Validation Report August 2016

Developmental validation of the Investigator® 24plex GO! Kit

The QIAGEN® Investigator 24plex 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 FTA paper, and crude buccal swab lysates.

The performance of the Investigator 24plex 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 22 polymorphic STR markers recommended by the CODIS (Combined DNA Index System) Core Loci Working Group, the European Network of Forensic Science Institutes (ENFSI) and the European DNA Profiling Group (EDNAP), D1S1656, D2S441, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D22S1045, CSF1PO, FGA [FIBRA], TH01 [TC11], TPOX, vWA, SE33 [ACTBP2], DYS391 and the gender-specific Amelogenin (3). These genetic loci have been characterized in numerous studies by other laboratories (4–6). As a special feature, the Investigator 24plex GO! Kit contains an internal PCR control (Quality Sensor QS1 and QS2), which provides helpful information about the efficiency of the PCR and the presence of PCR inhibitors. The internal Quality Sensor is enclosed in the Primer Mix and amplified simultaneously with the polymorphic STR markers. For further information about the Quality Sensor please refer to the *Investigator 24plex GO! Kit Handbook*.

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 the $MgCl_2$ concentration (page 3). The robustness of the assay regarding variations in PCR cycling conditions and on a range of different thermal cyclers was investigated (pages 3–7). The effects of using increased or decreased PCR cycle numbers were analyzed (page 5). Sensitivity was addressed by amplifying dilution series of blood on FTA paper or buccal swab lysates (page 9). Cross-reactivity with non-human DNA (page 15) was also assessed. The reproducibility of the results was verified (page 17). This validation study covers a fully automated workflow for blood

or buccal cells on FTA cards using the STAR Q Punch AS Instrument, based on the Hamilton® easyPunch™ STARlet system (page 29). The fully automated workflow for epithelial cells on swabs was validated using the STAR Q Swab Instrument, based on Hamilton's STARlet platform (page 33). The stability of the kit components was validated with regard to repeated freeze-thawing (page 38), and transport (page 39).

Results of developmental validation

The validation study was performed at the QIAGEN R&D department. All of the electropherograms shown were generated on an Applied Biosystems® 3500™ Genetic Analyzer. The standard conditions specified in the *Investigator 24plex GO! Kit Handbook* were used for electrophoresis. Unless stated otherwise, an Applied Biosystems® GeneAmp® PCR System 9700 with a Gold-plated Silver 96-Well Sample Block Module was used for amplification. Data were analyzed using Applied Biosystems GeneMapper® *ID-X* software, v1.2.

Whatman® EasiCollect™ indicator cards were used to transfer buccal cells on FTA® paper. Investigator STR GO! Punch Buffer was used to improve the amplification efficiency of buccal cell samples on FTA paper, as recommended in the handbook. Whatman FTA cards were used for blood samples. Puritan polyester or 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 an optimal performance in terms of robustness, specificity and reproducibility. The influence of critical buffer components on the amplification efficiency was tested.

For example, various concentrations of $MgCl_2$, one of the critical buffer components, were evaluated with blood on FTA (Figure 1) and buccal swab samples (Figure 2). The assay yielded robust results within a $MgCl_2$ concentration range of ±15% of the optimum.

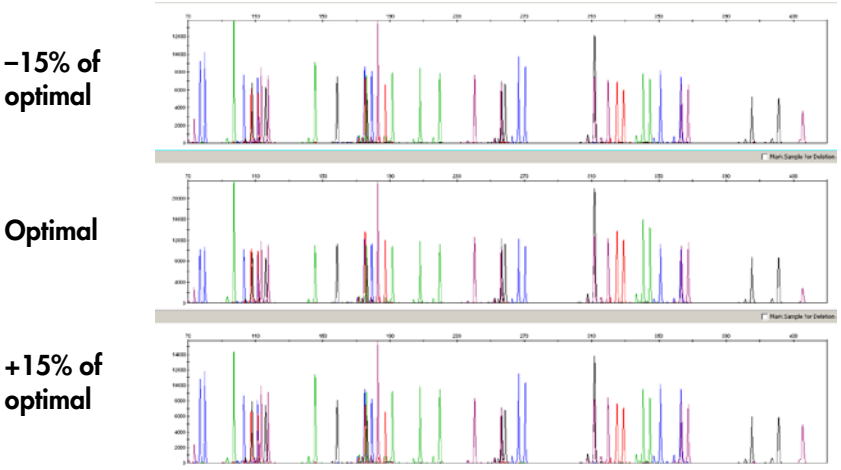


Figure 1. Influence of $MgCl_2$ concentration. Examples of blood on FTA sample triplicates are shown.

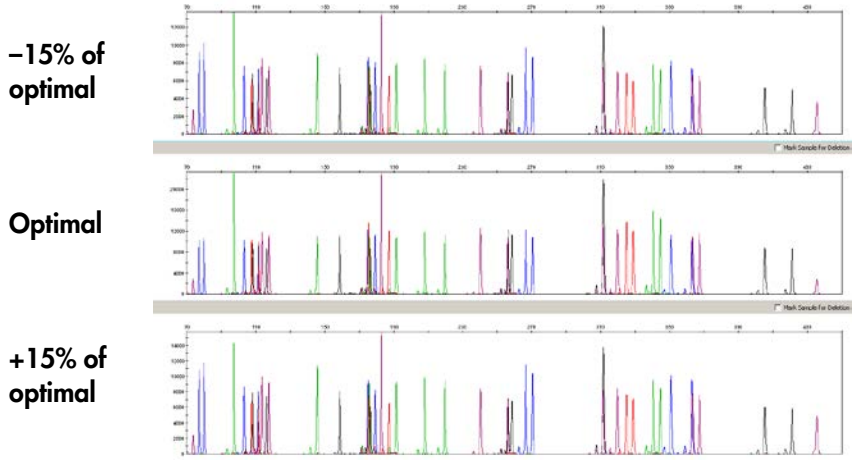


Figure 2. Influence of $MgCl_2$ concentration. Examples of buccal swab sample triplicates are shown.

Effect of PCR annealing temperature variations

Specificity, sensitivity and robustness are affected by the annealing temperature (T_m). Since the actual T_m may vary depending on cycler conditions, the assay was validated in a range surrounding the optimal T_m of the Investigator 24plex GO! Kit reaction (first 3 cycles at 64°C, following 27 cycles at 61°C).

Table 1. Standard cycling protocol recommended for all DNA samples

Temperature	Time	Number of cycles
98°C*	30 s	3 cycles
64°C	40 s	
72°C	5 s	
96°C	10 s	variable cycles
61°C	40 s	
72°C	5 s	
68°C	2 min	
10°C	∞	–

* Hot-start to activate DNA polymerase.

Annealing temperatures between -4°C and +3°C around the optimal annealing temperature of 64°C / 61°C were applied to the amplification of blood on FTA, buccal swab samples. The annealing temperature of the first 3 cycles and of the following 27 cycles were varied to the same extend. PCR was performed on an Eppendorf® Mastercycler® ep instrument (Figure 3). Reactions using annealing temperatures between -4°C and +3°C resulted in full profiles. Good interlocus balance was observed for the temperature range of -3°C to +1°C. However, the average peak height of markers was best for the conditions closest to the actual annealing temperature of 64°C / 61°C (Figure 3 and Figure 4). No dropouts were observed in the tested range applying a threshold of 100 RFU. No nonspecific PCR products were observed. In order to ensure optimal performance of the assay, we strongly recommend regular calibration of thermal cyclers.

Similar sets of experiments were performed to set optimal temperatures and hold-times for all PCR protocol steps (data not shown).

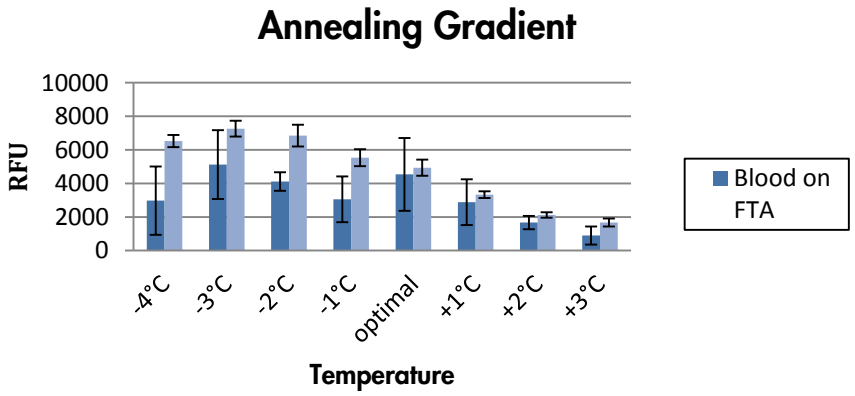


Figure 3. Effect of variations in the PCR annealing temperature. Blood on FTA and buccal swab lysates from a single donor each were analyzed in triplicates. 25 PCR cycles were used for blood on FTA, 27 cycles for swab lysates.

