QIAGEN Validation Report

Developmental validation of the Investigator® ESSplex SE GO! Kit

The QIAGEN Investigator ESSplex SE 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 ESSplex SE 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 the 15 polymorphic STR markers recommended by the ENFSI and the European DNA Profiling Group (EDNAP) as the new European Standard Set of loci (D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045, FGA [FIBRA], TH01 [TC11], and vWA), plus SE33 [ACTBP2], and the gender-specific Amelogenin (3). These genetic loci have been characterized in numerous studies by other laboratories (4–6). 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 9–10).

Cross-reactivity with non-human DNA was also assessed (pages 14–15). The reproducibility of the results was verified (pages 16–25). This validation study covers a fully automated workflow for blood or buccal cells on FTA paper using the Hamilton easyPunch STARlet system (page 26).

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 ESSplex SE 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).

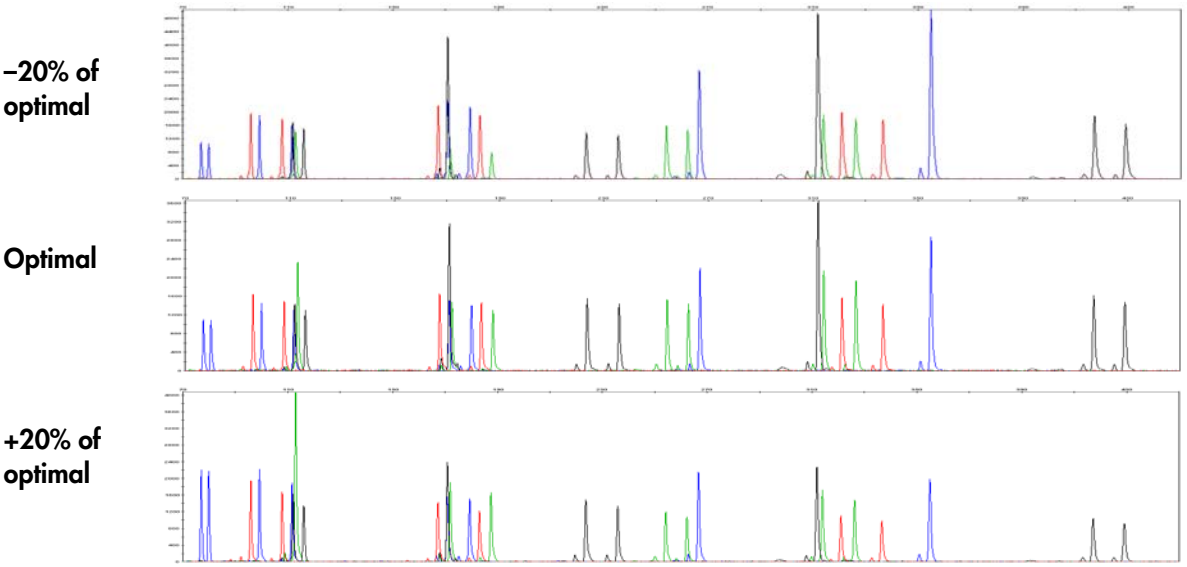


Figure 1. Influence of $MgCl_2$ concentration. Examples of blood on FTA paper analyzed in triplicate.

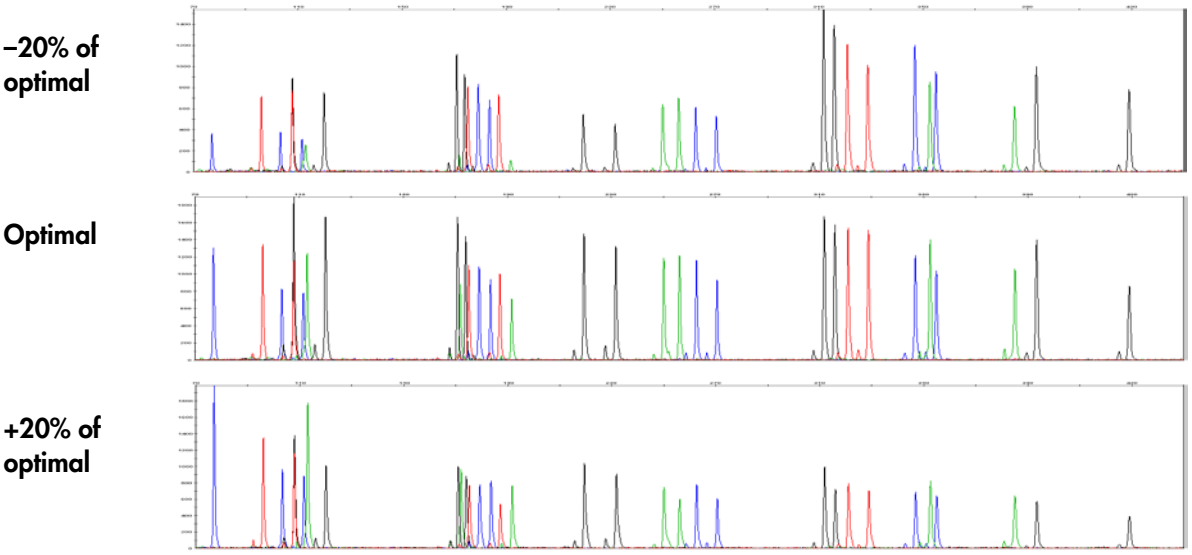


Figure 2. Influence of $MgCl_2$ concentration. Examples of a buccal swab sample analyzed in triplicate.

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 ESSplex SE GO! reaction (61°C). Annealing temperatures between 57°C and 65°C were used for the amplification of DNA from 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, alleles of markers D12S391 and D2S1338 dropped below 100 RFU. Overall signal heights were at a comparable level at 58°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.

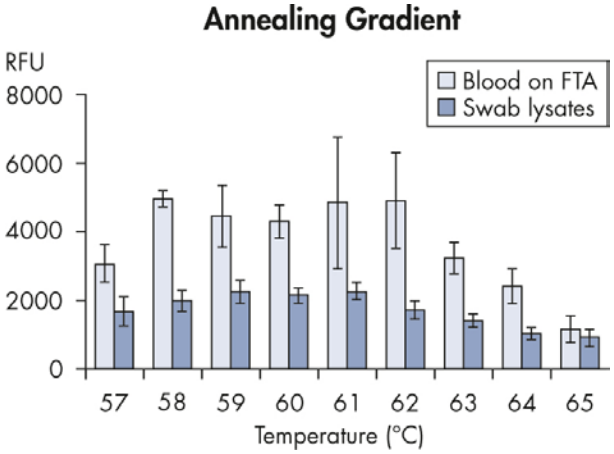


Figure 3. Effect of variations in the PCR annealing temperature. Blood on FTA paper and buccal swab lysates from a single donor were analyzed in triplicate using 25 PCR cycles for blood on FTA paper and 28 cycles for swab lysates.

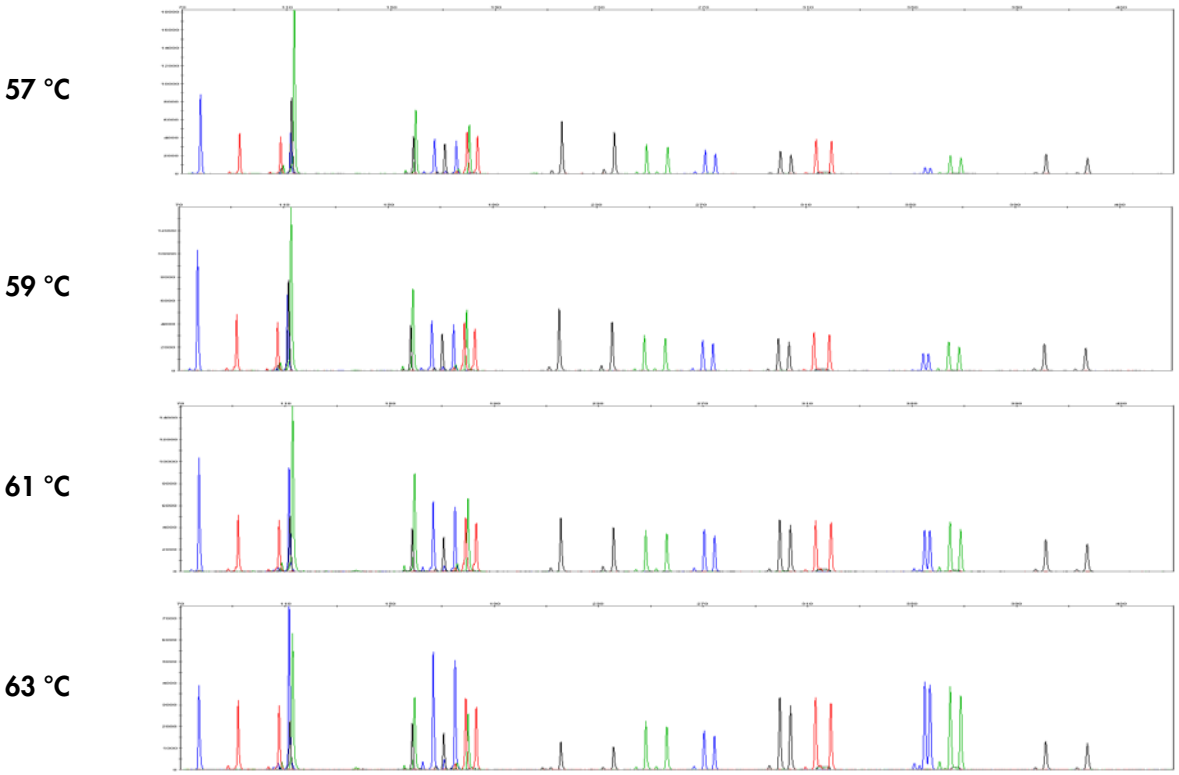


Figure 4. Variations in the PCR annealing temperature. Representative electropherograms for reactions using annealing temperatures of 57–63°C for blood on FTA paper.

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 with each cycle added.

