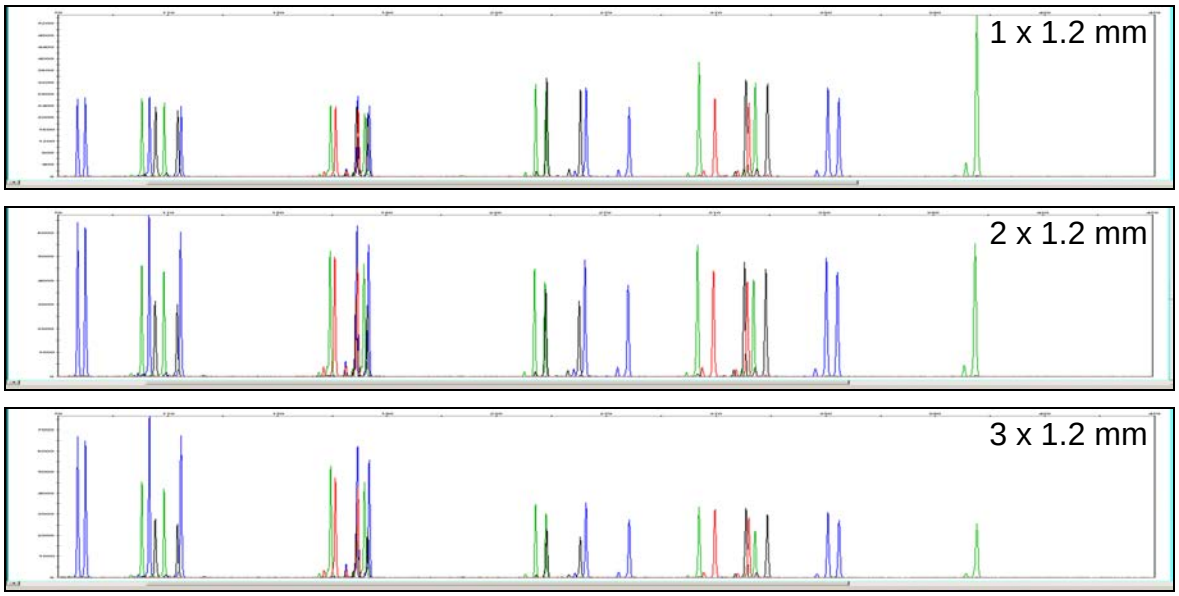


Figure 11. Sample input, blood on FTA paper. Amplification of DNA from 1, 2, or 3 punches of 1.2 mm.

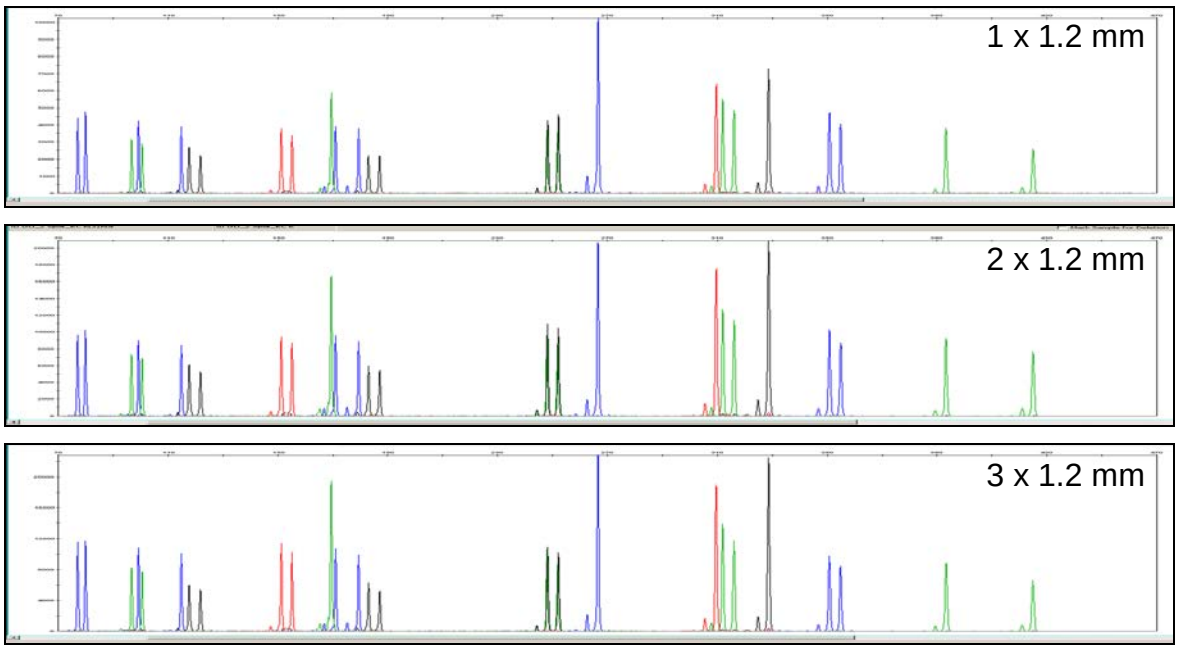


Figure 12. Sample input, buccal cells on FTA paper. Amplification of DNA from 1, 2, or 3 punches of 1.2 mm.

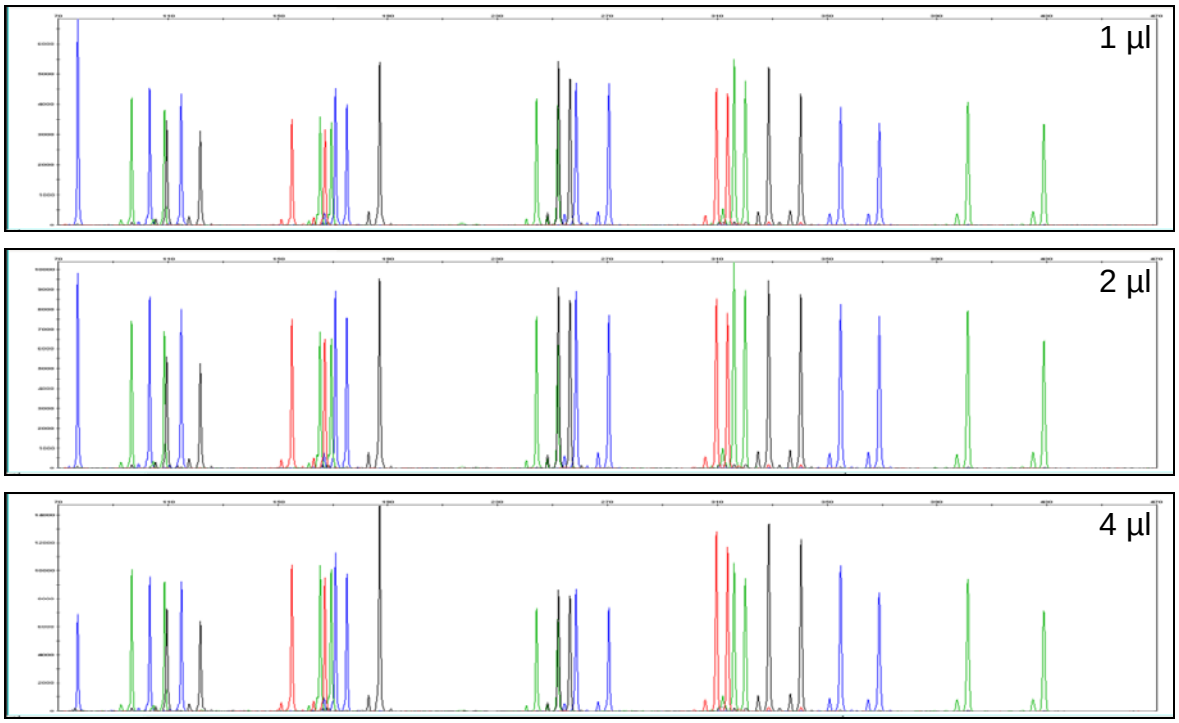


Figure 13. Sample input, buccal cell lysates. Amplification of DNA from 1, 2, or 4 µl of a buccal swab lysate.