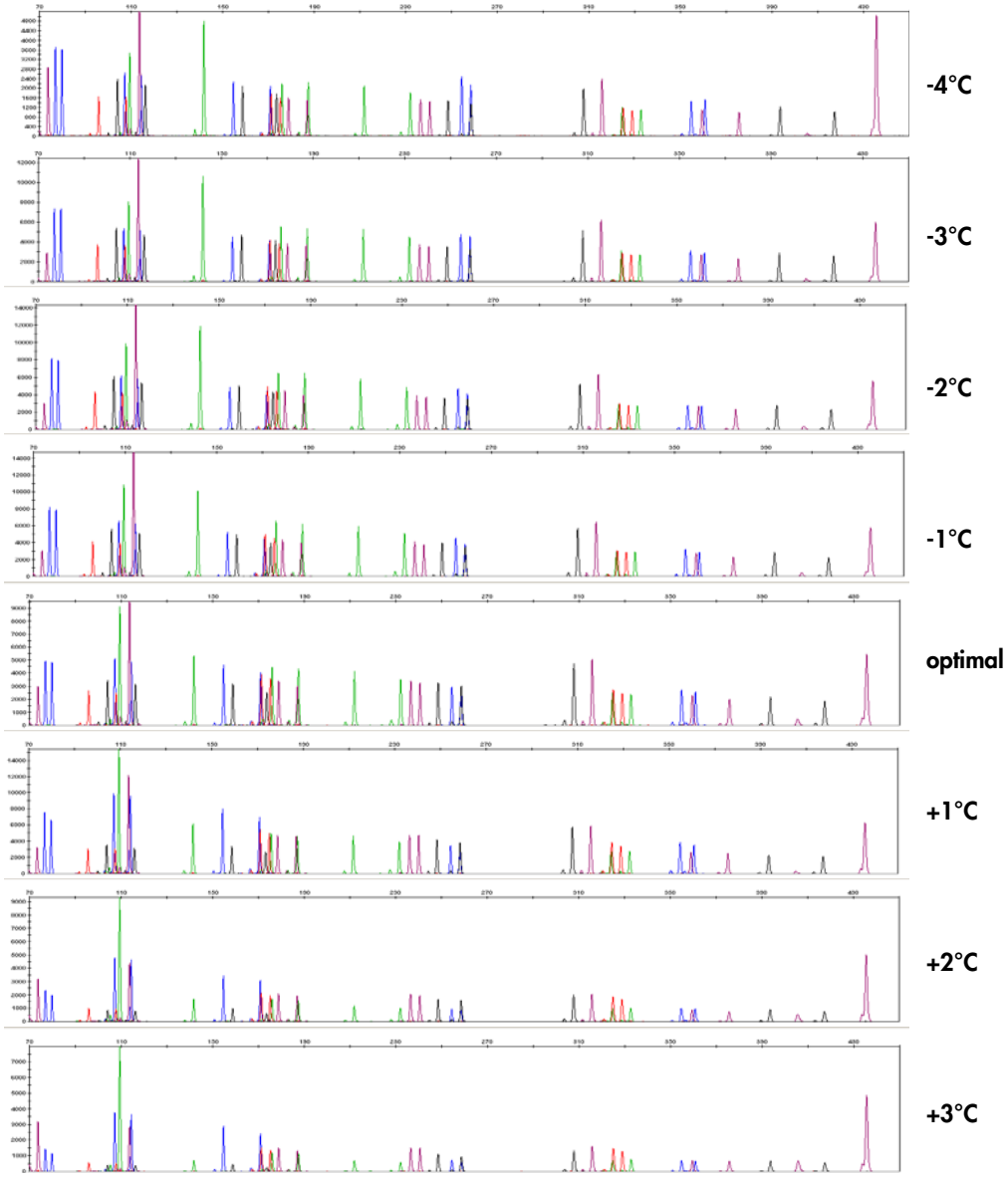


Figure 4. Variations in the PCR annealing temperature. Blood on FTA samples were amplified on an Eppendorf Mastercycler ep. Representative electropherograms for reactions using annealing temperatures between -4°C and +3°C are shown. Note that the y-axis was scaled for best fit.

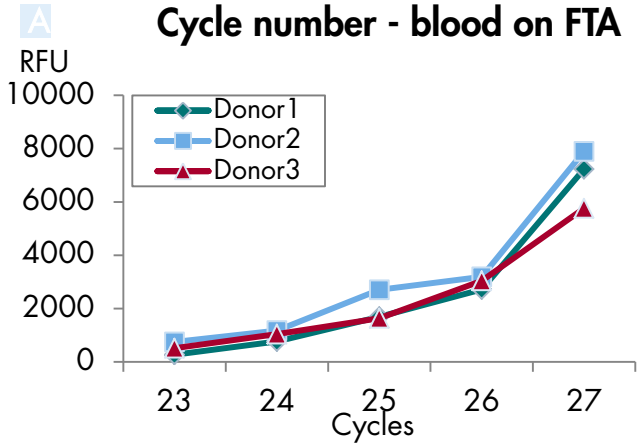
Effect of different cycle numbers

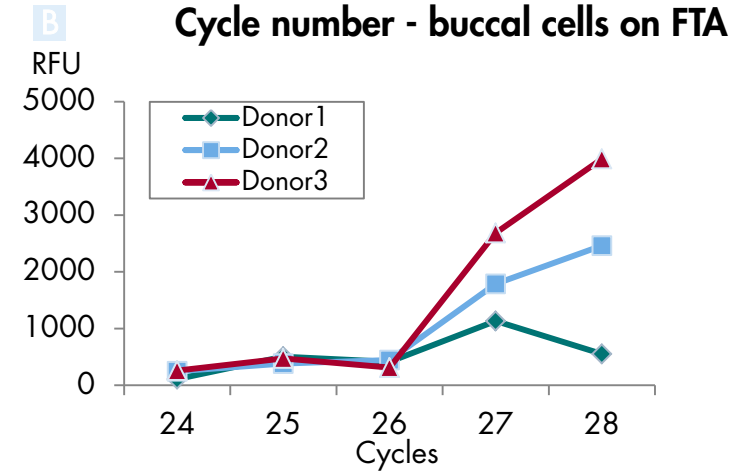
The amount of sample material present for direct amplification varies between different reference sample types, donors, collection procedures and storage conditions. Therefore, for optimal results

it is important to evaluate a representative batch of samples and to adapt reaction conditions if necessary. We recommend adjusting the number of PCR cycles to adapt the reaction conditions to varying DNA template amounts. Cycle numbers can be either increased to enhance amplification signals, or decreased when the DNA sample is abundant.

Blood or buccal cells on FTA and buccal swab lysates were amplified using the cycle number recommended as starting point for evaluation, +/- two PCR cycles (Figure 5). Samples from three donors were analyzed in five replicates each. As expected, average signal heights increase with each cycle added.

We recommend reviewing routine use data periodically to ensure conditions chosen during evaluation still provide best possible pass rates.





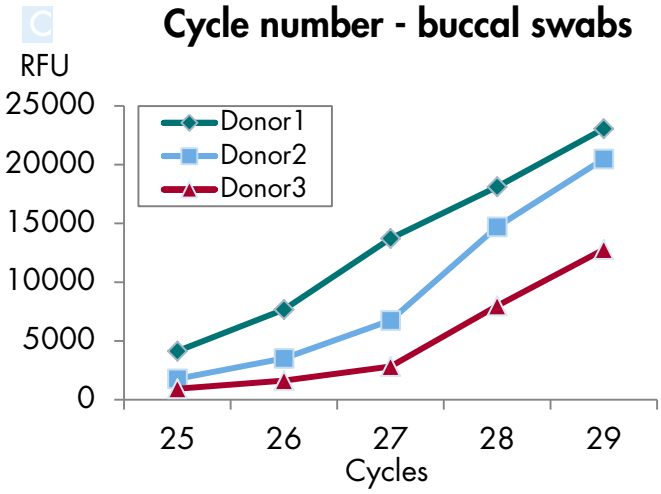


Figure 5. Effect of different cycle numbers on mean peak height. Samples were run in five replicates per donor. A) Blood on FTA paper. B) Buccal cells on FTA paper. C) Buccal swab lysates.

Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator 24plex GO! Kit to demonstrate kit robustness, independent of the instrument (Figure 6). Blood on FTA and buccal swab lysates from five different donors were run in four replicates each. 25 PCR cycles were used for blood on FTA and 27 cycles for swab lysates. The following thermal cyclers were evaluated:

- GeneAmp PCR System 9700 with Aluminum 96-Well Sample Block Module (Applied Biosystems Inc., Foster City, CA, USA)
- GeneAmp PCR System 9700 with Silver or Gold-plated Silver 96-Well Sample Block Module (Applied Biosystems Inc., Foster City, CA, USA)
- Veriti® 96-Well Thermal Cycler (Applied Biosystems Inc., Foster City, CA, USA)
- Eppendorf® Mastercycler® ep (Eppendorf AG, Hamburg, Germany)
- 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)

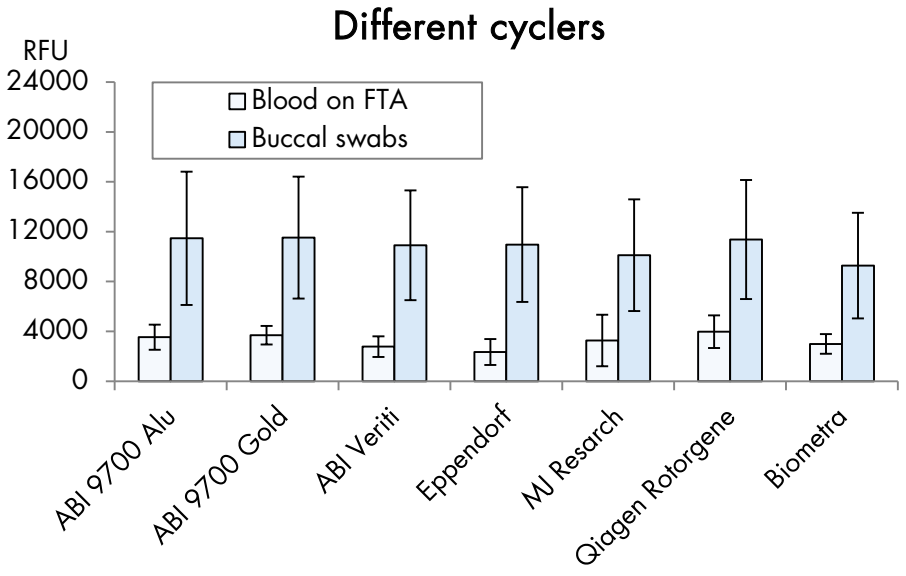


Figure 6. Performance on different PCR thermal cyclers. Average signal heights of 4 replicates across 5 different donors are shown. Error bars indicate the standard deviation between different donors.

Comparable mean peak heights were obtained for all of the tested PCR cyclers. No significant differences in intra- and inter-locus balance or non-specific amplification was observed on any of the thermal cyclers.

Effect of different genetic analyzers

The Investigator 24plex GO! Kit uses a six color setup with fluorescence-labeled primers with the following dyes 6-FAM™, BTG, BTY, BTR2 and BTP plus the BTO labeled size standard. All of the electropherograms shown were generated on an Applied Biosystems 3500 Genetic Analyzer, with the standard conditions specified in the *Investigator 24plex GO! Kit Handbook*. Further genetic analyzers that are equipped to analyze a six color setup, for example, an upgraded Applied Biosystems 3130 Genetic Analyzer, have not been validated yet.

Several Applied Biosystems 3500 Genetic Analyzers at different locations were tested, in order to demonstrate the robustness of the Investigator 24plex GO! Kit. The data shown in Figure 7 were generated at three different sites using aliquots of the same PCR products. Blood on FTA and buccal swab samples from three different donors were each amplified in four replicates. The reactions took place under standard conditions.

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 (Figure 7). Variations reflected differences in sensitivity between individual instruments, for example, due to

laser power, array life and matrix calibration effects. Limitations of genetic analyzer sensitivity must be addressed by individual laboratories during an internal validation study using the instrumentation in their laboratory.