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

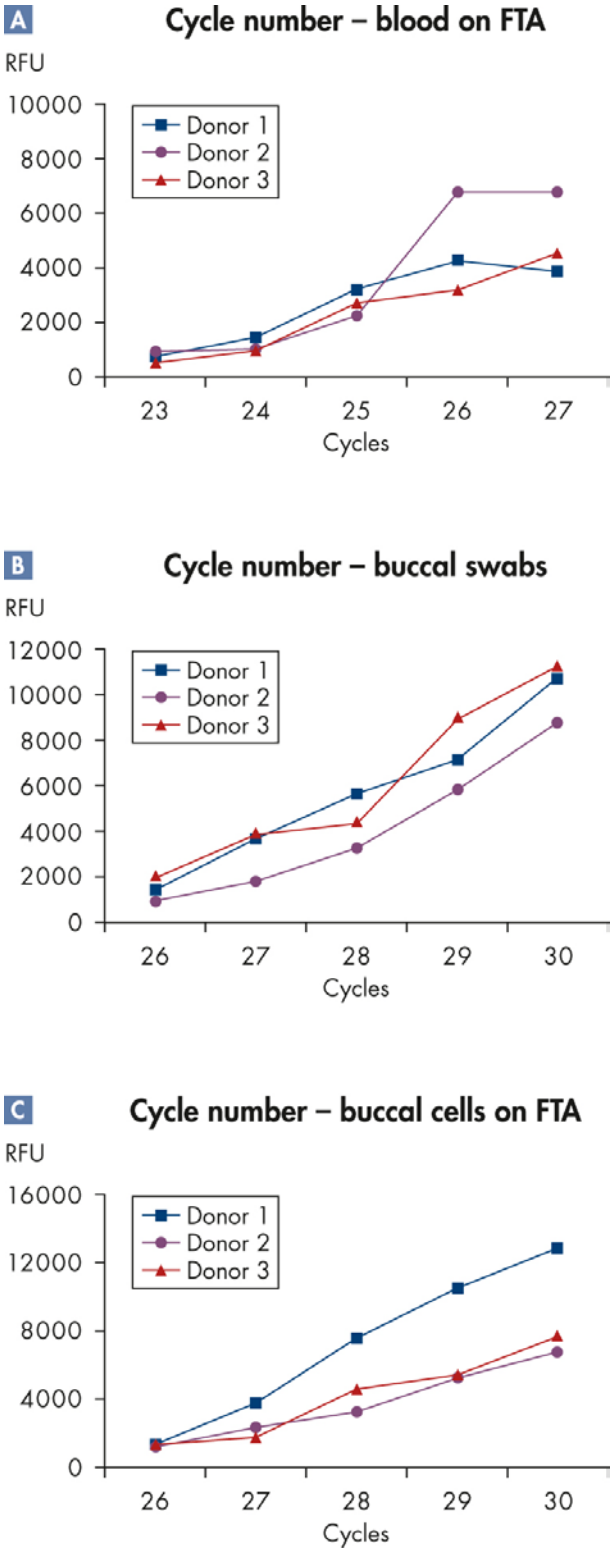


Figure 5. Effect of different cycle numbers on mean peak height. Samples were run in 5 replicates per donor. **[A]** Blood on FTA paper. **[B]** Buccal swabs. **[C]** Buccal cells on FTA paper.

Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator ESSplex SE 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-ThermoBlock (Biometra Biometra biomedizinische Analytik GmbH, Göttingen, Germany)

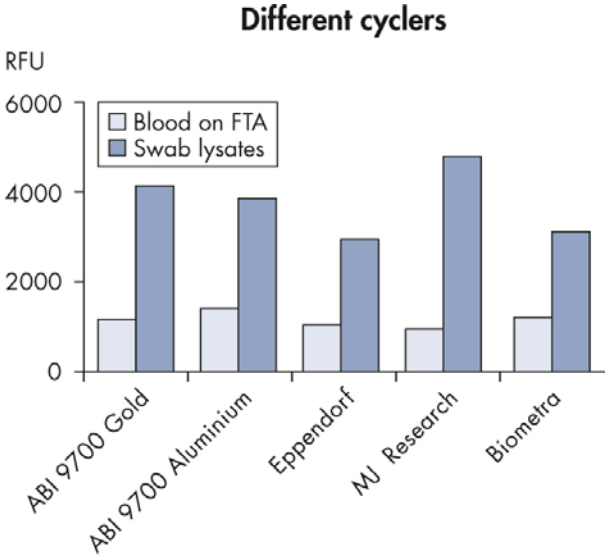


Figure 6. Performance on different PCR thermal cyclers. Average signal heights for blood on FTA paper and buccal swab lysates from 5 different donors, run in 5 replicates each.

Effect of different genetic analyzers

Two different genetic analyzers were tested with the Investigator ESSplex SE 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, were run on a GeneAmp PCR System 9700 with Gold-plated Silver 96-Well Block and 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 (Figure 7). However, average signal heights were lower on the 3130 Genetic Analyzer (Figure 8). Therefore, limitations of genetic analyzer sensitivity must be addressed by individual laboratories during an internal validation study using the instrumentation in their laboratory.

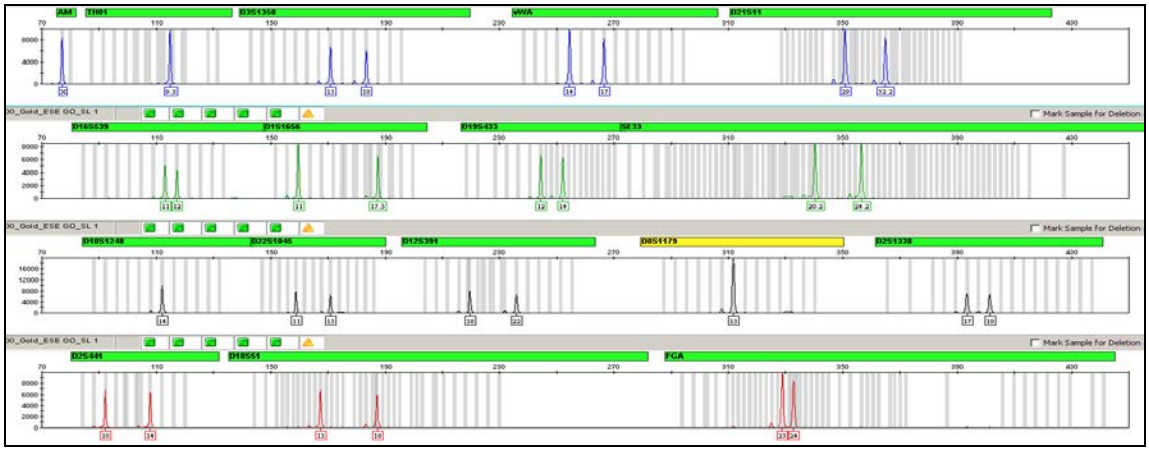


Figure 7. Analysis of PCR products on different genetic analyzers. DNA from blood on FTA paper was amplified on a GeneAmp PCR System 9700. Samples were run according to the *Investigator ESSplex SE GO! Handbook*. Analysis was performed on an Applied Biosystems 3500 Genetic Analyzer.

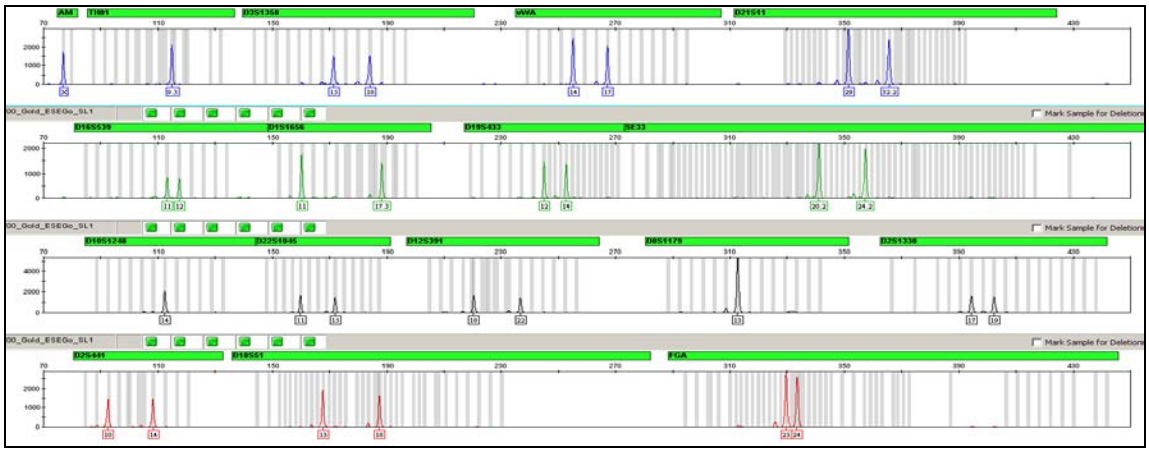


Figure 8. Analysis of PCR products on different genetic analyzers. Same sample as shown in Figure 7, analyzed on an Applied Biosystems 3130 Genetic Analyzer.

Sensitivity

The Investigator ESSplex SE 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.

The obtained signal heights correlated well with the dilution factor of the sample material (Figure 9). Full profiles were achieved for all samples down to a 1:27 dilution. Individual alleles dropped below the 50 RFU thresholds for both blood on FTA paper and buccal cell lysates at a dilution of 1:81 (Figure 10). However, the full profiles from these samples could be recovered by increasing the number of PCR cycles.

Issues with very high sample amounts

Fluorescence intensity may go off the scale depending on the instrumentation and settings used for capillary electrophoresis. "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.

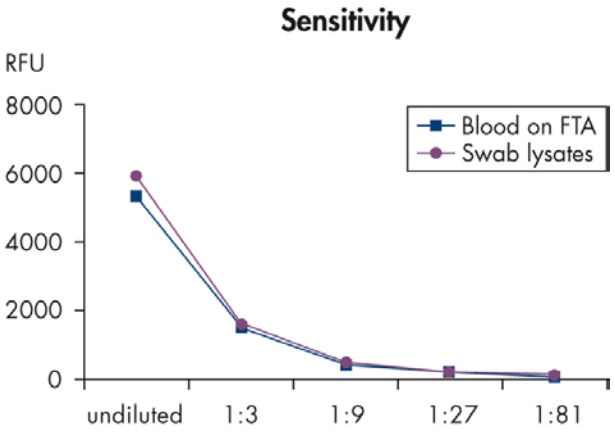


Figure 9. Sensitivity study. A serial dilution of a blood sample on FTA paper or a buccal swab lysate.