Aged samples

DNA was amplified from blood on FTA paper stored at room temperature for 18 months and crude lysates from buccal swabs stored at –20°C for 12 months (3 replicates of 3 different donors). No sample degradation or other negative impacts of storage were observed (Figures 14 and 15 show typical electropherograms).

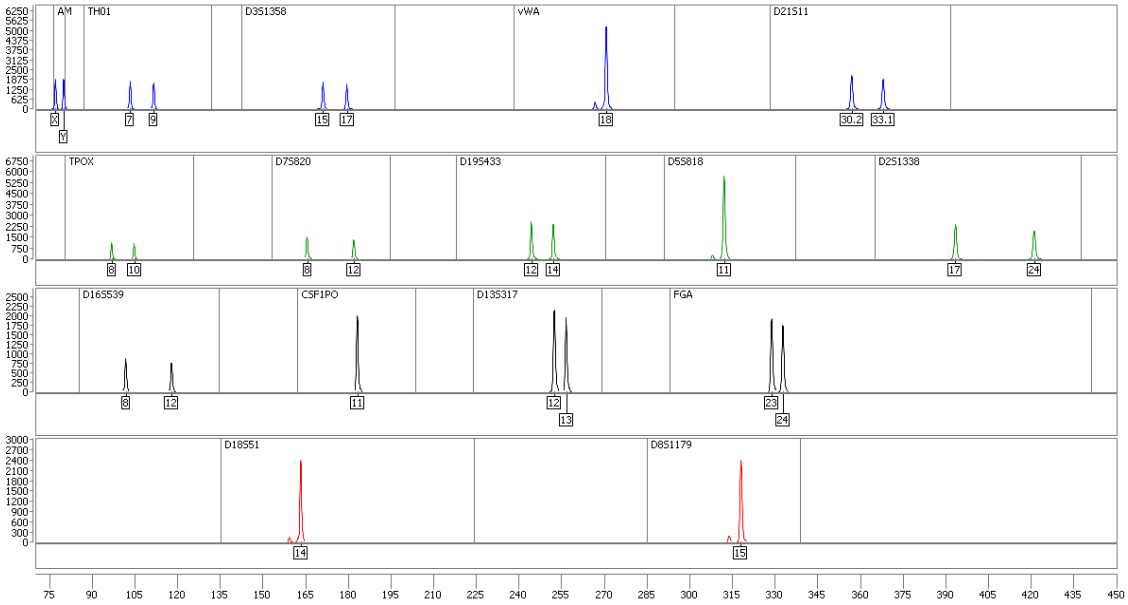


Figure 14. Aged samples. Example of blood on FTA paper stored for 18 months at room temperature.

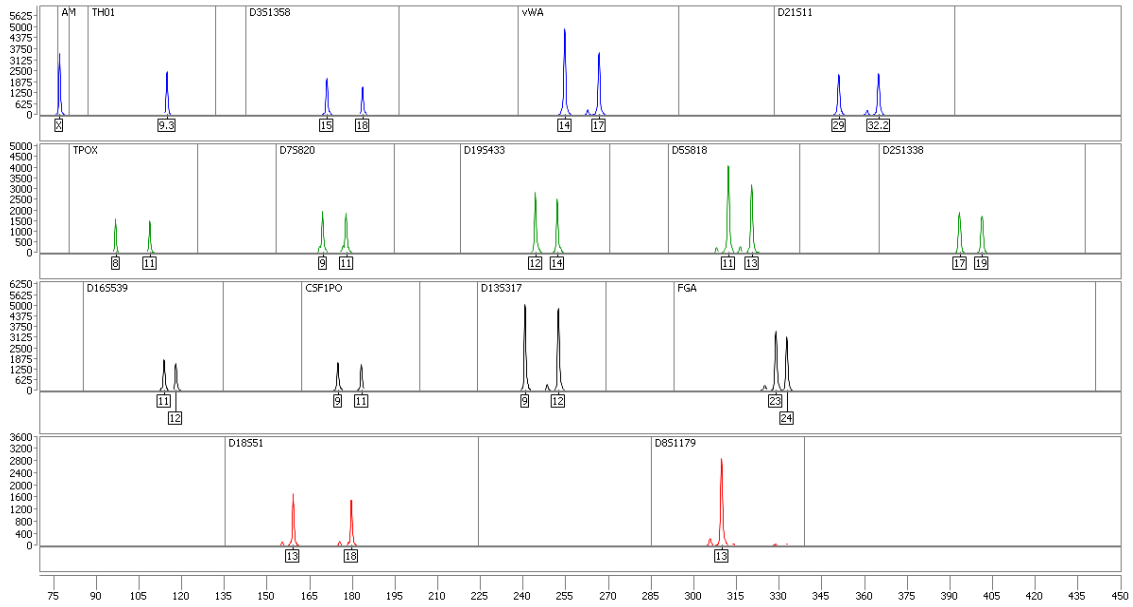


Figure 15. Aged samples. Example of buccal swab lysate stored for 12 months at –20°C.

Species specificity

To verify Investigator IDplex GO! species specificity for human DNA, the assay was run using purified human DNA and DNA from common pets, farm animals, and some primates species (Figure 16). As expected for primate DNA, amplification of some products was possible. Chimpanzee, bonobo, orangutan, and to a lesser degree, gorilla gave rise to several peaks within marker ranges in all channels, some of which match the size of human STR products. Macaque DNA produced an Amelogenin X-peak and further off-ladder peaks in the FAM, BTG and BTR panel. No DNA from other species tested gave rise to reproducible peaks above 50 RFU.