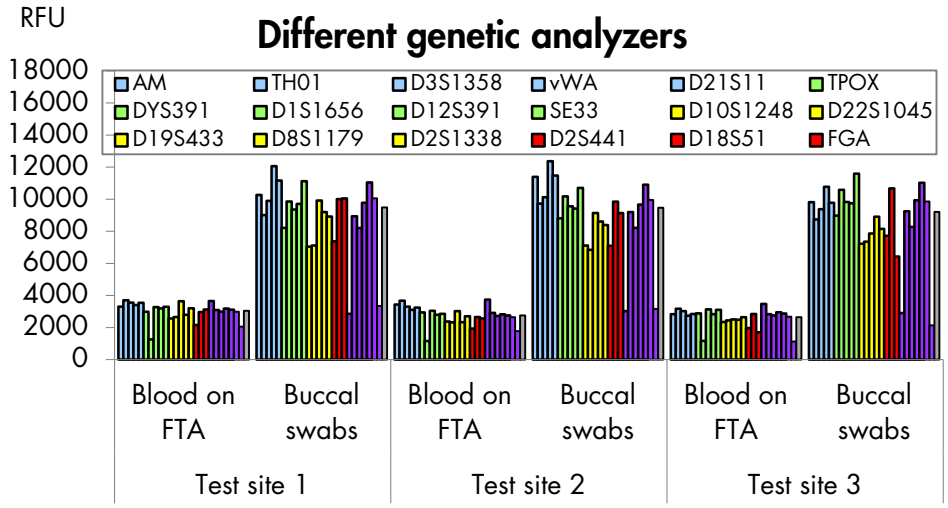


Figure 7. Analysis of PCR products on different Applied Biosystems 3500 Genetic Analyzers. Blood on FTA and buccal lysate from 3 different donors were amplified on a GeneAmp PCR System 9700 in four replicates. Samples were run according to the *Investigator 24plex GO! Kit Handbook*. Aliquots of the same PCR were delivered to three different test sites and analyzed on Applied Biosystems® 3500 Genetic Analyzers. Average peak heights of the different donors and replicates are shown.

Sensitivity

The Investigator 24plex GO! Kit is designed to work robustly over a range of sample input amounts. Dilution series of blood were spotted on FTA paper and amplified using 25 PCR cycles. Swab lysates were diluted and amplified using 27 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 8). Down to 1:81 fold dilutions all samples provided full profiles for buccal cell lysates. Using blood on FTA as template, all samples provided full profiles for 1:3 fold dilutions. For 1:9 fold dilutions, individual alleles dropped below the 50 RFU thresholds (Figure 9). Note however that full profiles from these samples could be recovered by using increased PCR cycle numbers.

Issues with very high sample amounts

Depending on the instrumentation and settings used for capillary electrophoresis, fluorescence intensity 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 becomes 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 re-amplifying a sample using less amplification cycles or re-injecting at a shorter injection time. Due to inherent sample-to-sample variation of direct amplification, the result of a re-injection has a higher predictability compared to re-amplification. This holds in particular true for buccal cells transferred to paper samples, which frequently show considerable punch to punch variability even if indicator cards are used.

Issues with very low sample amounts

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

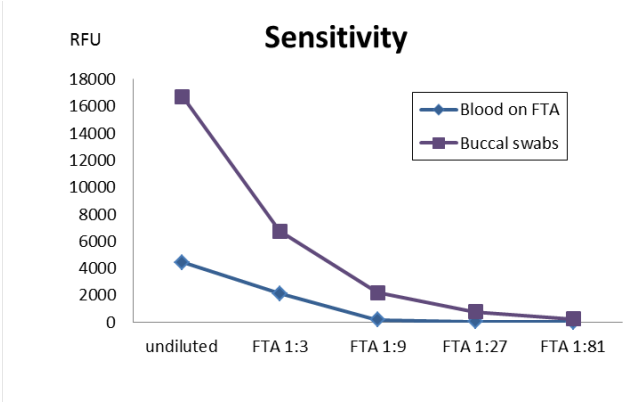


Figure 8. Sensitivity study. Serial dilutions of blood samples on FTA cards or buccal swab lysates were analyzed.

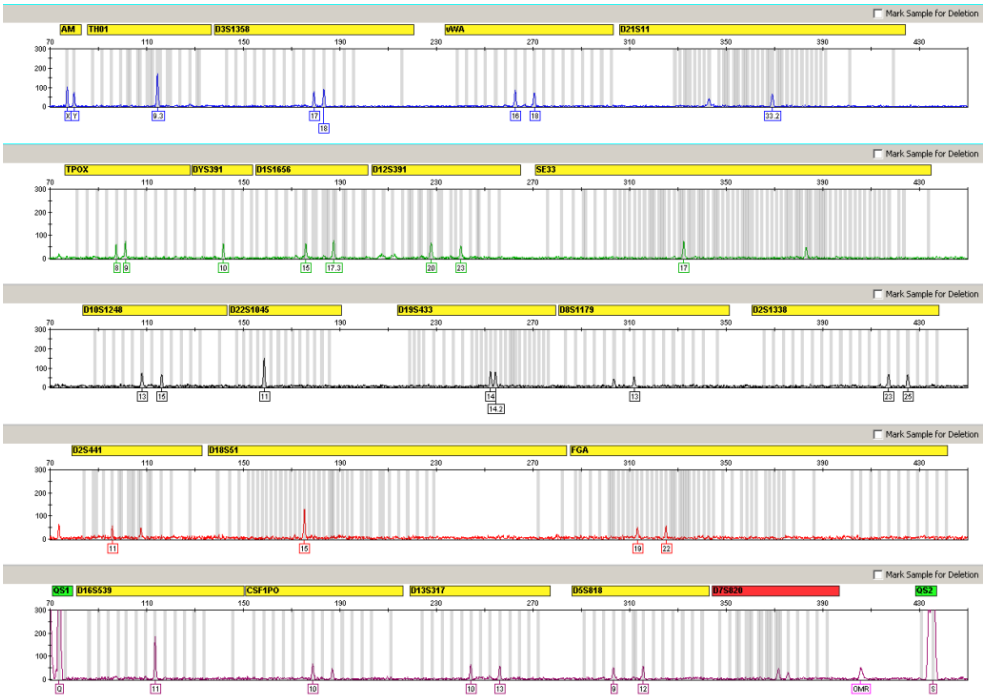


Figure 9. Sensitivity study. Example of a blood on FTA sample using blood that has been diluted 1:9-fold prior to spotting. Several alleles dropped below 50 RFU.

Stability – Sample input

The robustness towards varying amounts of sample input was tested. For blood or buccal cells on FTA paper, one, two or three punches of 1.2 mm diameter were used as samples. All buccal cells on FTA paper were amplified with the additive Investigator STR GO! Punch Buffer, as recommended in the handbook. Buccal swabs were prepared with the Investigator STR GO! Lysis Buffer, and 1, 2 or 4 µl of crude lysates were used. For all sample types triplicates of three different donors were run.

Increased sample amounts in general still provided full DNA profiles, but the balance of markers may be negatively affected. In particular, introduction of three punches of blood on FTA frequently results in reduced amplification of the longer STR markers and of the Quality Sensor QS2, as well as shoulder formation of marker D18S51 (Figure 10). These effects indicate inhibition of the PCR by the increased amount of FTA paper.

With increasing sample input of buccal cells on FTA paper the overall signal intensity escalates (Figure 11). Introduction of three punches of FTA might lead to marginal inhibition effects.

When using 4 µl buccal swab lysate as sample, in most cases the overall signal intensity increases (Figure 12). We recommend using sample amounts as indicated in the handbook and to increase PCR cycle numbers if signals are too low.

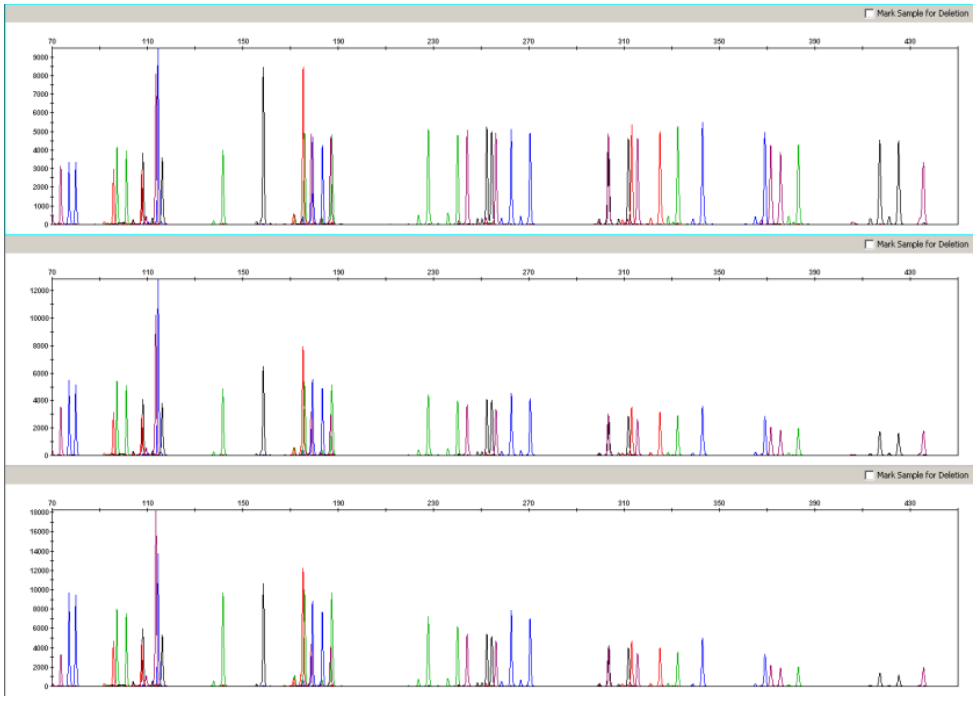


Figure 10. Sample input blood on FTA. 1, 2 or 3 punches of 1.2 mm were used for amplification.