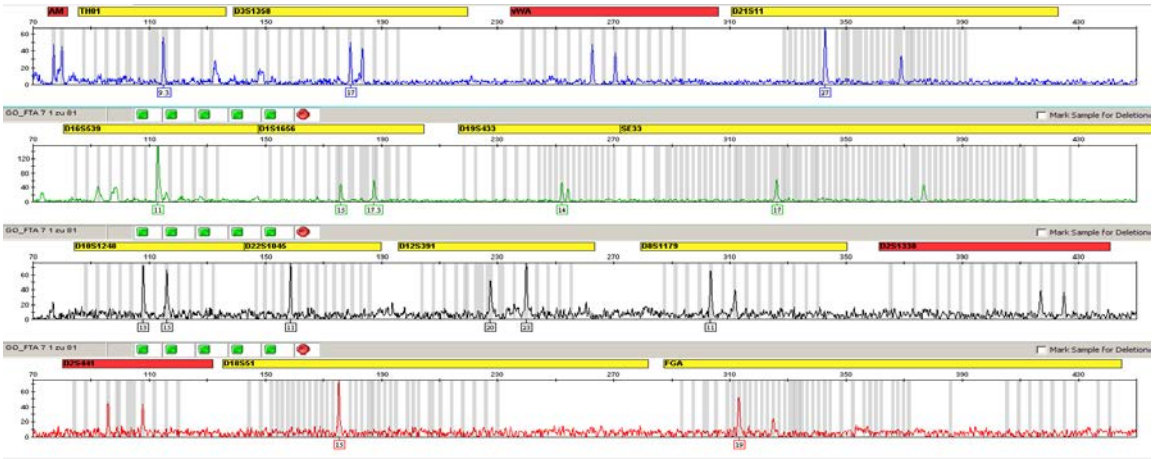


Figure 10. Sensitivity study. Example of blood on FTA paper diluted 1:81 prior to spotting. Several alleles dropped below 50 RFU.

Stability

Sample input

The robustness of the assay 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 lysate were used (Figures 11–13 show example electropherograms). For all sample types, 3 replicates from 3 donors were run. Although increased sample amounts in general 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 and D2S441. Both effects indicate inhibition of 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.

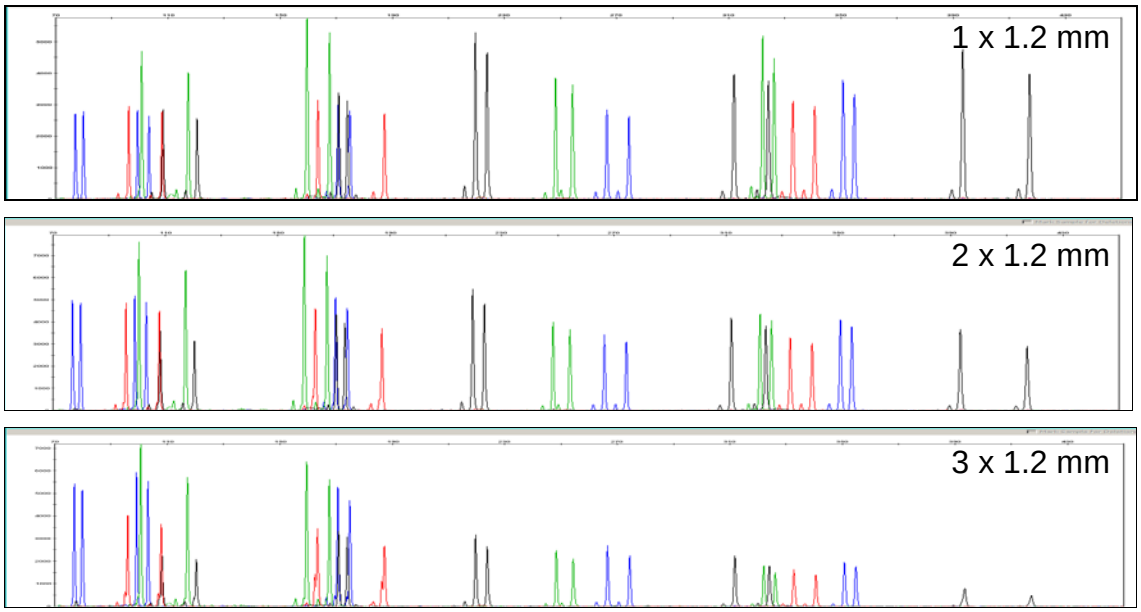


Figure 11. Sample input, blood on FTA paper. Amplification of DNA from 1, 2, or 3 punches of 1.2 mm.

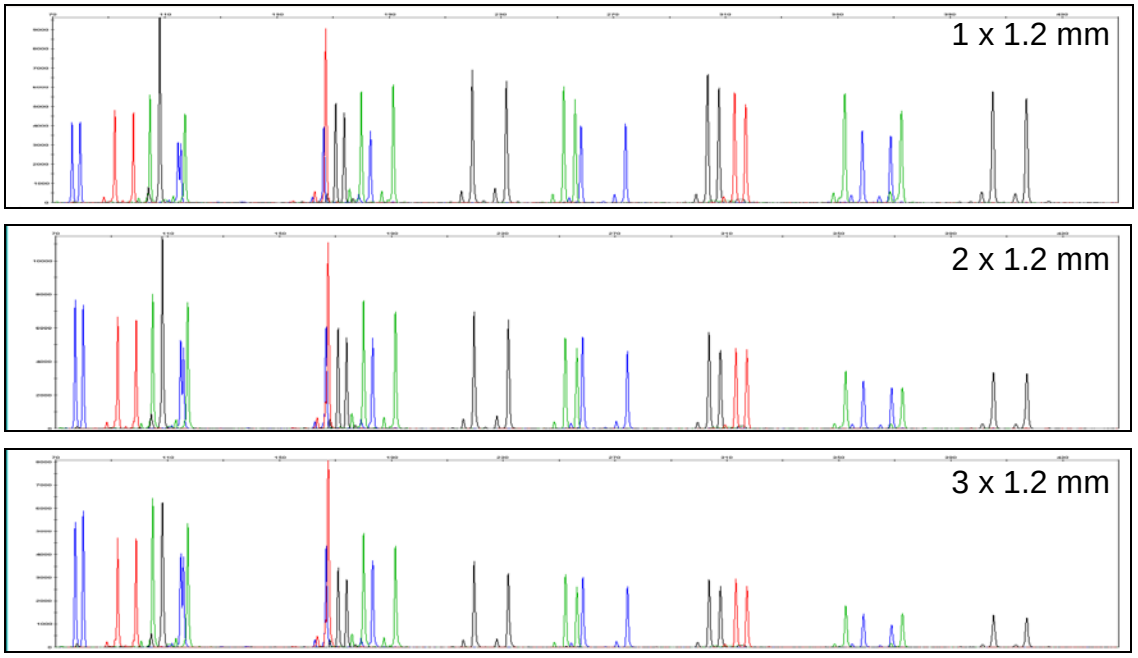


Figure 12. Sample input, buccal cells on FTA paper. Amplification of DNA from 1, 2, or 3 punches of 1.2 mm.

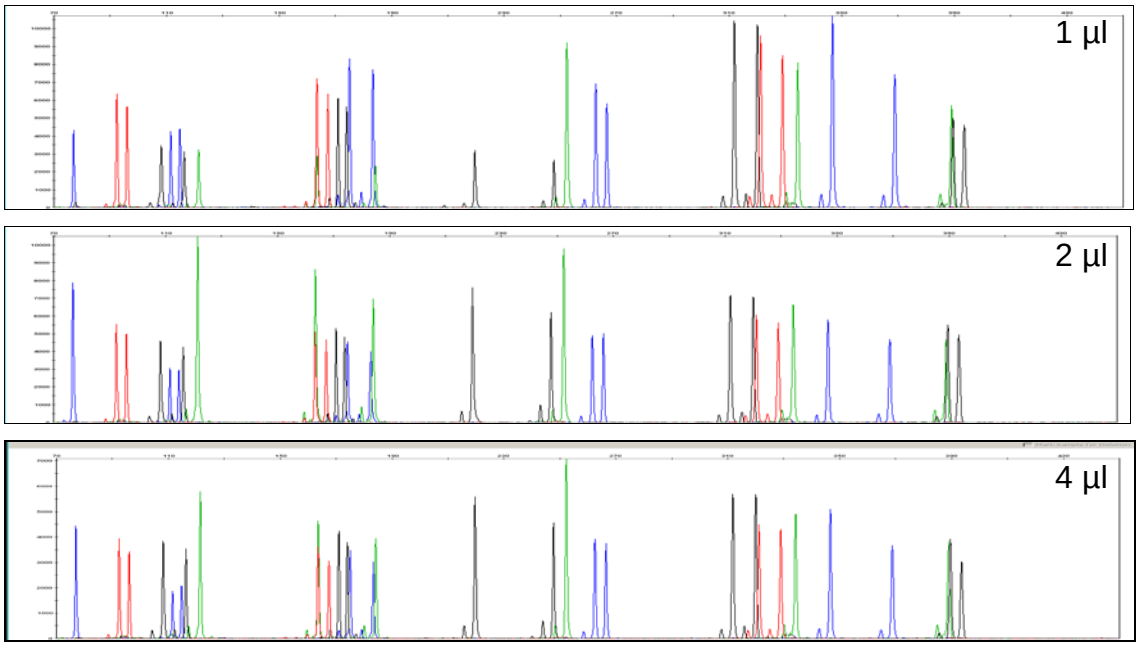


Figure 13. Sample input, buccal cell lysates. Amplification of DNA from 1, 2, or 4 µl of a buccal swab lysate.