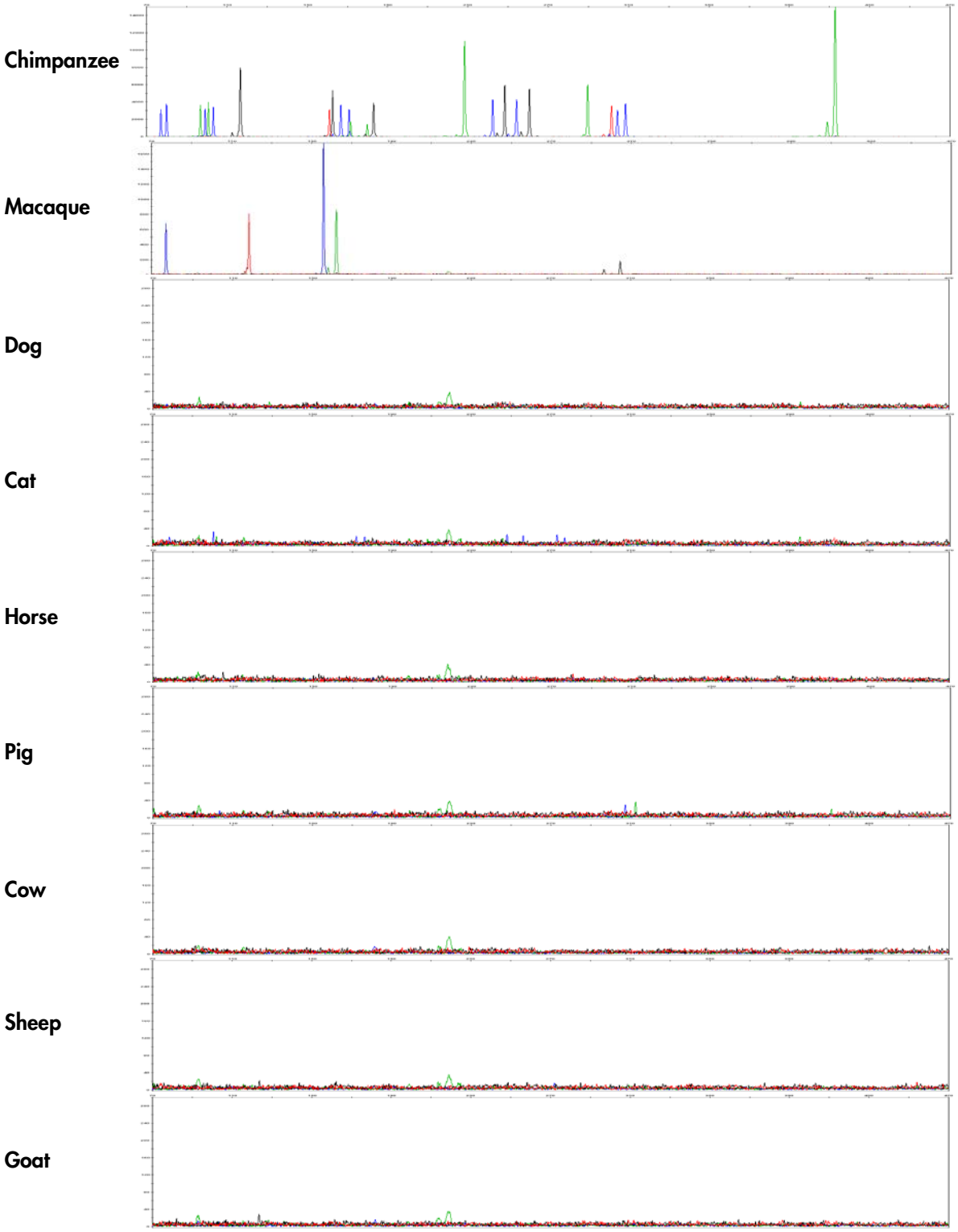


Figure 16. Representative results of the species specificity assessment. Primate DNA (500 pg) and DNA (2.5 ng) from all other species was used as template.

Reproducibility — concordance test

All primer binding sites of the Investigator IDplex GO! Kit are the same as in the Investigator IDplex Plus assay. Additional primers have been introduced to recover known primer binding site mutations of vWA and D16S539 (Figure 17). The mutations are present at elevated frequencies in populations of African origin, and affect the primer binding sites of vWA and D16S539. An 8 bp deletion in the vWA flanking region due to repeat sequence structure provides an almost perfect match, differing in just a single base. An additional SNP primer has been introduced to recover amplification of the affected alleles. A true SNP mutation is present in the binding region of the D16S539 marker.

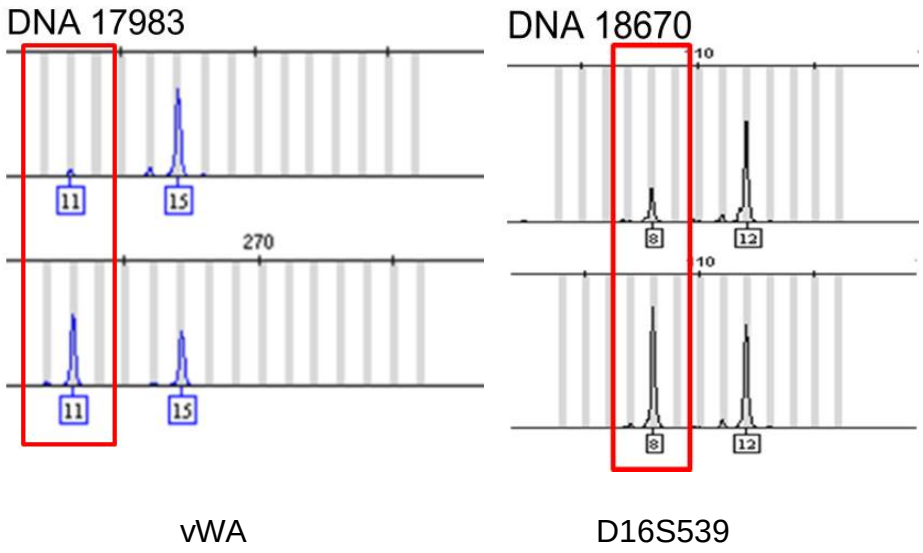


Figure 17. Analysis of DNA carrying SNP mutations. Details for the electropherograms from reference samples carrying known mutations pf vWA or D16S539 of African origin. Samples were amplified with (lower panel) or without (upper panel) additional SNP primers.

Concordance with IDplex Plus was tested using samples of blood on FTA paper from an internal pool of 100 donors; no discordant results were obtained. To test the performance with samples collected on untreated paper, blood from 19 different donors was applied to Whatman 903 Specimen Collection Paper. Punches of 1.2 mm were taken in 5 replicates for each donor and amplified using the same conditions as for blood on FTA paper. All samples gave full profiles and no obvious differences in profile quality were observed compared to treated paper (Figure 18 and Table 1).

Stutter peak heights are characteristic for each marker and the number of repeat motifs of an allele. TH01, in general, shows the lowest stutter ratio of all STR markers of the Investigator IDplex GO! Kit. Smaller alleles display lower stutter levels than longer alleles of the same marker. Only forward stutter positions were analyzed, as backward stutters usually are of very low height, although present (Figure 19 and Table 2).

In order to prove robustness for other sample types, EasieCollect cards from 38 different donors, Sarstedt cotton swabs from 36 different donors, and Puritan Polyester swabs from 35 different donors were processed in 5 replicates each. All samples gave full profiles (typical profile examples are shown in Figures 20–24). All FTA card sample processing was automated on a Hamilton easyPunch system (see section "Reproducibility — automated reaction setup").