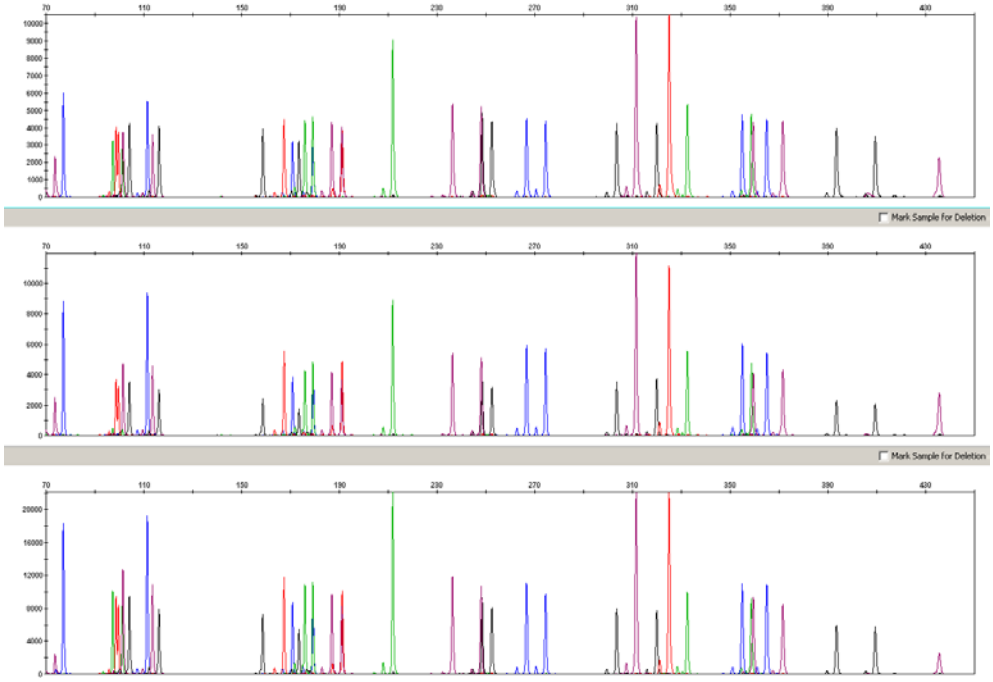


Figure 11. Sample input buccal cells on FTA. 1, 2 or 3 punches of 1.2 mm were used for amplification.

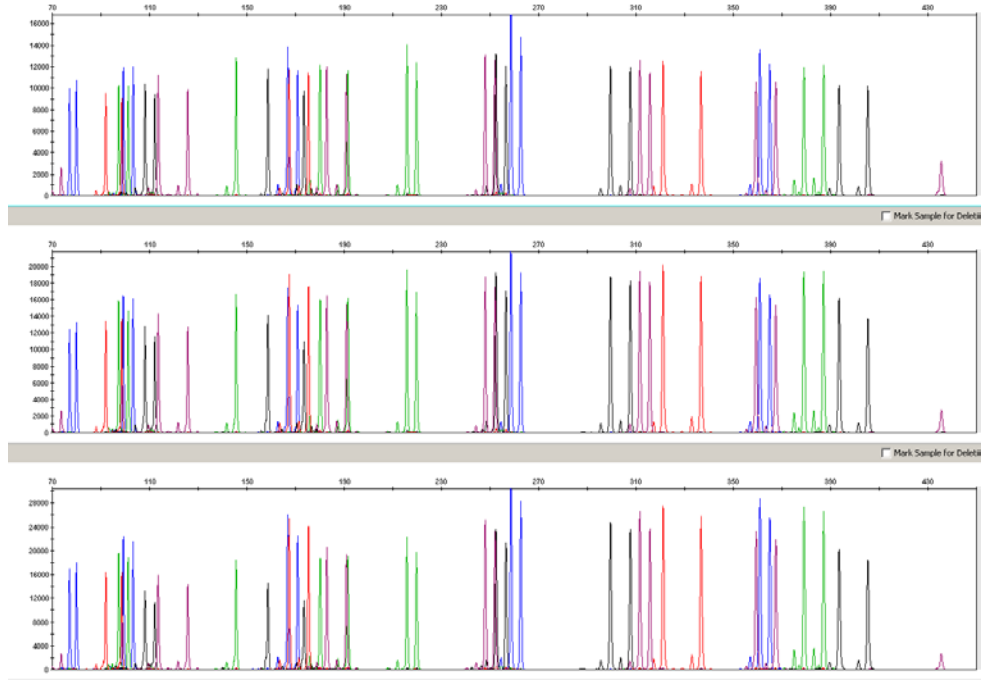


Figure 12. Sample input buccal cell lysates. 1, 2, or 4 µl of a buccal swab lysate were used for amplification.

Aged samples

Blood on FTA samples stored at room temperature for 15 months, and crude lysates from buccal swabs stored at -20°C for 5 months were used. For each sample type, 3 replicates of 3 different donors were amplified. Figure 13 and Figure 14 show typical electropherograms. No sample degradation or other negative impact of storage was observed.

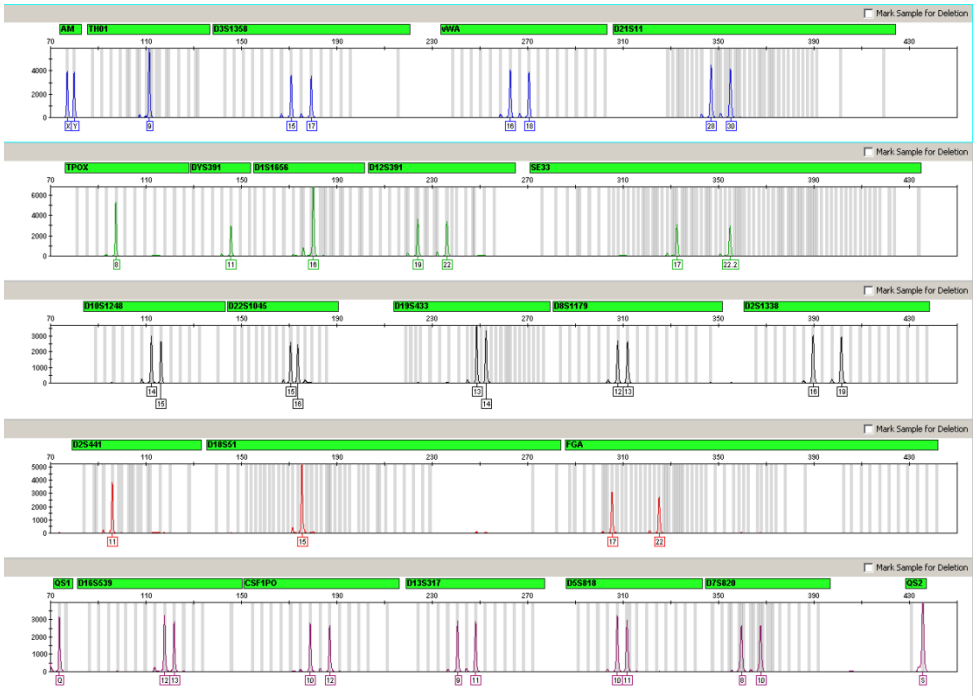


Figure 13. Aged samples. Example of a blood on FTA sample that was stored for 15 months at room temperature.

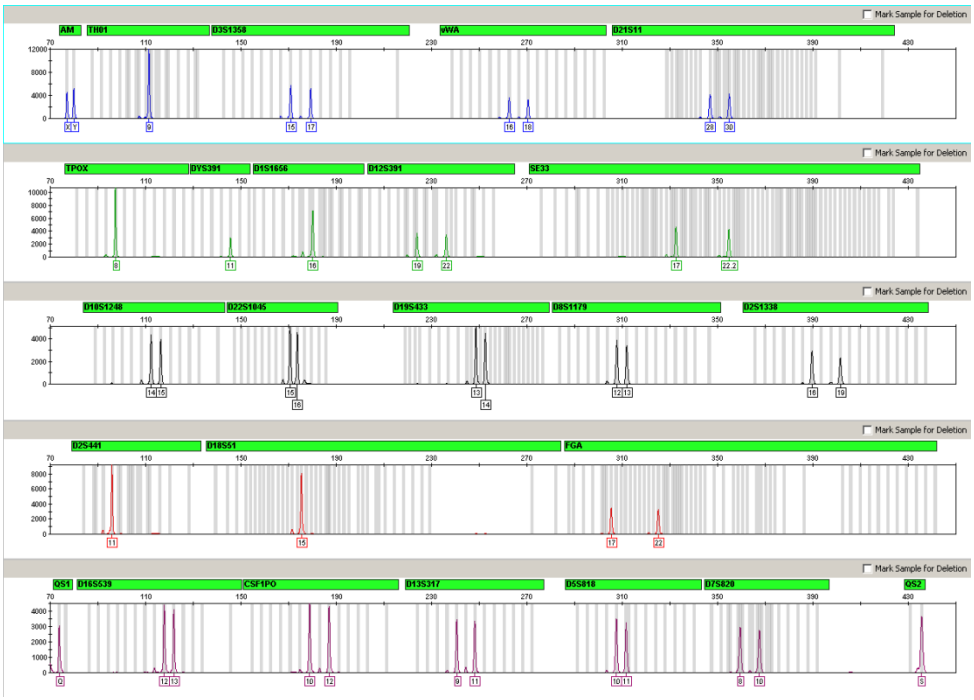


Figure 14. Aged samples. Example of buccal swab lysate sample that was stored for 5 month at -20°C.

Species specificity

To verify Investigator 24plex GO! Kit species specificity for human DNA, DNA from other species was also tested. The test was run on purified DNA using 27 PCR cycles.

Besides common pets and farm animals, some primates were also tested. As expected for primates, amplification of some products is possible. Chimpanzee, bonobo, orangutan and to a lesser degree gorilla, give rise to several peaks within marker ranges in all channels, some of which match the size of human STR products. Macaque DNA produced 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. Typical examples are shown (Figure 15). Quality sensor QS1 produced an artificial off-ladder plus stutter at 97.5 bp and QS2 produced an artificial off-ladder minus stutter at 406 bp. In general, these artificial peaks may be present in samples with DNA from any kind of species, including human DNA, as well as in samples without any template DNA.