Aged samples

DNA was amplified from blood on FTA paper stored at room temperature for 19 months and crude lysates from buccal swabs stored at –20°C for 14 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).

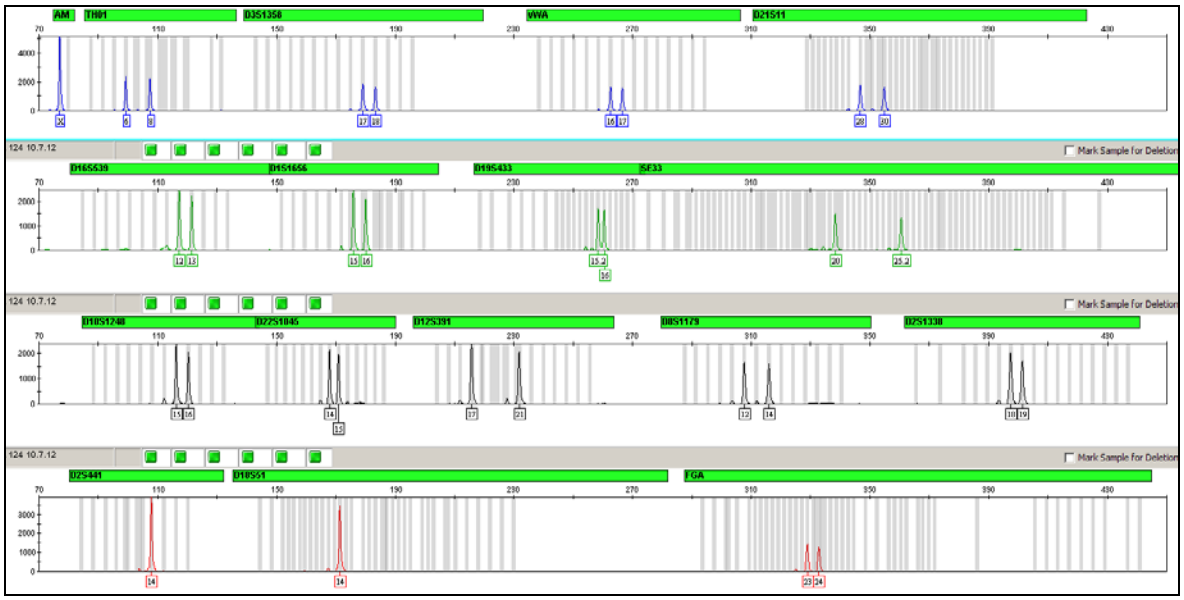


Figure 14. Aged samples. Example of blood on FTA paper stored for 19 months at room temperature.

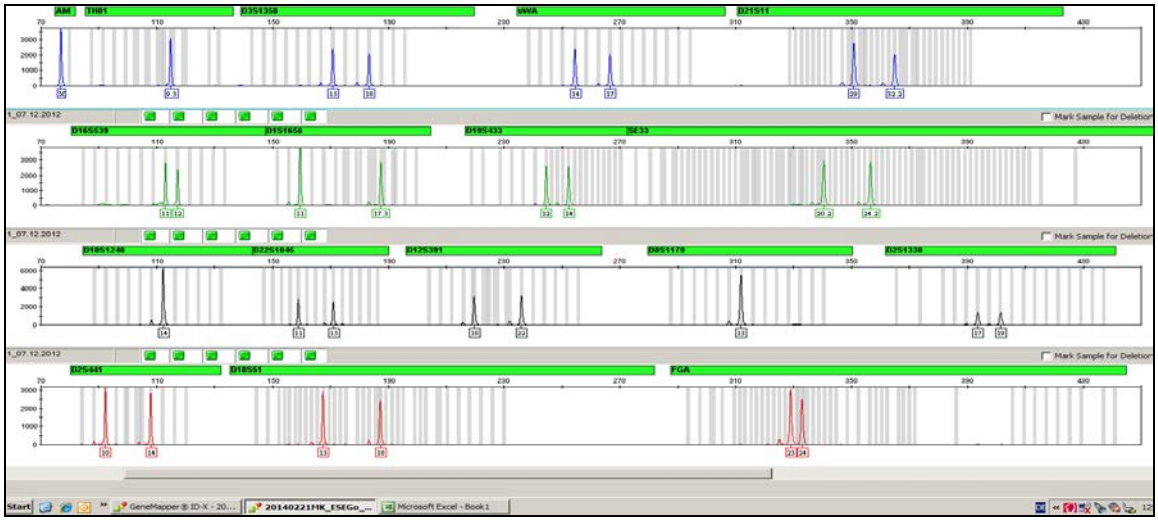


Figure 15. Aged samples. Example of buccal swab lysate stored for 14 months at –20°C.

Species specificity

To verify Investigator ESSplex SE GO! species specificity for human DNA, the assay was run using purified human DNA and DNA from common pets, farm animals, and some primate species (Figure 16). As expected for primate DNA, amplification of some products was possible. Chimpanzee, bonobo, orangutan, and to a lesser degree, gorilla DNA 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. Porcine DNA produced a single peak of about 300 bp that was detected in the FAM and BTG panel between 50 and 150 RFU. No DNA from the other species tested gave rise to reproducible peaks above 50 RFU.

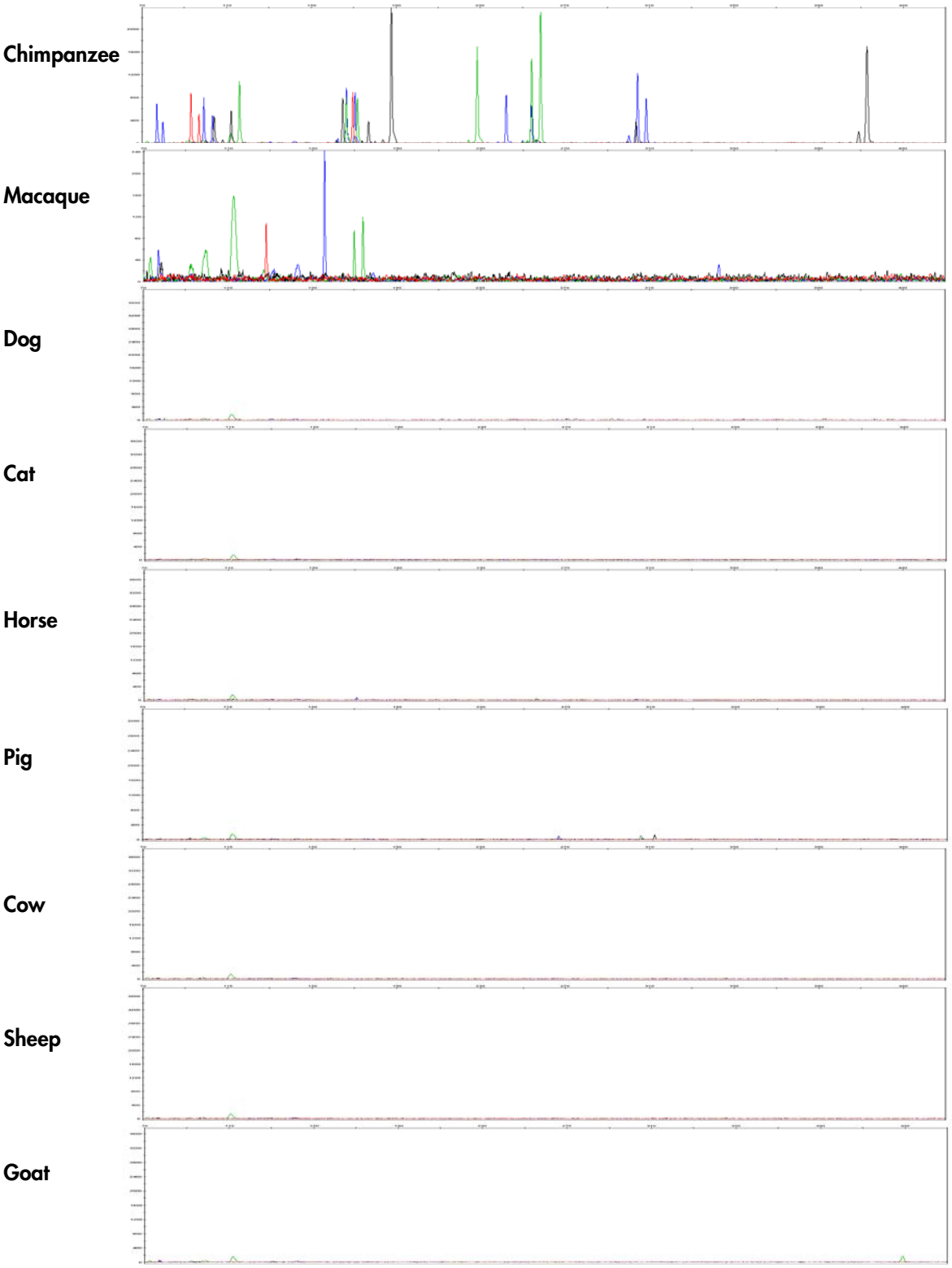


Figure 16. Representative results from a species specificity assessment. Primate DNA (500 pg) and DNA (2.5 ng) from all other species was used as template.

Reproducibility — concordance test

All primer binding sites of the Investigator ESSplex SE GO! Kit are the same as in the Investigator ESSplex SE Plus assay. Additional primers have been introduced to recover known primer binding site mutations of vWA, D16S539, and SE33 (Figure 17). 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. Similarly, for SE33 a 4 bp deletion creates a single base mismatch that can be overcome with another SNP primer. A true SNP mutation is present in the binding region of the D16S539 marker.