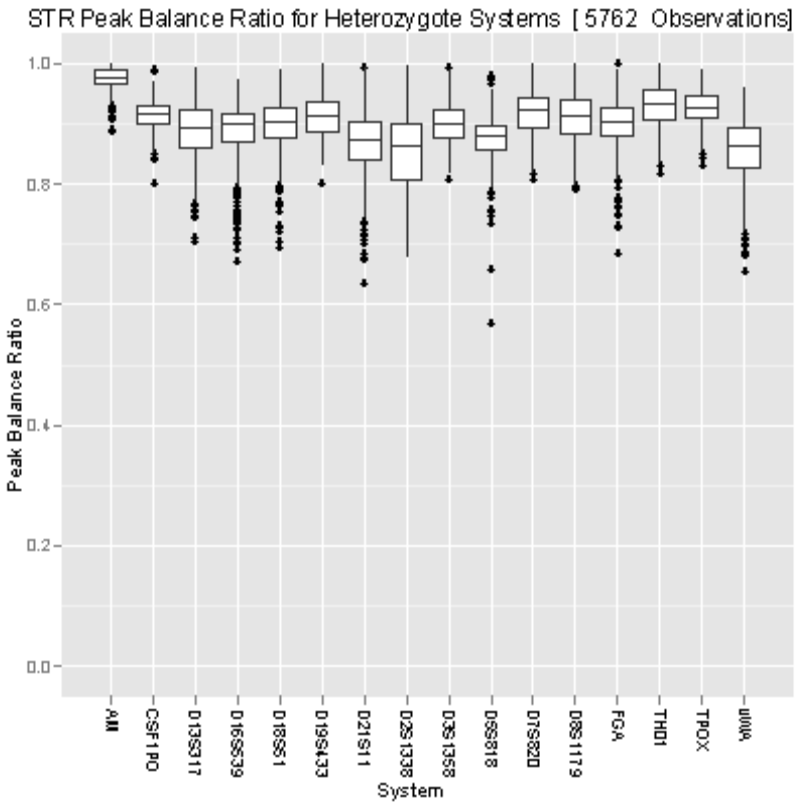
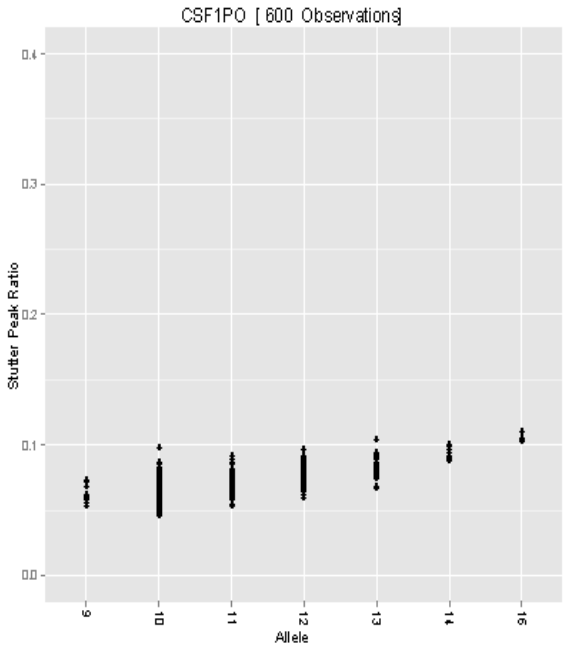
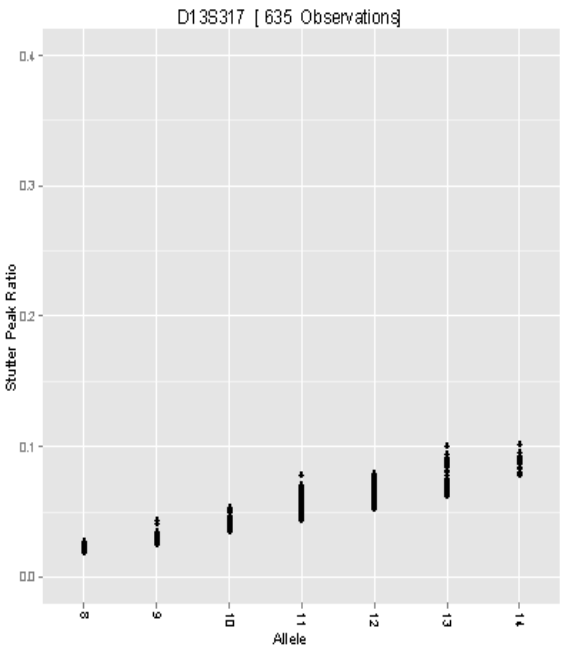


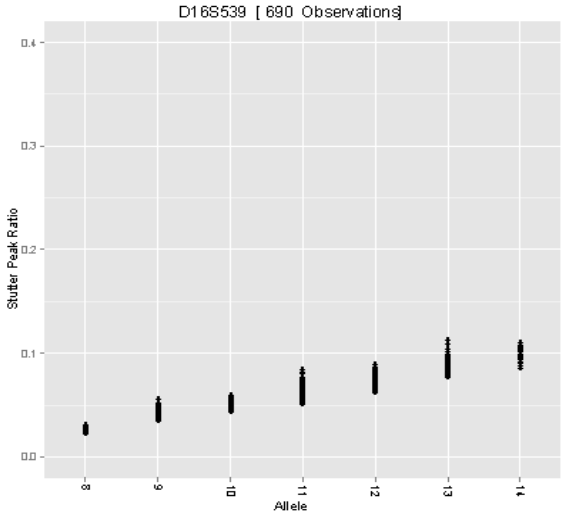
Figure 18. Box plot analysis of intra-locus peak height ratios. Values refer to the lower of the 2 peaks in a heterozygous sample. Boxes represent the middle 50% (interquartile range, [IQR]) of data, and lines inside the boxes represent the median. Whiskers show data within 1.5 IQR, and dots represent outliers.

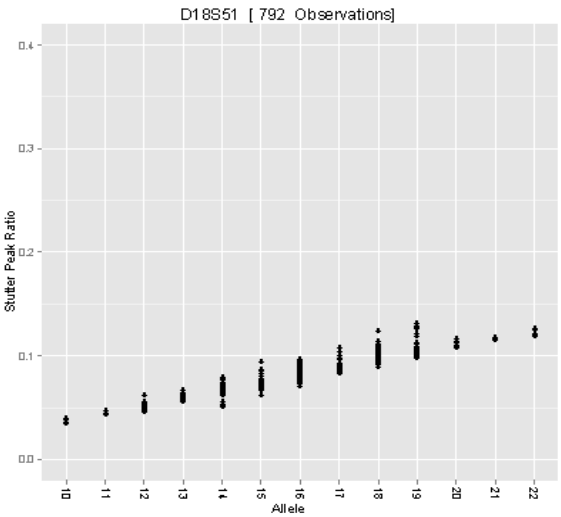
Table 1. Heterozygote peak height ratios

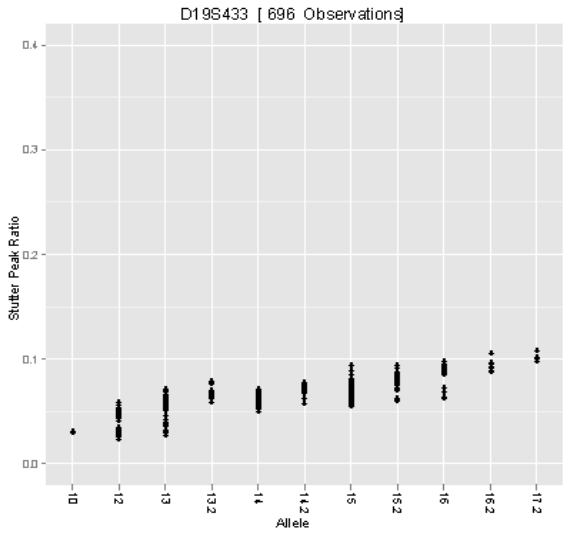
System	Observations	Mean (%)	Min (%)	Max (%)
CSF1PO	358	91.42	80.16	99.06
D13S317	365	88.73	70.46	99.29
D16S539	404	87.92	67.01	97.45
D18S51	404	89.52	69.39	98.81
D19S433	388	91.33	80.02	99.97
D21S11	362	86.86	63.37	99.50
D2S1338	425	85.60	68.21	99.49
D3S1358	385	89.99	80.78	99.29
D5S818	297	87.16	57.02	98.12
D7S820	390	91.97	80.61	99.92
D8S1179	399	90.96	79.20	99.93
FGA	408	89.77	68.58	99.93
TH01	324	93.22	81.81	99.93
TPOX	277	92.51	82.93	98.87
vWA	358	85.34	65.40	96.11