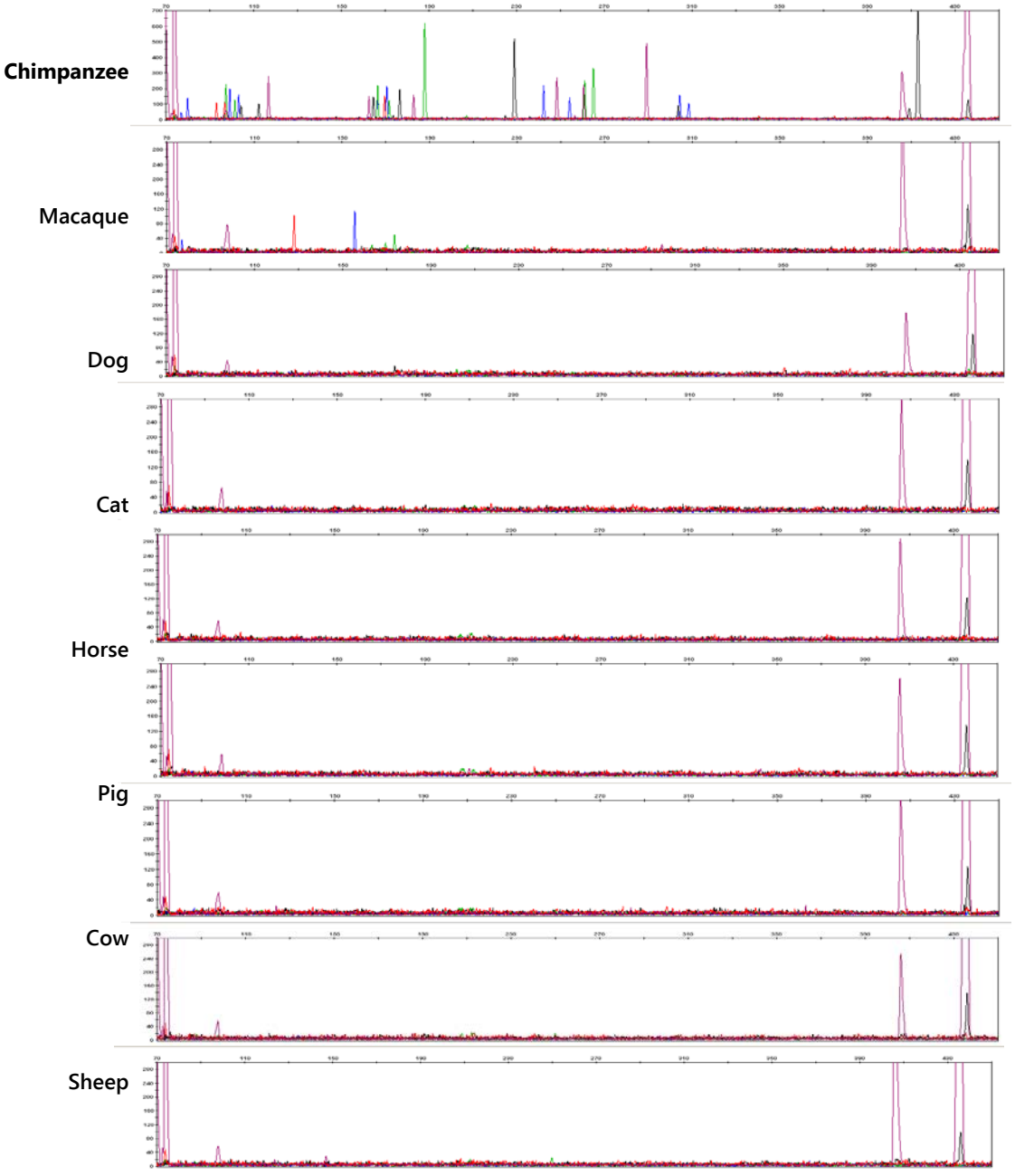


Figure 15. Representative results of the species specificity assessment. As template, 500 pg primate DNA and 2.5 ng DNA from all other species was used in a 27-cycle amplification.

Reproducibility – Concordance test

The Investigator 24plex GO! Kit contains the same primer sequences as the Investigator 24plex QS Kit. To demonstrate the concordance of the Investigator 24plex QS Kit, the NIST institute compared the results of the new kit to the NIST final data set with 656 unrelated individuals (NIST U.S. population set (650 samples) and SRM 2391c (6 samples)). Therefore, the results of the NIST concordance study are valid for both kits.

A full concordance was assessed with NIST SRM 2391c certified values. Out of the total data set no null alleles were observed. One discordant result for D7S820 (1 sample out of 656 samples) was detected. The Investigator 24plex QS Kit result for D7S820 is concordant to kits, like Identifiler®, PP Fusion, AmpFℓSTR ® Profiler Plus®, but it is discordant to other kits, like PowerPlex® 16, AmpFℓSTR MiniFiler™, IDplex Plus. Out of 29,520 alleles compared, one discordant call was observed, with a 0.003% discordance.

In conclusion, the concordance between the Investigator 24plex QS Kit and the NIST final data set is accounted to 99.997%.

Robustness – Comprehensive test of different sample types

In order to proof the robustness for different sample types, the following samples were processed in five replicates each:

Table 2. Samples used to provide evidence of robustness

Sample type	Material	Number of donors
Blood on FTA card	Whatman FTA Micro Card	57
Blood on FTA card	Whatman EasiCollect	38
Blood on non-FTA card	903 Protein Saver Snap-Apart Card, Whatman	19
Buccal cells on FTA card	Whatman EasiCollect card	19
Buccal cells on Polyester swab	Puritan Sterile Cotton tipped Applicators	19
Buccal cells on cotton swab	Sarstedt Forensic Swab in a transport tube with ventilation membrane	19

The reactions took place under standard conditions specified in the *Investigator 24plex GO! Kit Handbook*. All samples gave full profiles. Typical profile examples are shown (Figure 16–21).

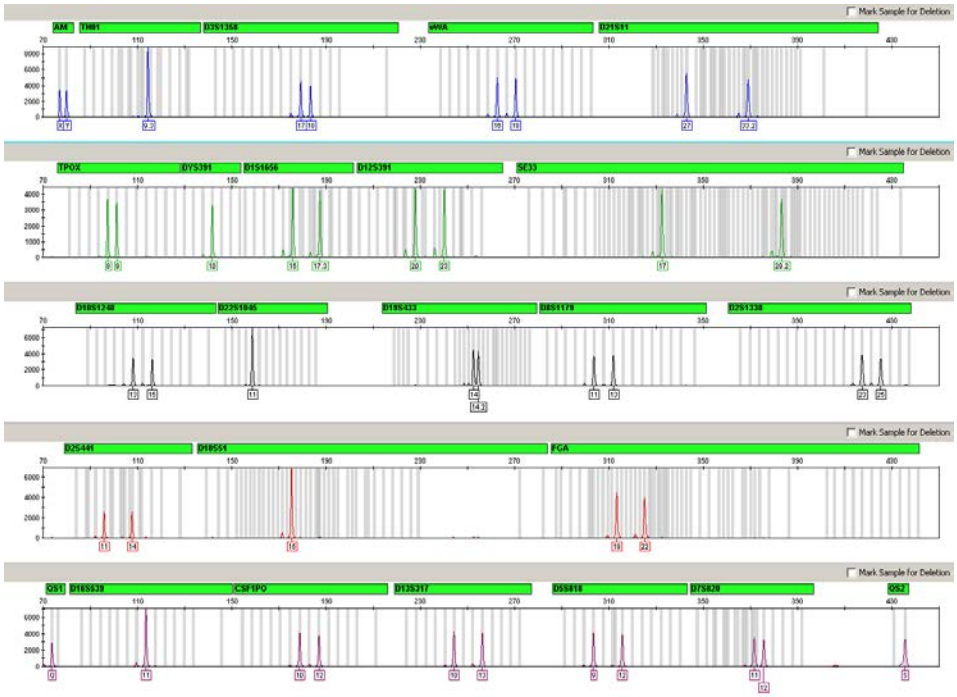


Figure 16. Representative electropherogram for a blood on Whatman FTA Micro Card sample.

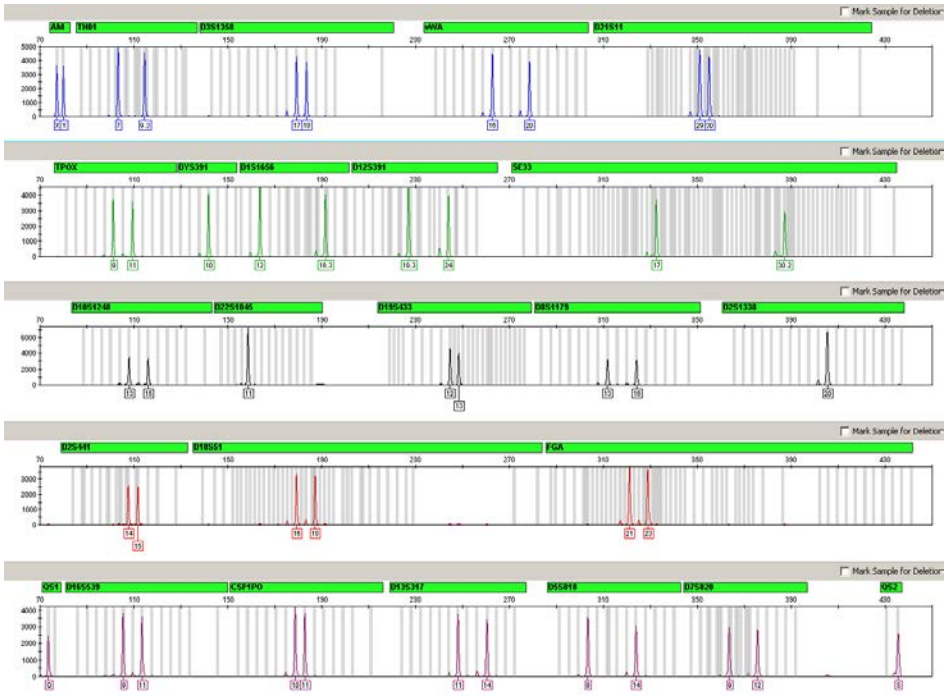


Figure 17. Representative electropherogram for a blood on Whatman EasiCollect sample.

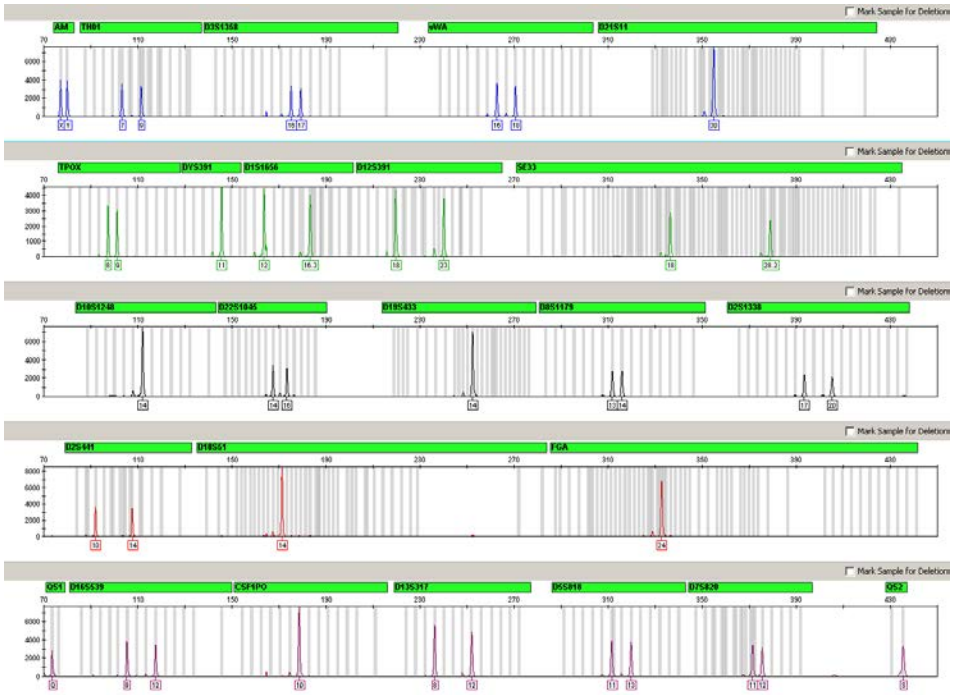


Figure 18. Representative electropherogram for a blood on Whatman 903 collection paper sample.