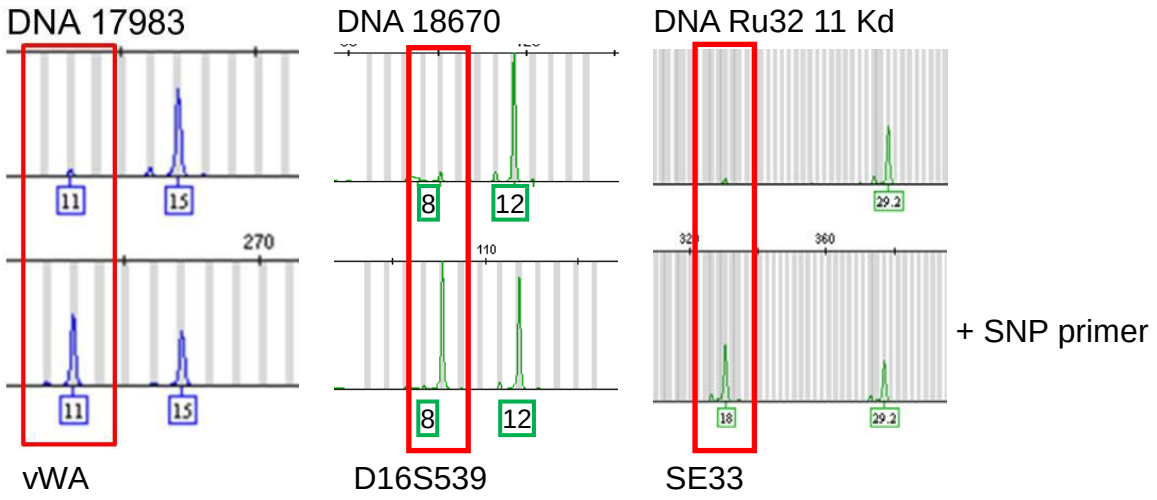


Figure 17. Analysis of DNA carrying SNP mutations. Details for the electropherograms from reference samples carrying known mutations of African origin.

Concordance with ESSplex SE Plus was previously tested using samples of blood on FTA paper from an internal pool of 95 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 ESSplex SE 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). Note that marker D22S1045 shows a significantly elevated backward stutter. This is intrinsic and due to the fact that the marker consists of trinucleotide instead of tetranucleotide

repeats. This may also lead to unexpectedly high forward stutter peaks if alleles differ by two repeat units, and the backward stutter of the smaller allele overlaps with the forward stutter of the longer allele.

In order to prove robustness for other sample types, EasiCollect 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").

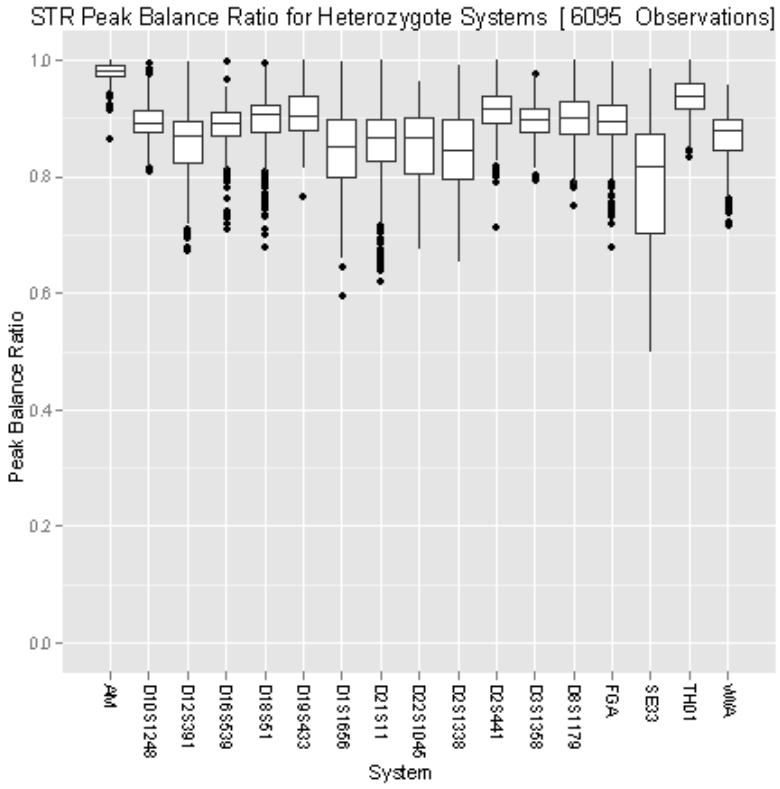
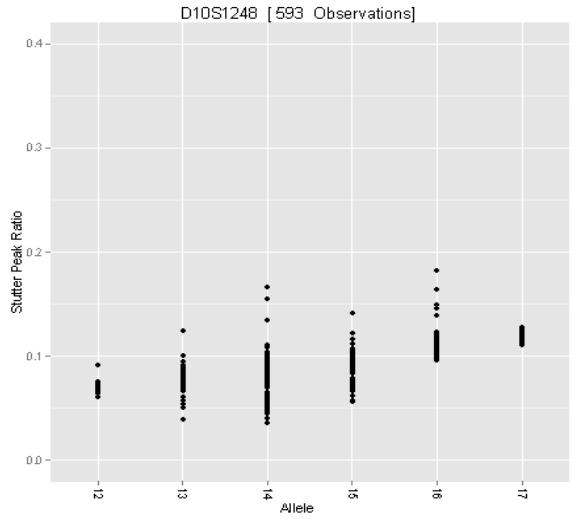
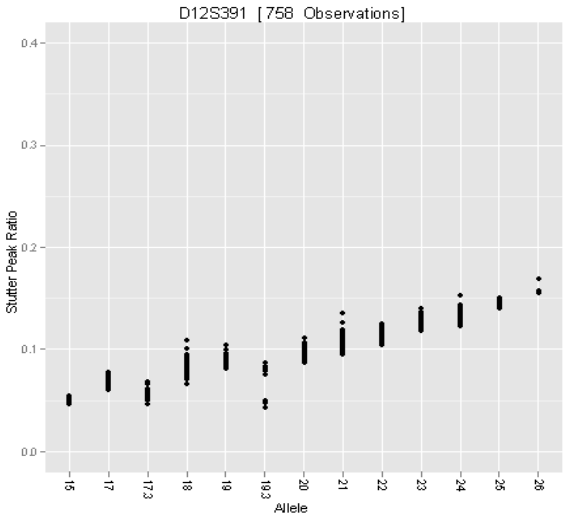


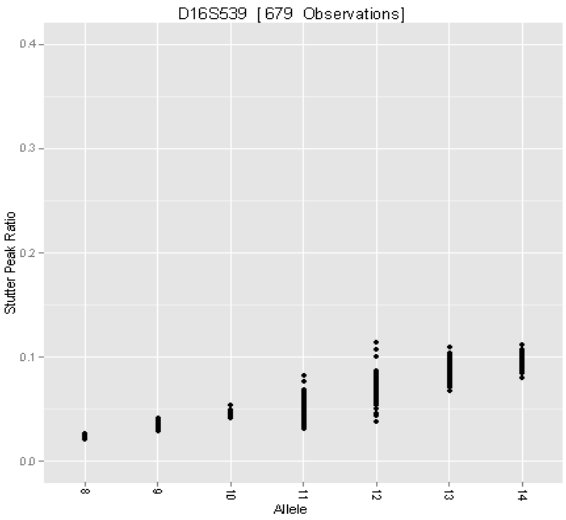
Figure 18. Box plot analysis of intra-locus peak height ratios. Values refer to the lower of the 2 peaks in a heterozygous sample. Boxes represent the middle 50% (interquartile range, [IQR]) of data, and lines inside the boxes represent the median. Whiskers show data within 1.5 IQR, and dots represent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

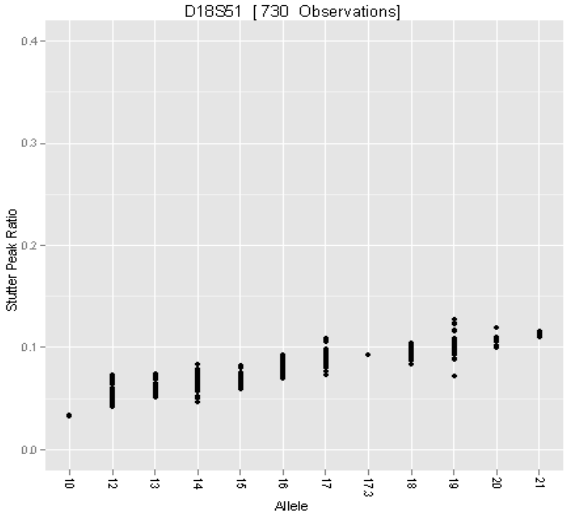
Table 1. Heterozygote peak height ratios

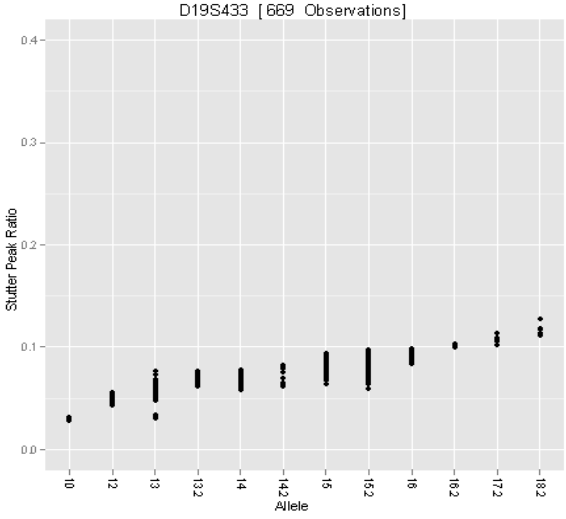
System	Observations	Mean (%)	Min (%)	Max (%)
D10S1248	365	89.61	81.05	99.63
D12S391	393	85.98	67.19	99.65
D16S539	368	88.61	71.13	99.79
D18S51	370	89.35	67.97	99.38
D19S433	373	90.95	76.58	100.00
D1S1656	419	84.87	59.45	99.89
D21S11	348	85.72	62.10	99.97
D22S1045	336	85.29	67.92	96.18
D2S1338	393	84.26	65.64	99.17
D2S441	314	91.25	71.24	100.00
D3S1358	346	89.64	79.41	97.82
D8S1179	366	90.01	74.95	99.97
FGA	352	89.37	68.01	99.80
SE33	393	78.93	50.29	98.43
TH01	298	93.76	83.38	99.94
vWA	345	86.84	71.59	95.78