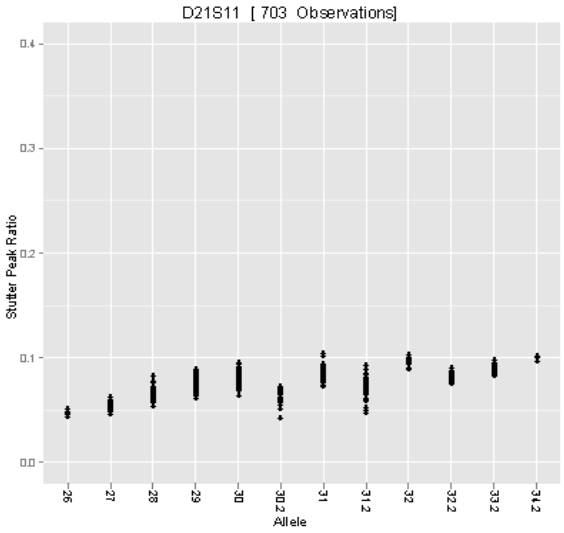

CSF1PO [600 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
9
10
11
12
13
14
16
Allele

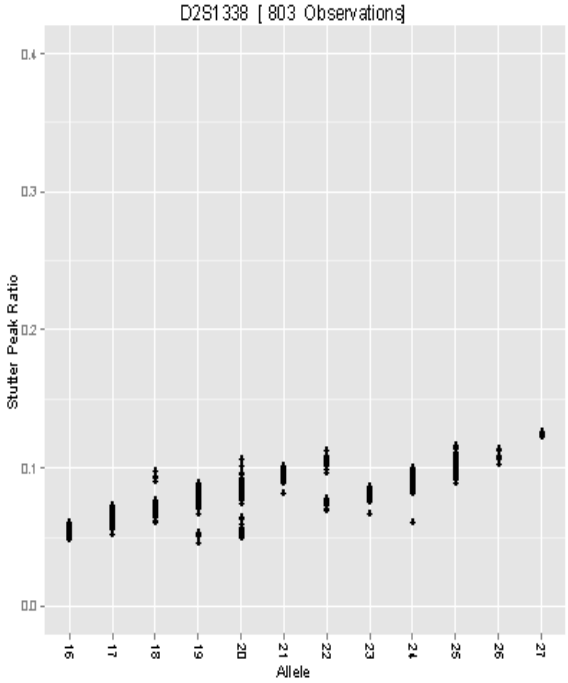

D13S317 [635 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
8
9
10
11
12
13
14
Allele

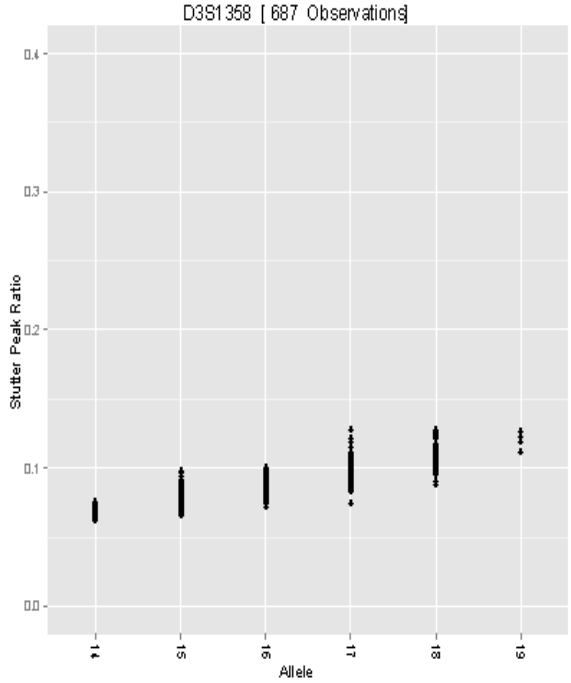

D16S539 [690 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
8
9
10
11
12
13
14
Allele


D18S51 [792 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
10
11
12
13
14
15
16
17
18
19
20
21
22
Allele


D19S433 [696 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
10
12
13
13.2
14
14.2
15
15.2
16
16.2
17.2
Allele


D21S11 [703 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
26
27
28
29
30
30.2
31
31.2
32
32.2
33.2
34.2
Allele


D2S1338 [803 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
16
17
18
19
20
21
22
23
24
25
26
27
Allele


D3S1358 [687 Observations]
Stutter Peak Ratio
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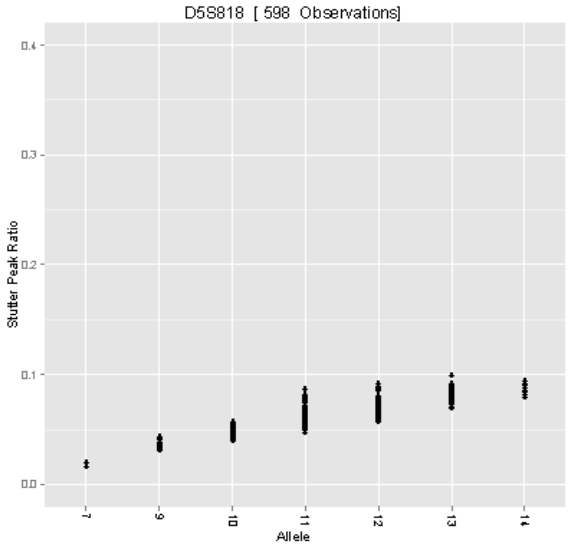
0.3
0.4
14
15
16
17
18
19
Allele


D5S818 [598 Observations]
Stutter Peak Ratio
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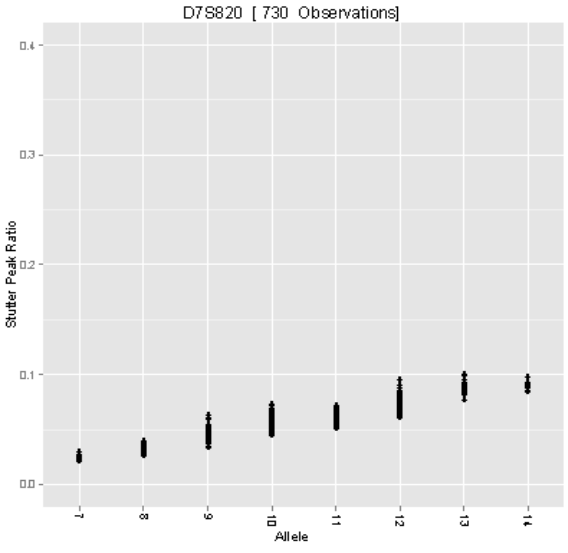
0.3
0.4
7
9
10
11
12
13
14
Allele


D7S820 [730 Observations]
Stutter Peak Ratio
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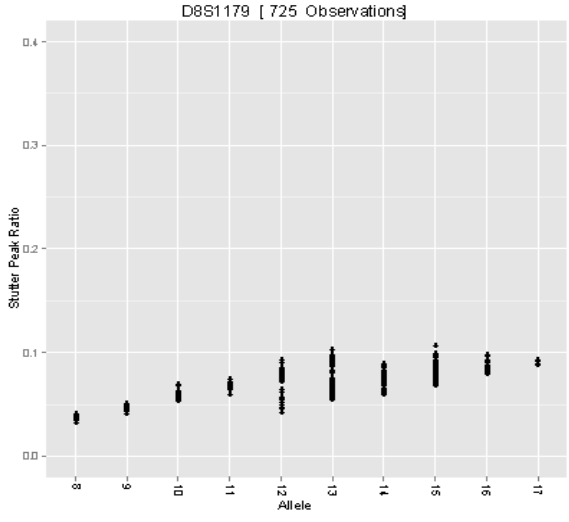
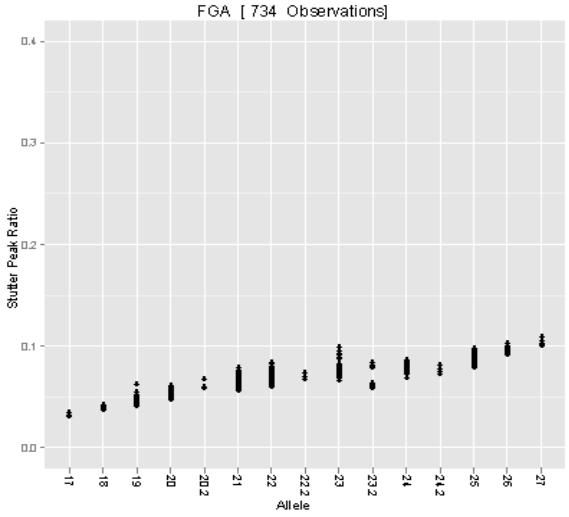
0.2
0.3
0.4
7
8
9
10
11
12
13
14
Allele