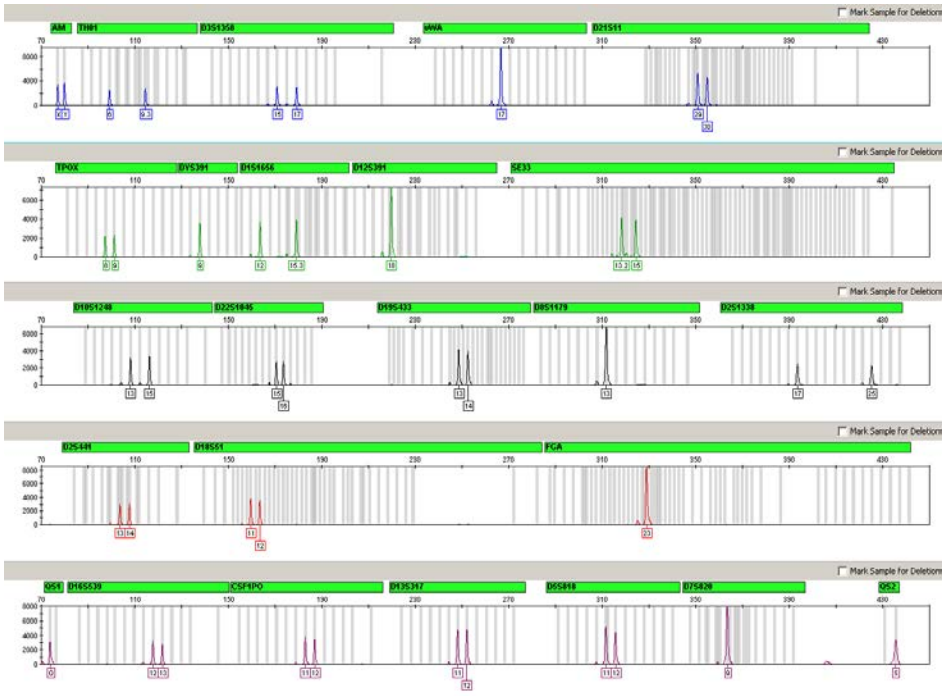


Figure 19. Representative electropherogram for a buccal cells on Whatman EasiCollect sample.

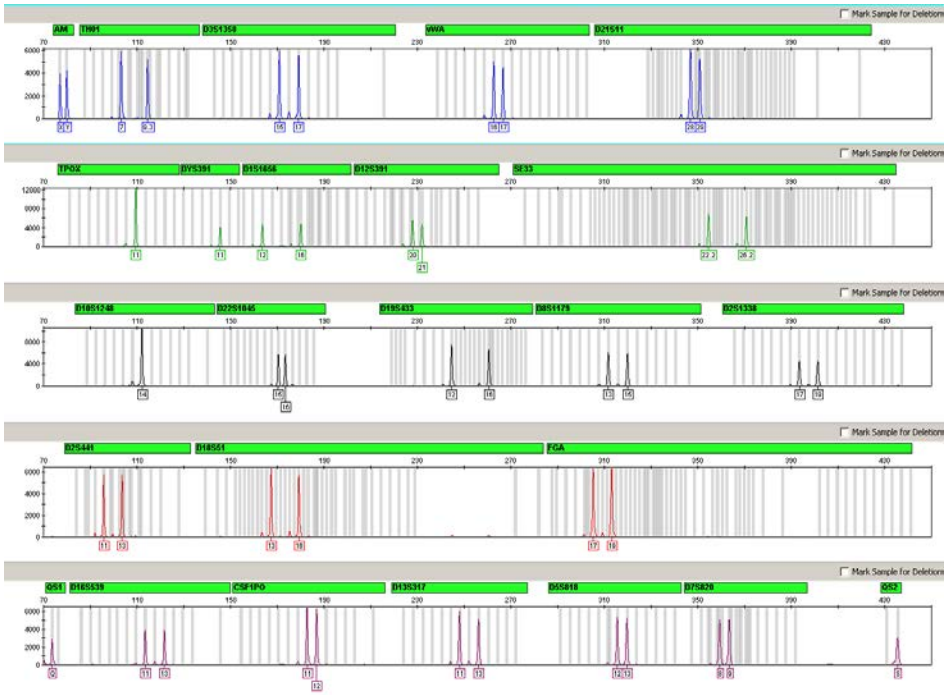


Figure 20. Representative electropherogram for a Puritan® polyester swab lysate sample.

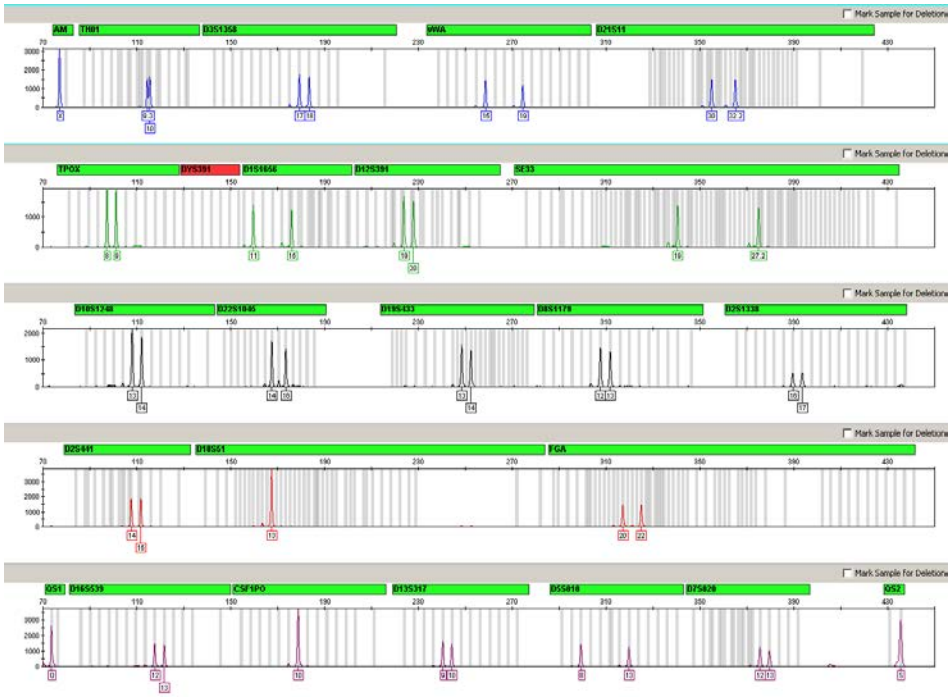


Figure 21. Representative electropherogram for a Sarstedt® cotton swab lysate sample. Note that the red label caused by the absence of the Y-chromosomal marker DYS391 is expected, since a female profile is shown.

As expected, the performance for direct amplification reactions shows a high variability from sample-to-sample. This is dependent on different factors, for example on the DNA amount given by certain donors (Figure 22). For highest first round success rates, each laboratory should determine the optimal cycle number by running a representative batch of samples.

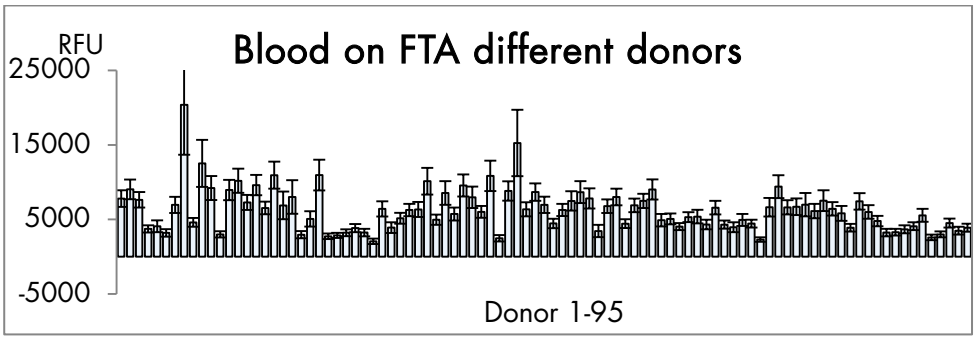


Figure 22. Robustness: Blood on FTA cards. Average peak heights across samples from 95 different donors processed in 5 replicates.

Peak height ratios of heterozygous markers (Figure 23; Table 3) were analyzed for all 171 direct PCR samples, each in 5 replicates. Analyzed sample types are stated above.

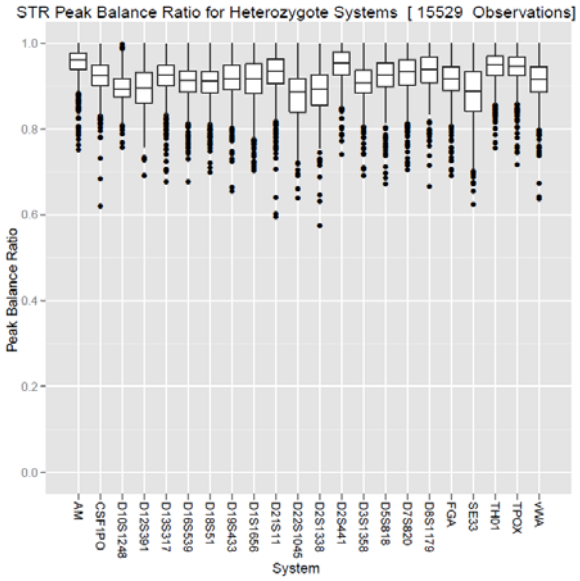


Figure 23. Box plot analysis of intra-locus peak height ratios. Values refer to the lower of the 2 peaks in a heterozygous sample. Boxes represent the middle 50% (interquartile range, [IQR]) of data, lines inside the boxes the median. Whiskers show data within 1.5 IQR, dots represent outliers.