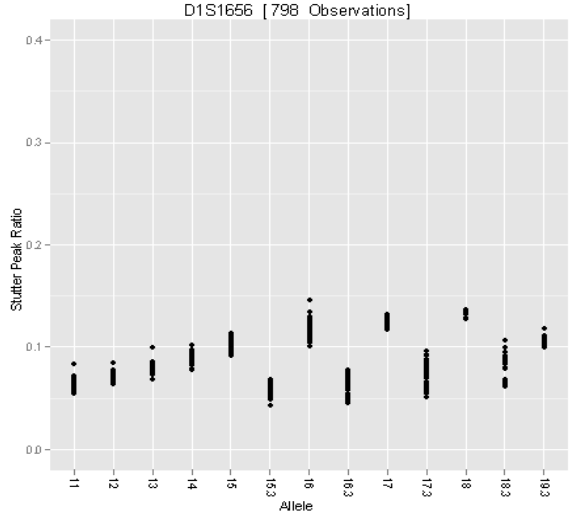

D10S1248 [593 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
12
13
14
15
16
17
Allele

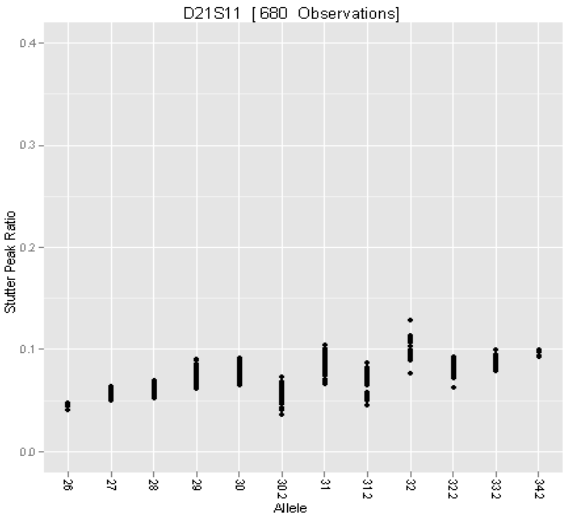

D12S391 [758 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
15
17
17.3
18
19
19.3
20
21
22
23
24
25
26
Allele

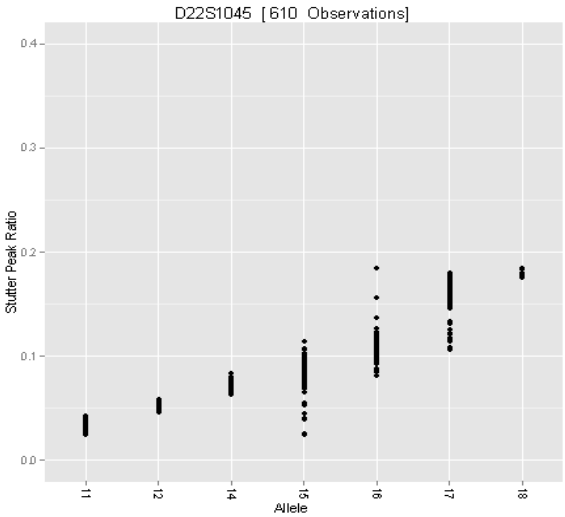

D16S539 [679 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
8
9
10
11
12
13
14
Allele

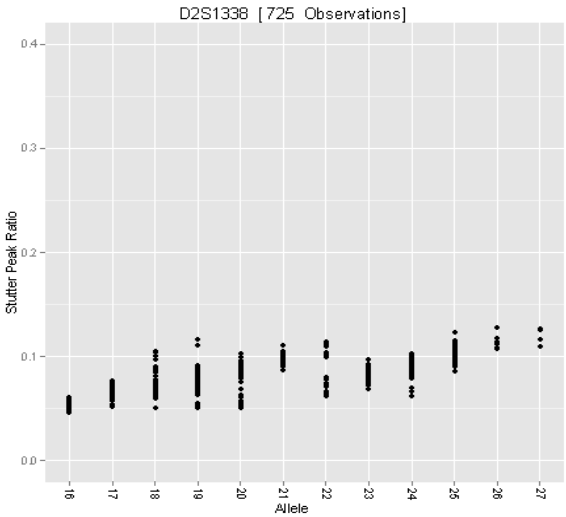

D18S51 [730 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
10
12
13
14
15
16
17
17.3
18
19
20
21
Allele

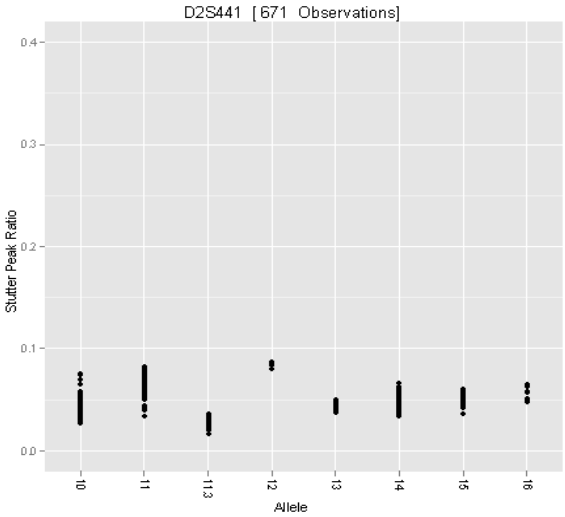

D19S433 [669 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
10
12
13
13.2
14
14.2
15
15.2
16
16.2
17.2
18.2
Allele

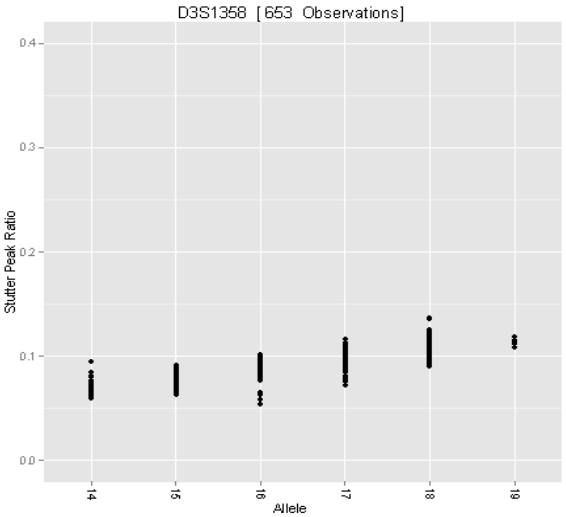

D1S1656 [798 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
11
12
13
14
15
15.3
16
16.3
17
17.3
18
18.3
19.3
Allele

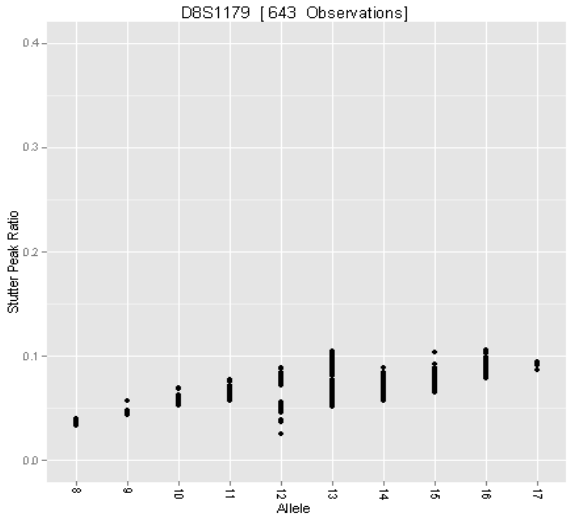

D21S11 [680 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
26
27
28
29
30
30.2
31
31.2
32
32.2
33.2
34.2
Allele


D22S1045 [610 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
11
12
14
15
16
17
18
Allele


D2S1338 [725 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


D2S441 [671 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
10
11
11.3
12
13
14
15
16
Allele


D3S1358 [653 Observations]
Stutter Peak Ratio
0.0
0.1
0.2
0.3
0.4
14
15
16
17
18
19
Allele


D8S1179 [643 Observations]
Stutter Peak Ratio
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0.2
0.3
0.4
8
9
10
11
12
13
14
15
16
17
Allele