D8S1179 [725 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
8
9
10
11
12
13
14
15
16
17
Allele


FGA [734 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
17
18
19
20
20.2
21
22
22.2
23
23.2
24
24.2
25
26
27
Allele

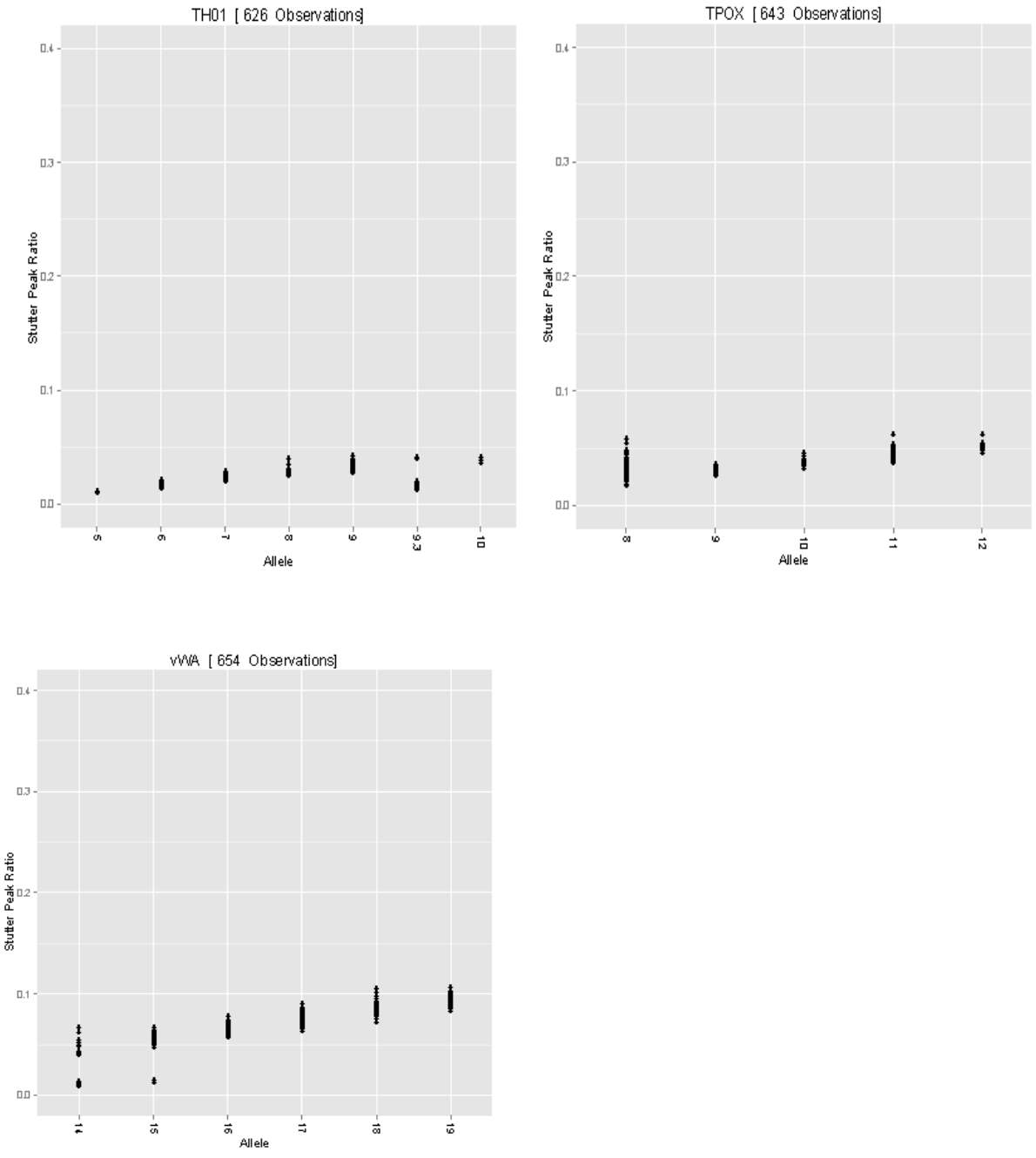


Figure 19. Stutter peak height ratios. Peak heights of forward stutters compared to main peaks were analyzed for 100 blood samples on FTA paper from different donors in 5 replicates.

Table 2. Stutter peak height ratios

System	Observations	Stutter Mean (%)	Stutter Min (%)	Stutter Max (%)
CSF1PO	600	7.0	4.6	18.2
D13S317	635	5.3	1.8	16.3
D16S539	690	6.6	2.2	11.3
D18S51	792	7.6	3.5	17.2
D19S433	696	6.4	2.3	12.4
D21S11	703	7.6	4.2	10.9
D2S1338	803	8.0	4.6	12.6
D3S1358	687	8.7	6.2	12.8
D5S818	598	6.4	1.6	10.3
D7S820	730	5.6	2.1	11.4
D8S1179	725	7.0	3.3	11.5
FGA	734	6.9	3.1	11.3
TH01	626	2.1	1.0	4.2
TPOX	643	3.5	1.8	6.2
vWA	654	7.1	0.9	12.3

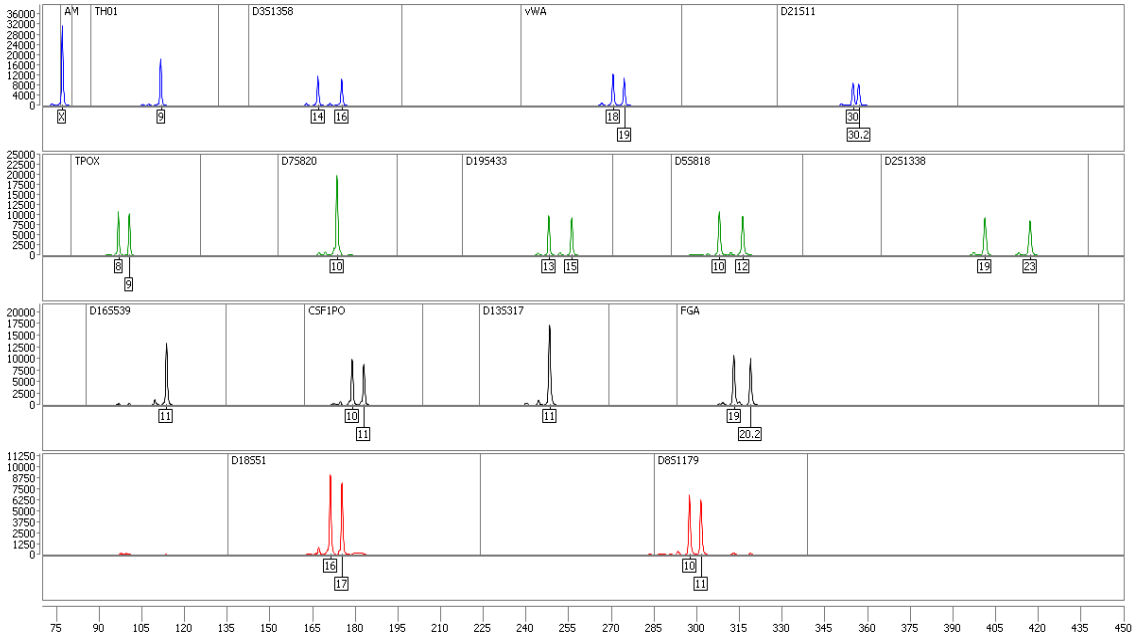


Figure 20. Representative electropherogram for a blood sample on FTA paper.