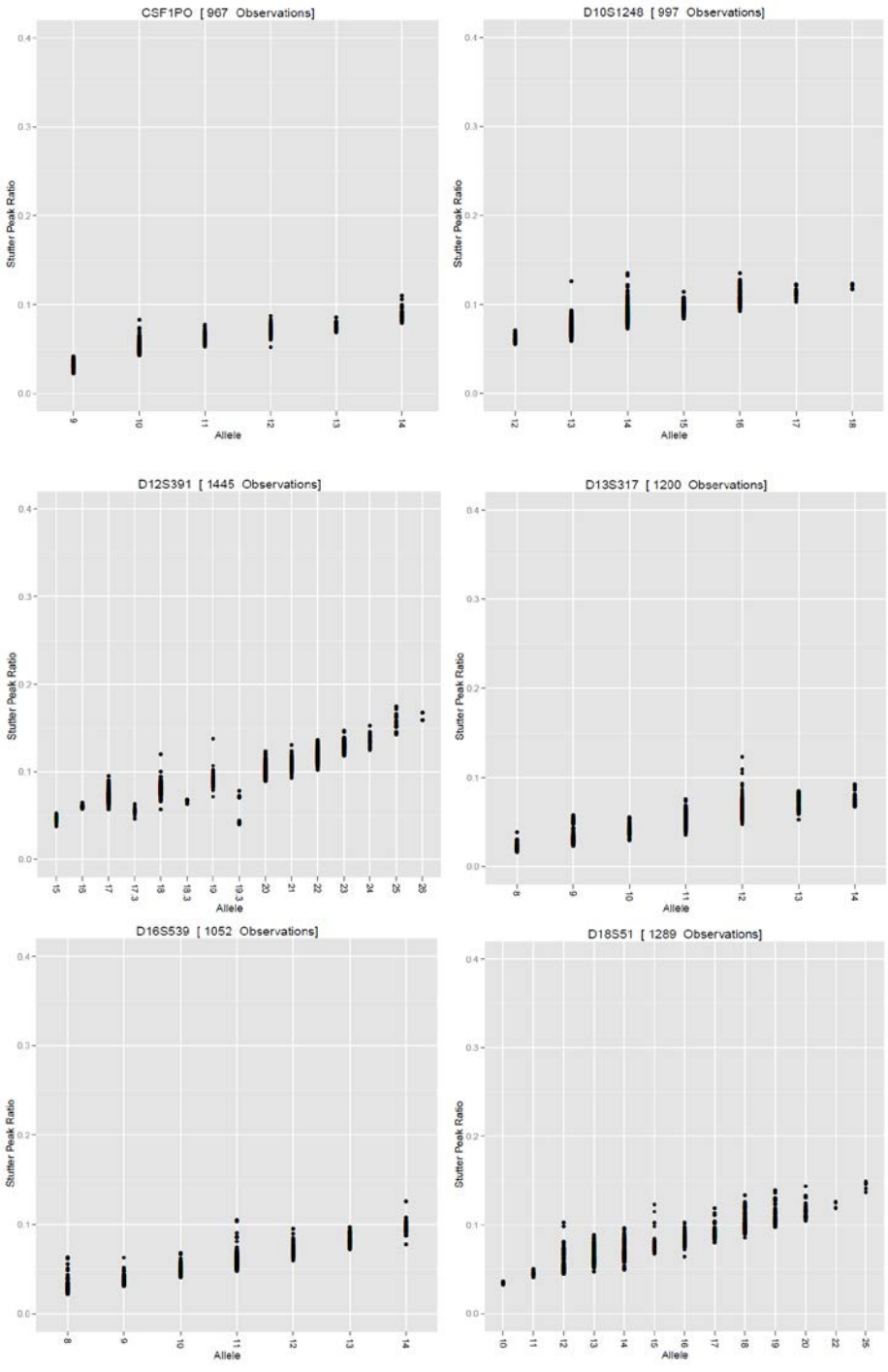
Table 3. Heterozygote peak height ratios. Observed intralocus balance for 171 direct PCR samples from different donors in 5 replicates.

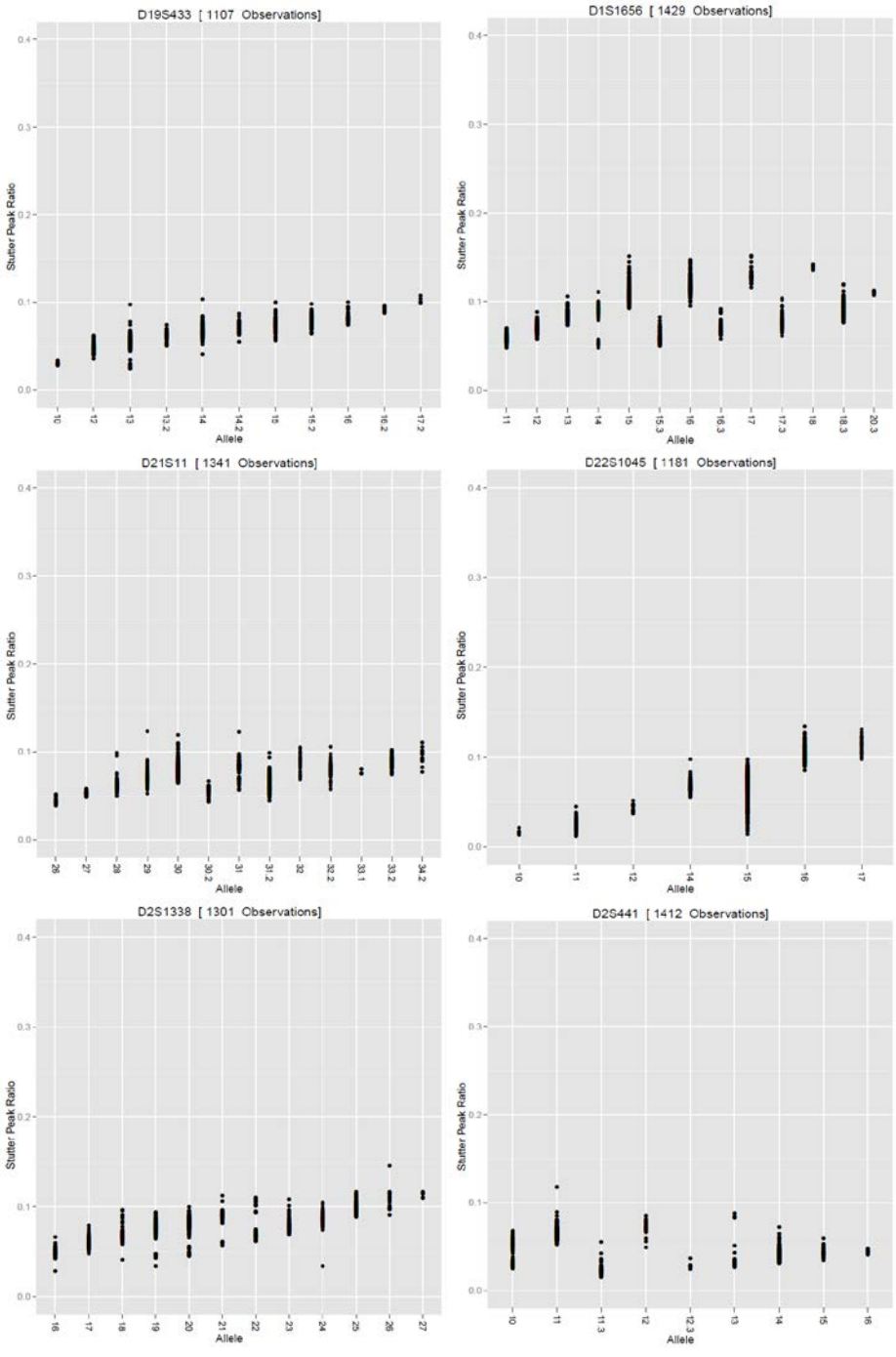
System	Observations	Mean (%)	Min (%)	Max (%)
AM	402	95.0	75.2	100
CSF1PO	625	92.3	61.9	99.9
D10S1248	672	89.6	75.6	99.8
D12S391	766	89.4	69.2	100
D13S317	701	92.0	67.7	99.9
D16S539	802	90.9	67.7	99.9
D18S51	734	90.6	70.0	99.9
D19S433	712	91.6	65.4	100
D1S1656	818	91.2	70.2	100
D21S11	740	92.9	59.5	100
D22S1045	745	87.8	63.9	99.9
D2S1338	753	88.8	57.6	99.9
D2S441	762	94.8	74.0	100
D3S1358	762	90.8	69.1	100
D5S818	635	92.3	67.0	99.9
D7S820	750	92.6	70.4	100
D8S1179	642	93.4	66.6	100
FGA	815	91.4	69.1	100
SE33	836	88.3	62.4	99.9
TH01	597	94.2	75.6	100
TPOX	606	94.0	71.6	100
vWA	654	91.1	63.6	100