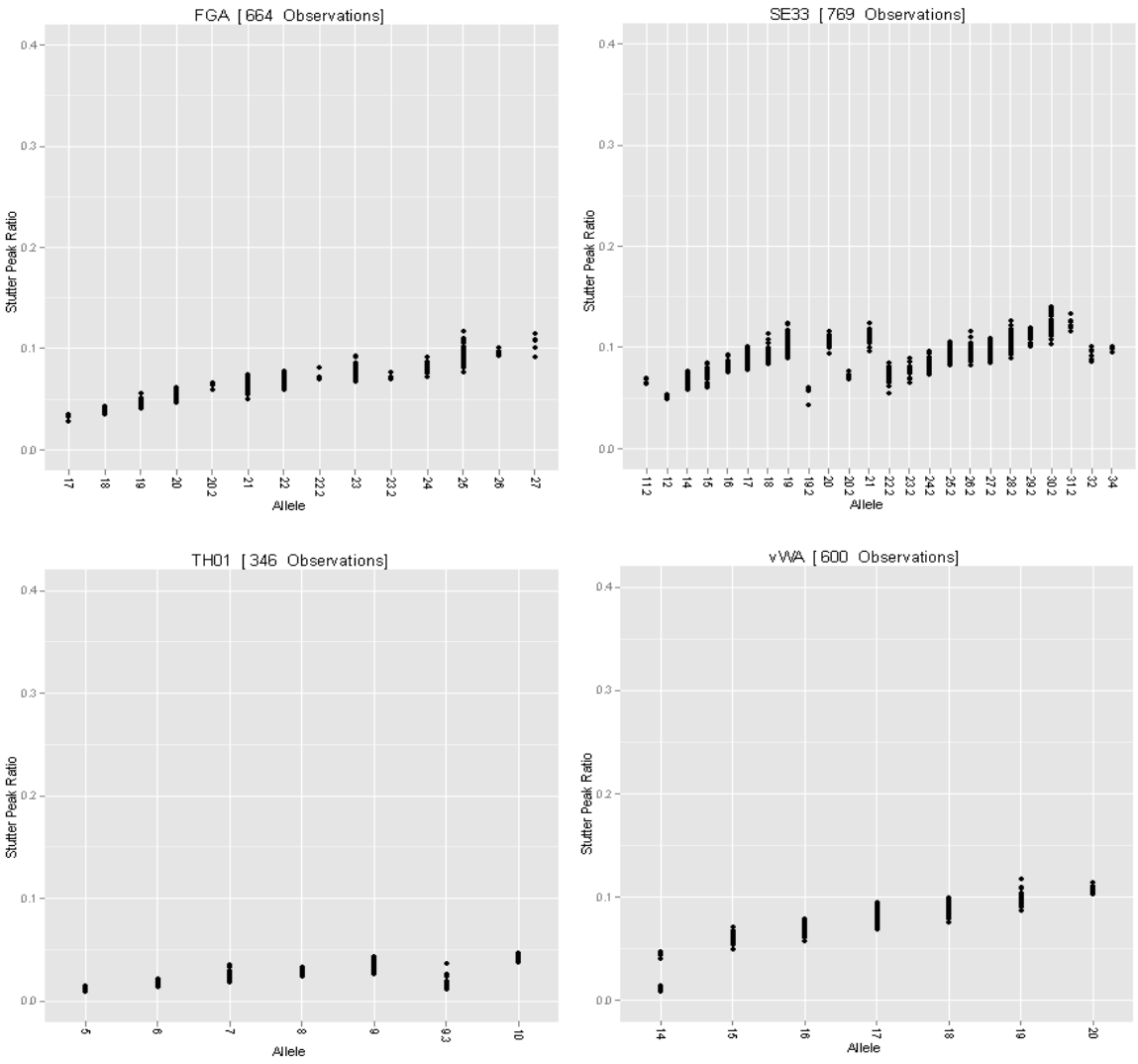

Figure 19. Stutter peak height ratios. Peak heights of forward stutters compared to main peaks were analyzed for 95 blood samples on FTA paper from different donors in 5 replicates.

Table 2. Stutter peak height ratios

System	Observations	Stutter Mean (%)	Stutter Min (%)	Stutter Max (%)
D10S1248	593	8.7	3.6	18.2
D12S391	798	9.4	4.4	16.9
D16S539	679	6.1	2.1	11.4
D18S51	730	7.1	3.2	12.7
D19S433	669	7.1	2.8	12.7
D1S1656	798	8.4	4.3	14.6
D21S11	680	7.4	3.6	12.9
D22S1045	610	8.1	2.4	18.4
D2S1338	725	8.1	4.6	12.8
D2S441	671	5.1	1.6	8.7
D3S1358	653	8.7	5.4	13.6
D8S1179	643	6.8	2.5	10.6
FGA	664	6.8	2.8	11.8
SE33	769	9.3	4.3	14.0
TH01	346	2.3	0.9	4.7
vWA	600	7.4	0.8	11.7

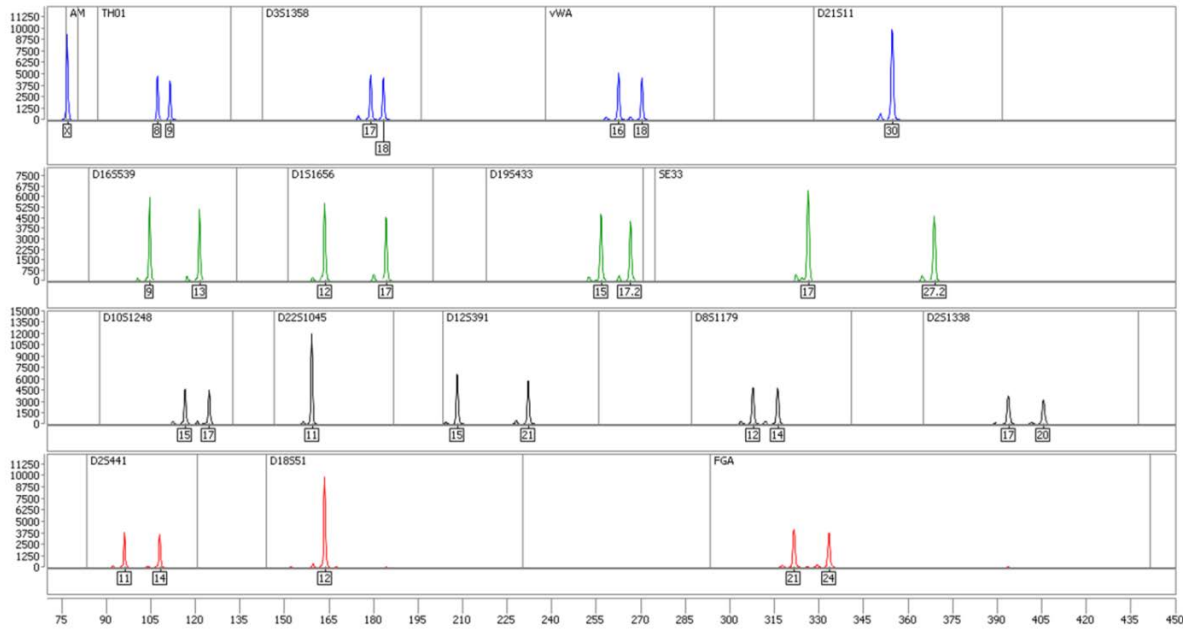

Figure 20. Representative electropherogram for a blood sample on FTA paper.

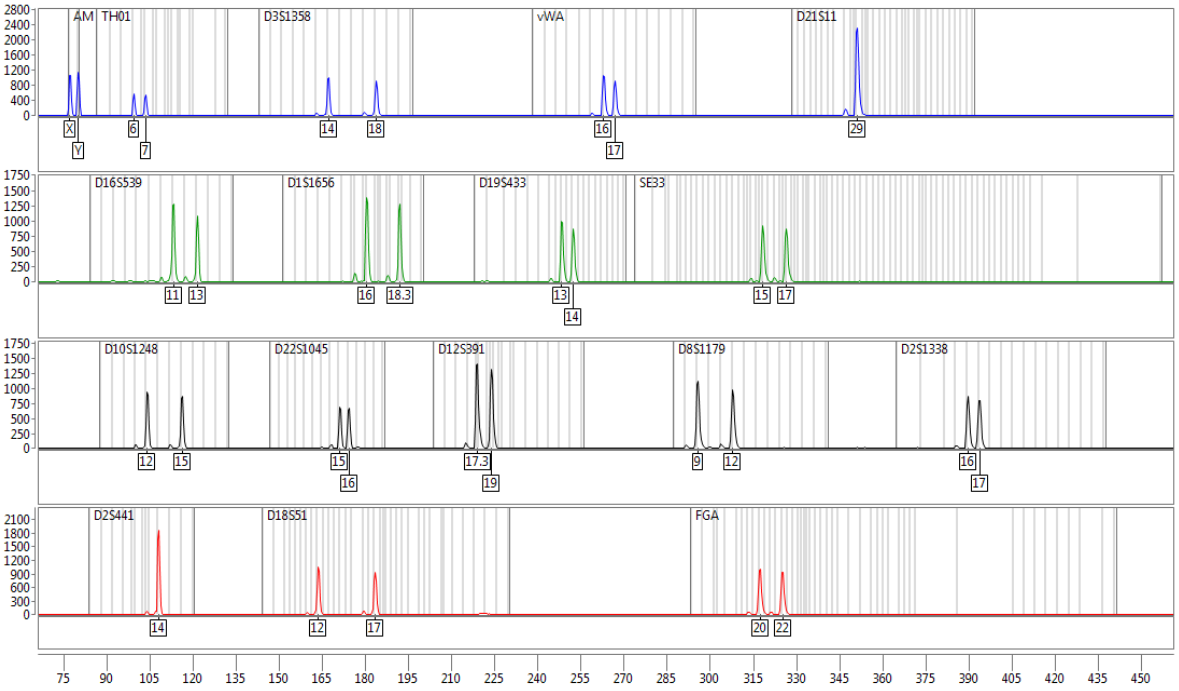

Figure 21. Representative electropherogram for a blood sample on Whatman 903 collection paper.

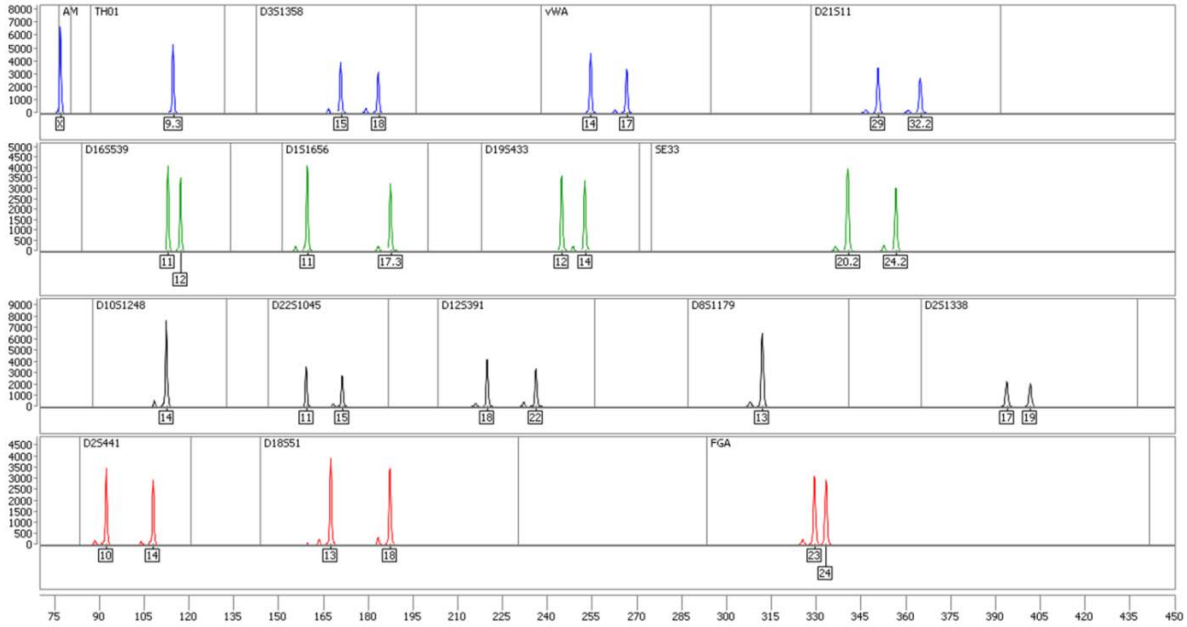

Figure 22. Representative electropherogram for a sample of buccal cells on Whatman EasiCollect.