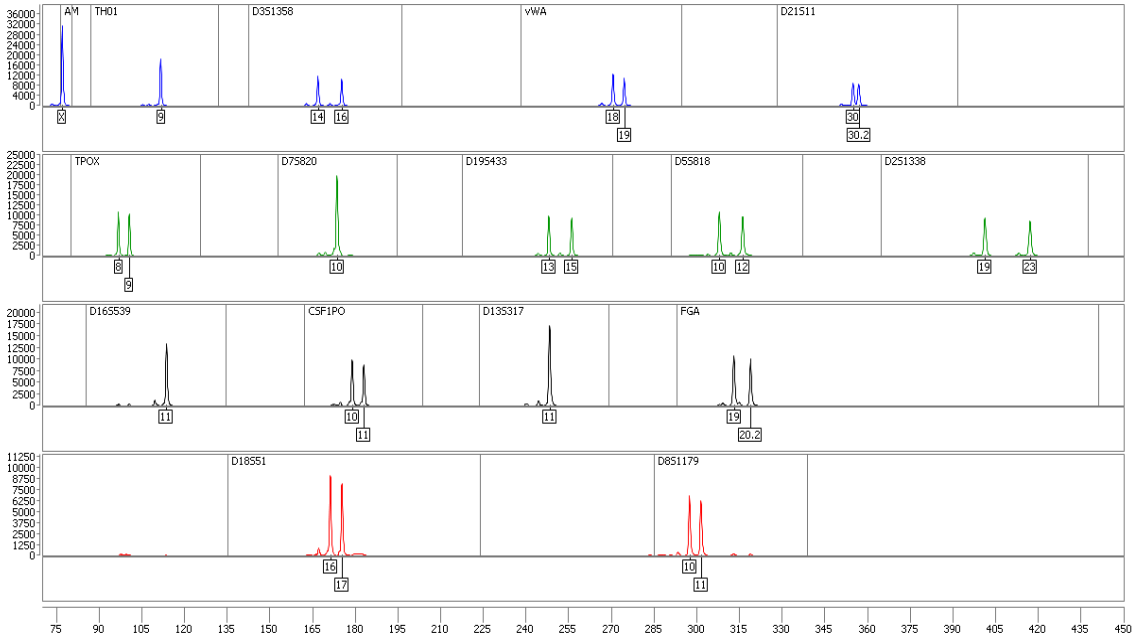


Figure 21. Representative electropherogram for a blood sample on Whatman 903 collection paper.

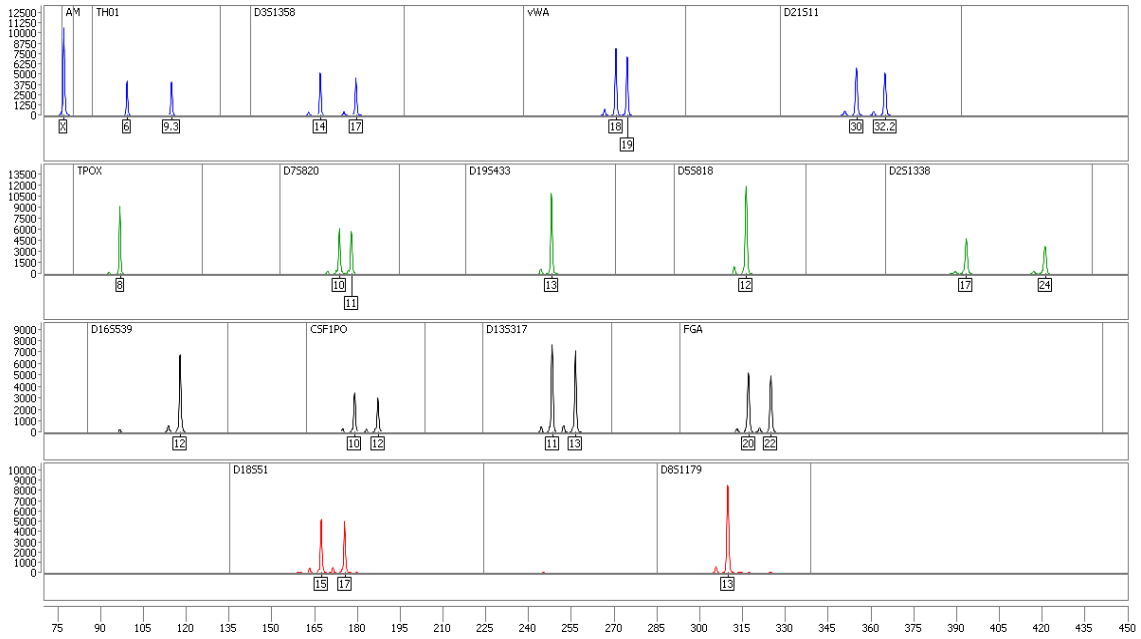


Figure 22. Representative electropherogram for a sample of buccal cells on Whatman EasiCollect.

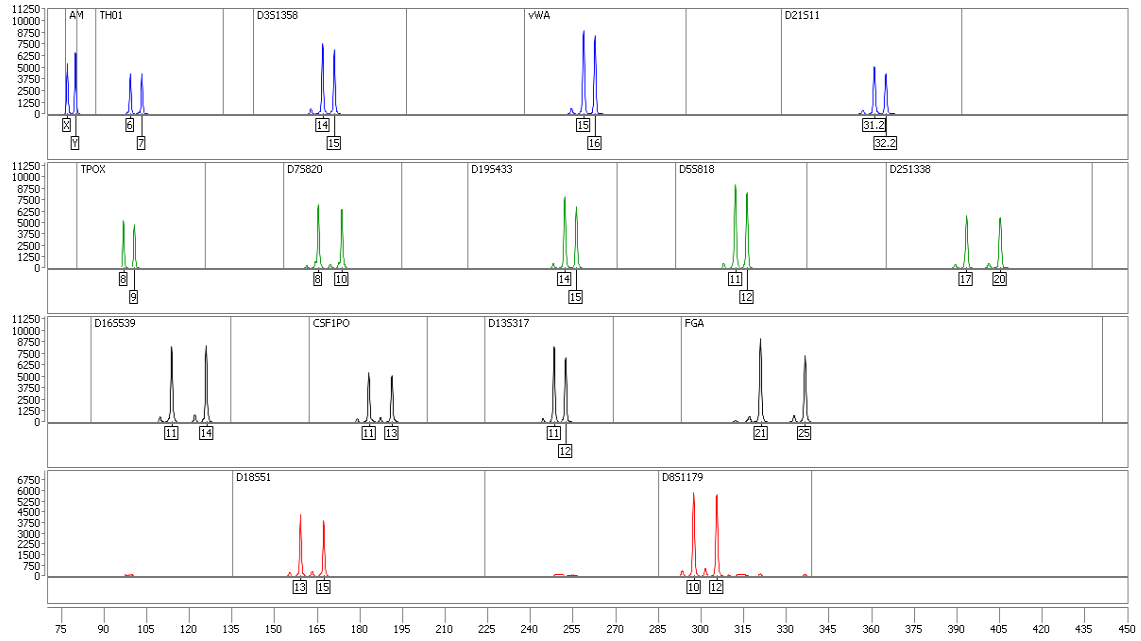


Figure 23. Representative electropherogram for a Puritan polyester swab lysate sample.