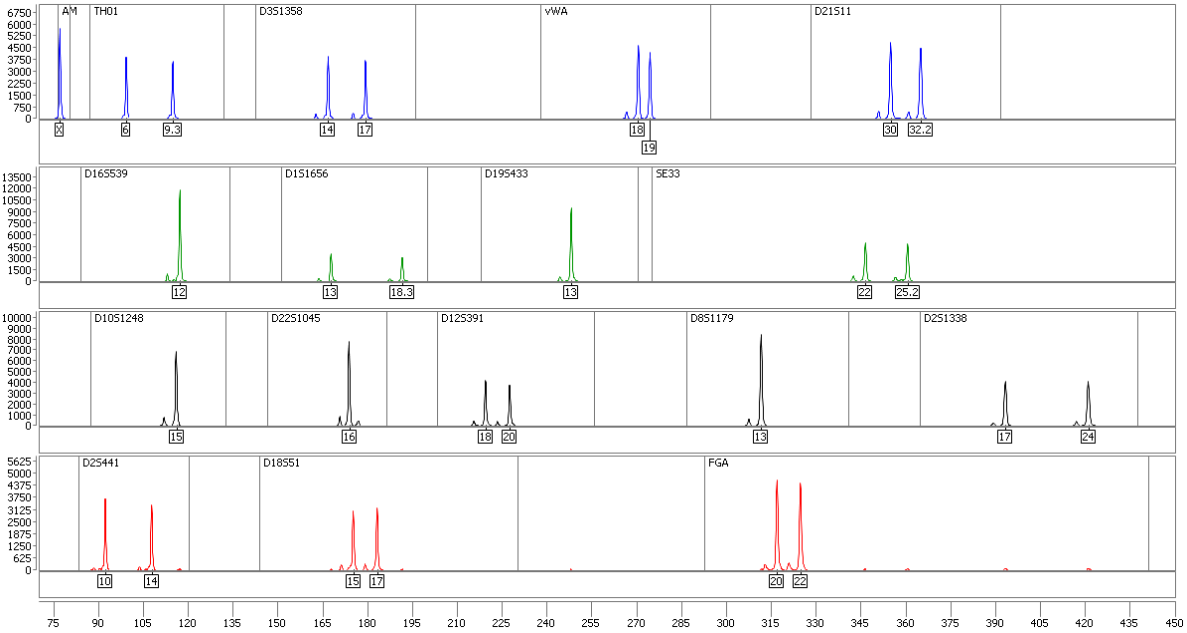

Figure 23. Representative electropherogram for a Puritan polyester swab lysate sample.

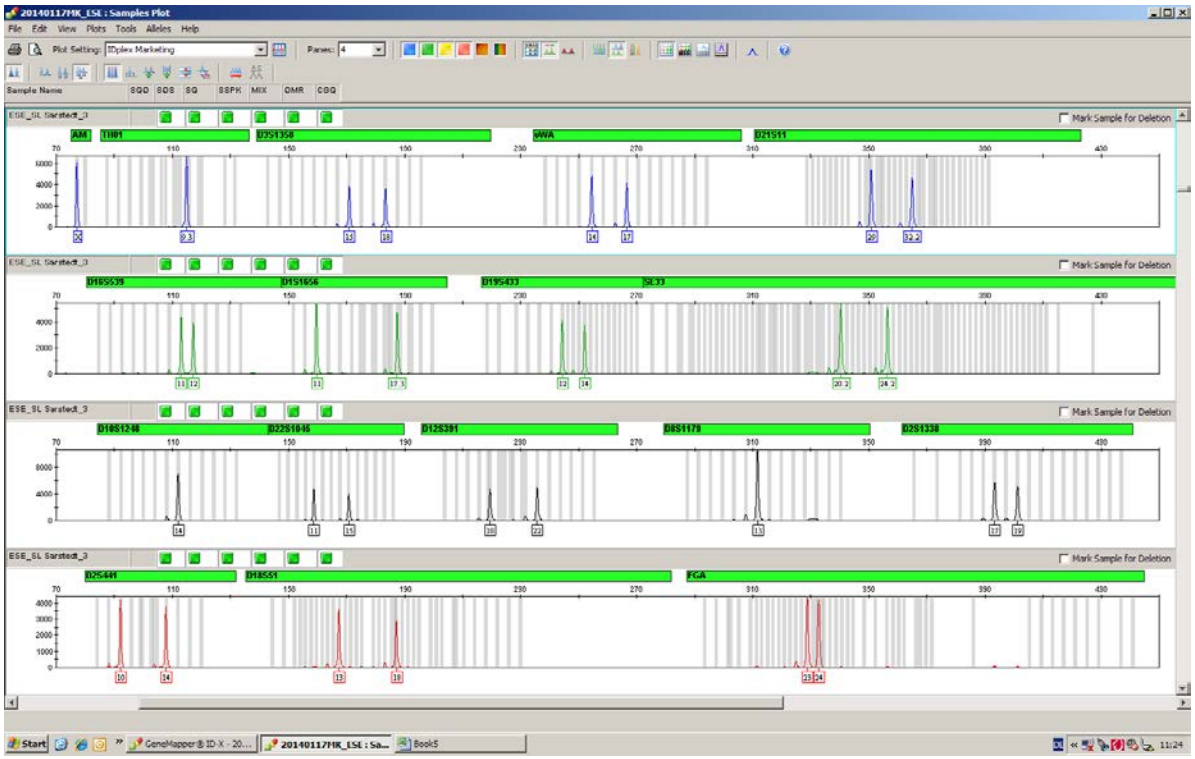

Figure 24. Representative electropherogram for a Sarstedt cotton swab lysate sample.

Reproducibility — automated reaction setup

Protocols were developed for fully automated punching of FTA paper and reaction setup for amplification with the QIAGEN Investigator ESSplex SE GO! Kit on the Hamilton easyPunch STARlet instrument. Blood on FTA paper (95 samples) and buccal cells on Whatman EasieCollect cards (38 samples) from different donors were processed in 5 PCR replicates (Figures 25 and 26). A single punch of 1.2 mm 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 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.

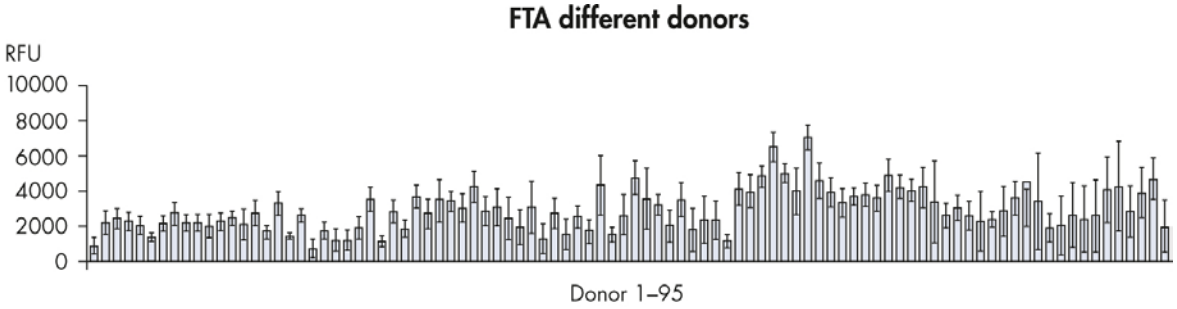

Figure 25. Blood on FTA paper. Average peak heights across samples from 95 different donors processed in 5 replicates.

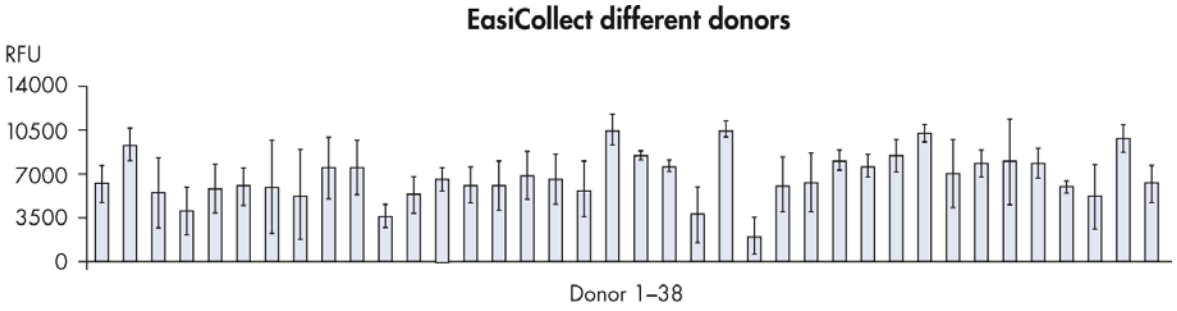

Figure 26. Buccal cells on FTA paper. Average peak heights across samples from 38 different donors processed in 5 replicates.

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.

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6. Lareu, M.V., et al. (1996) A highly variable STR at the D12S391 locus. J. Legal Med. **109**, 134.

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Product	Contents	Cat. no.
Investigator ESSplex SE GO Kit (200)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and RNase-free water	381566
Investigator ESSplex SE GO! Kit (1000)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and RNase-free water	381568
Investigator STR GO! Lysis Buffer (200)	Lysis buffer for 200 swab samples	386516

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