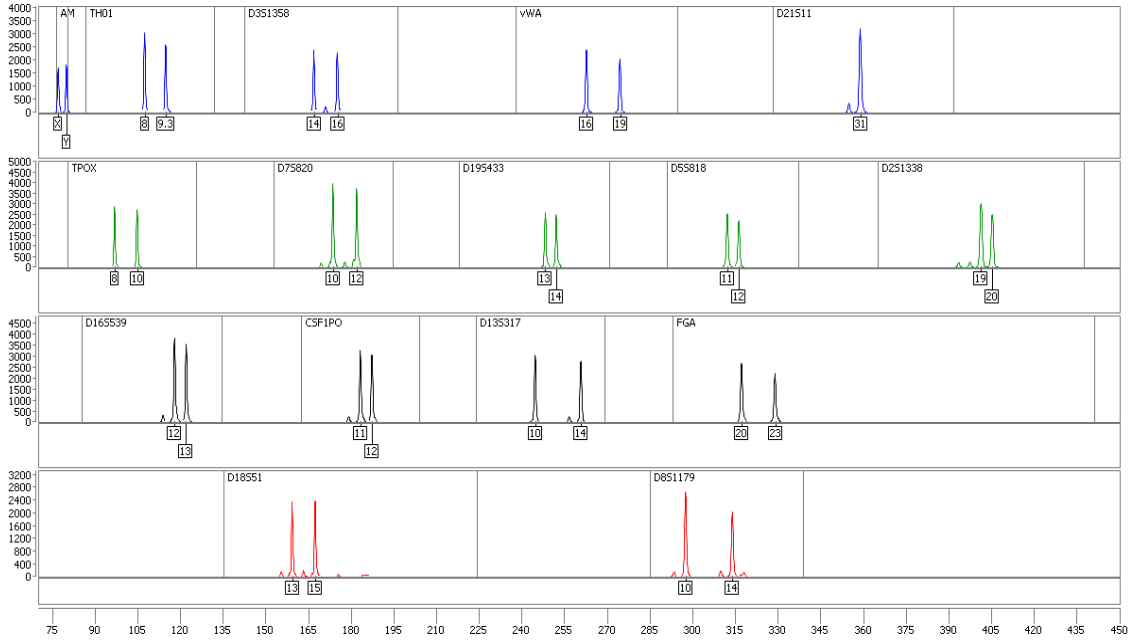


Figure 24. Representative electropherogram for a Sarstedt cotton swab lysate sample.

Reproducibility — automated reaction setup

Protocols were developed for fully automated punching of FTA paper and reaction setup for amplification with the QIAGEN Investigator IDplex GO! Kit on the Hamilton easyPunch STARlet instrument. Blood on FTA paper (100 samples) and buccal cells on EasieCollect cards (38 samples) from different donors were processed in 5 PCR replicates (Figures 25 and 26). A single punch of 1.2 mm size was taken from the center of the sampling area and placed in a 96-well PCR plate (BioRad Hard-Shell®), prior to the addition of 25 µl of PCR reagent master mix. Results obtained using the automated method were comparable to results from manually processed samples from the same donors. Variation between samples from different donors was within the expected range, and no incident of sample mix-up or missed samples was observed.

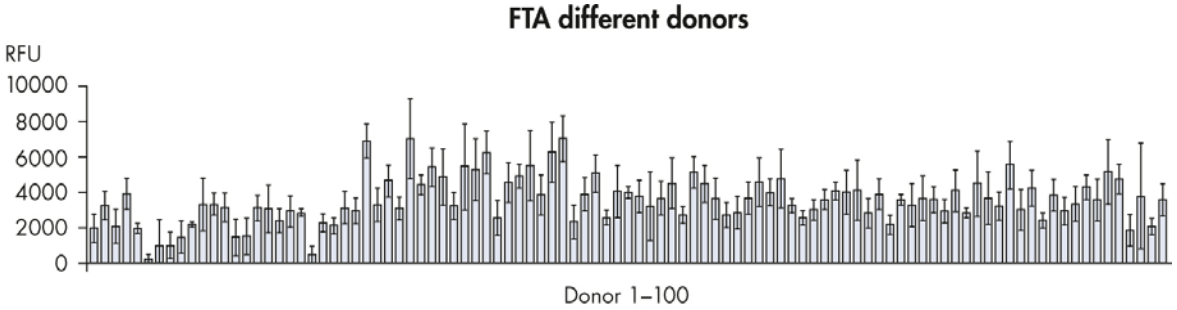


Figure 25. Blood on FTA paper. Average peak heights across samples from 100 different donors processed in 5 replicates.

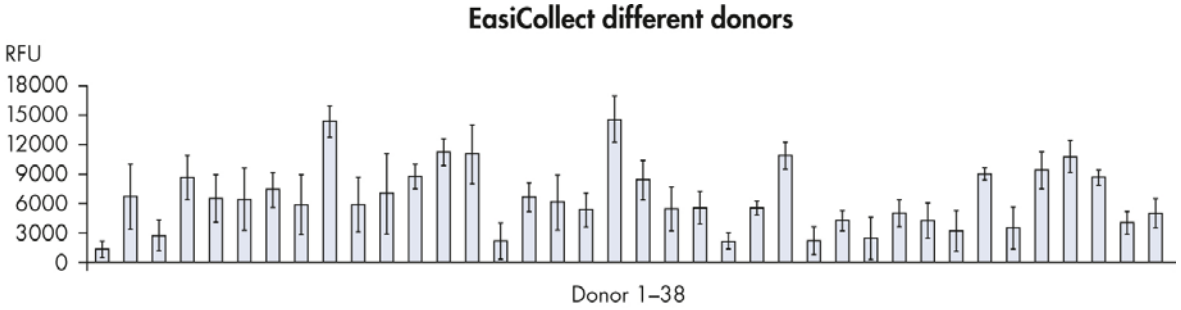


Figure 26. Buccal cells on FTA paper. Average peak heights across samples from 38 different donors processed in 5 replicates.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

Cited references

1. ENFSI Standing Committee for Quality and Competence (QCC). Validation and Implementation of (New) Methods. Ref. Code: QCC-VAL-001, Issue No. 001, 4 November 2006. http://www.enfsi.eu/get_doc.php?uid=144.
2. Revised Validation Guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM) Forensic Science Communications, July 2004, Volume 6, Number 3. www.cstl.nist.gov/strbase/validation/SWGDAM_Validation.doc.
3. Butler, J. (2006) Genetics and Genomics of Core Short Tandem Repeat Loci Used in Human Identity Testing. J. Forensic Sci. **51**, 253

Ordering Information

Product	Contents	Cat. no.
Investigator IDplex GO! Kit (200)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and RNase-free water	381636
Investigator IDplex GO! Kit (1000)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and RNase-free water	381638
Investigator STR GO! Lysis Buffer (200)	Lysis buffer for 200 swab samples	386516

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Service or your local distributor.

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