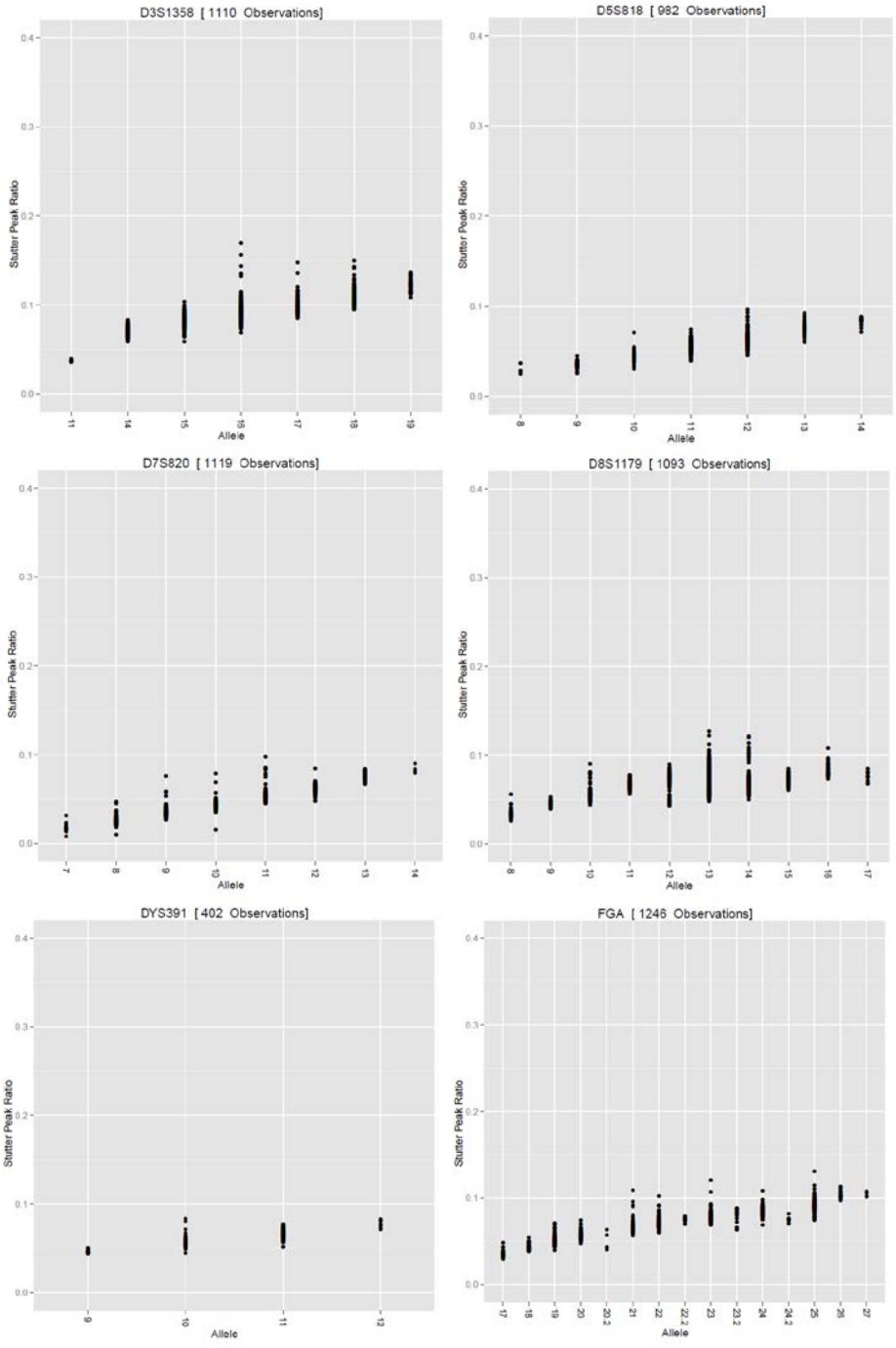
In addition, stutter peak heights were analyzed for all 171 direct PCR samples, each in 5 replicates. Analyzed sample types are stated above. Stutter peak heights are characteristic for each marker and the number of repeat motifs of an allele (Table 4). TH01 in general shows the lowest stutter ratio of all STR markers of the Investigator 24plex GO! Kit. Smaller alleles display lower stutter levels than longer alleles of the same marker (Figure 1). Stutter positions, where heterozygous alleles differ by two repeat units and the backward stutter of the smaller allele overlaps with the forward stutter of the longer allele, have been excluded from the analysis.

Table 4. Peak height ratios of forward stutters. Peak heights of forward stutters compared to main peaks were analyzed for 171 samples from different donors in five replicates.

System	Observations	Stutter mean (%)	Stutter min (%)	Stutter max (%)
CSF1PO	967	5.9	2.3	11.0
D10S1248	997	8.4	5.6	13.5
D12S391	1445	9.3	3.7	17.4
D13S317	1200	4.9	1.6	12.3
D16S539	1052	5.8	2.2	12.6
D18S51	1289	7.5	3.3	14.9
D19S433	1107	6.4	2.3	10.7
D1S1656	1429	8.7	4.7	15.2
D21S11	1341	7.3	3.9	12.3
D22S1045	1142	3.7	0.4	8.0
D2S1338	73	0.6	0.2	1.6
D2S441	926	1.1	0.3	2.7
D3S1358	820	0.9	0.2	3.7
D5S818	810	0.9	0.2	3.8
D7S820	637	0.6	0.2	1.6
D8S1179	719	0.9	0.2	3.2
DYS391	142	0.4	0.2	1.8
FGA	781	0.8	0.1	3.9
SE33	992	0.8	0.2	3.5
TH01	72	0.3	0.1	1.0
TPOX	647	0.7	0.1	3.8
vWA	503	0.5	0.1	1.6


CSF1PO [967 Observations]
D10S1248 [997 Observations]
D12S391 [1445 Observations]
D13S317 [1200 Observations]
D16S539 [1052 Observations]
D18S51 [1289 Observations]
Stutter Peak Ratio
Allele


D19S433 [1107 Observations]
D1S1656 [1429 Observations]
D21S11 [1341 Observations]
D22S1045 [1181 Observations]
D2S1338 [1301 Observations]
D2S441 [1412 Observations]
Stutter Peak Ratio
Allele


D3S1358 [1110 Observations]
D5S818 [982 Observations]
D7S820 [1119 Observations]
D8S1179 [1093 Observations]
DYS391 [402 Observations]
FGA [1246 Observations]
Stutter Peak Ratio
Allele

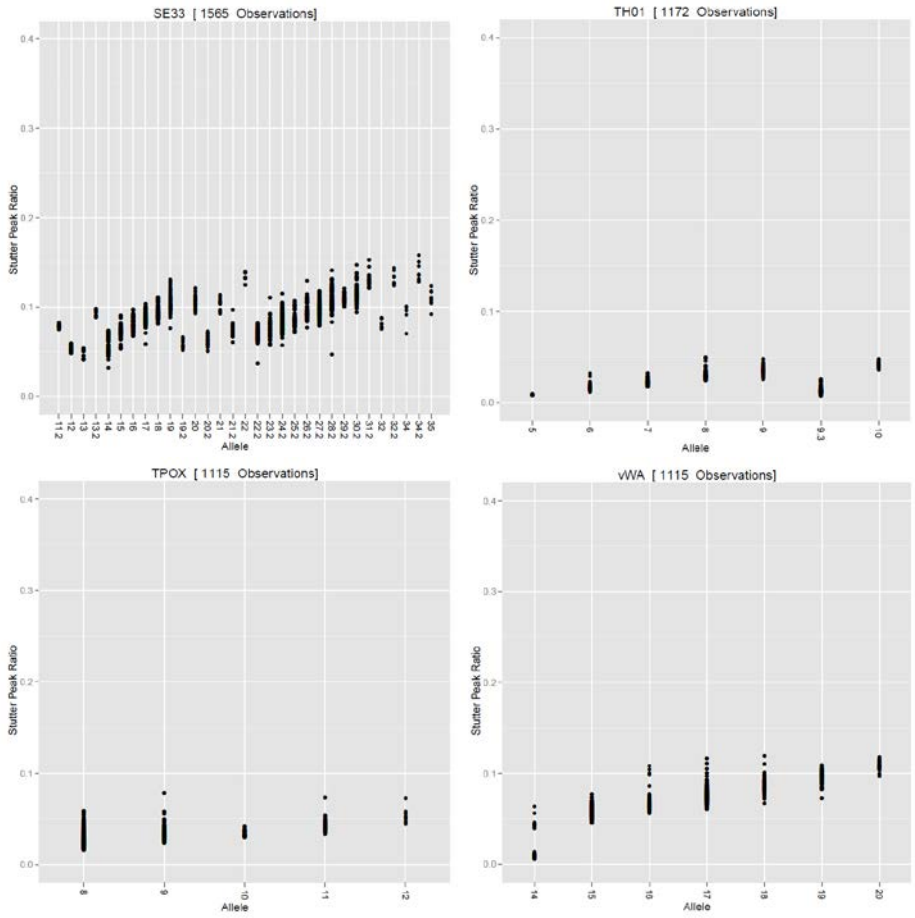


Figure 24. Peak height ratios of forward stutters. Peak heights of forward stutters compared to main peaks were analyzed for 171 samples from different donors in five replicates.

Backward stutters are usually of very low height, although present (Table 5). Stutter positions, where heterozygous alleles differ by two repeat units and the backward stutter of the smaller allele overlaps with the forward stutter of the longer allele, have been excluded from the analysis. Please note that marker D22S1045 shows a significantly elevated backward stutter (Figure 25). 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.

Table 5. Peak height ratios backward stutters. Peak heights of backward stutters compared to main peaks were analyzed for 171 samples from different donors in five replicates.

System	Observations	Stutter mean (%)	Stutter min (%)	Stutter max (%)
CSF1PO	855	1.0	0.4	3.5
D10S1248	443	0.5	0.2	1.8
D12S391	690	0.6	0.1	6.5
D13S317	896	0.8	0.1	4.7
D16S539	924	1.0	0.3	3.8
D18S51	937	1.1	0.2	3.6
D19S433	191	0.5	0.1	1.7
D1S1656	1196	1.1	0.3	4.8
D21S11	1079	1.0	0.2	6.2
D22S1045	1142	3.7	0.4	8.0
D2S1338	73	0.6	0.2	1.6
D2S441	926	1.1	0.3	2.7
D3S1358	820	0.9	0.2	3.7
D5S818	810	0.9	0.2	3.8
D7S820	637	0.6	0.2	1.6
D8S1179	719	0.9	0.2	3.2
DYS391	142	0.4	0.2	1.8
FGA	781	0.8	0.1	3.9
SE33	992	0.8	0.2	3.5
TH01	72	0.3	0.1	1.0
TPOX	647	0.7	0.1	3.8
vWA	503	0.5	0.1	1.6

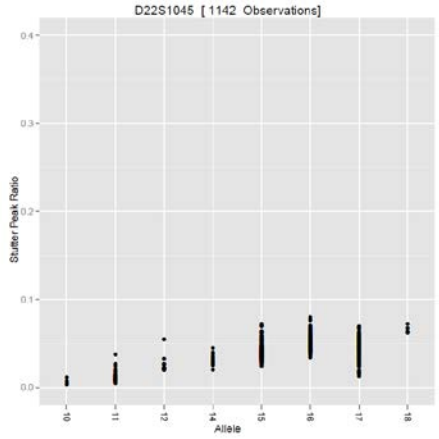


Figure 25. Peak height ratios of backward stutters of marker D22S1045. Peak heights of backward stutters compared to main peaks were analyzed.

Reproducibility – Automated reaction setup for FTA cards

Protocols were developed for fully automated punching of FTA cards and reaction setup for amplification with the QIAGEN Investigator 24plex GO! Kit on the STAR Q Punch AS Instrument. The protocols allow the processing of buccal cells on indicating FTA paper (GE Healthcare EasiCollect or Thermo NucleiCard) or blood on FTA paper. One punch of 1.2 mm size was punched from the center of the sampling area into a well of a 96-well PCR plate after the PCR reagent master mix had been distributed. Buccal cells were collected from 22 different donors in duplicates on EasiCollect Cards. These 44 positive samples were processed alternating with 44 empty cards to check for sample carryover. The protocol was set to take three cleaning punches from a separate card in between sample cards. A total of three runs were done using this setup. Amplification was done on an ABI 9700 instrument using 27 cycles. 130 out of 132 samples gave full profiles at a threshold of 200 RFU. A total of 6 expected alleles were missing from the remaining two samples. Of the negative samples, 95% did not show background above 50 RFU, and 4% produced background up to 100 RFU, with one sample above 100 RFU; no peaks above 200 RFU were observed.

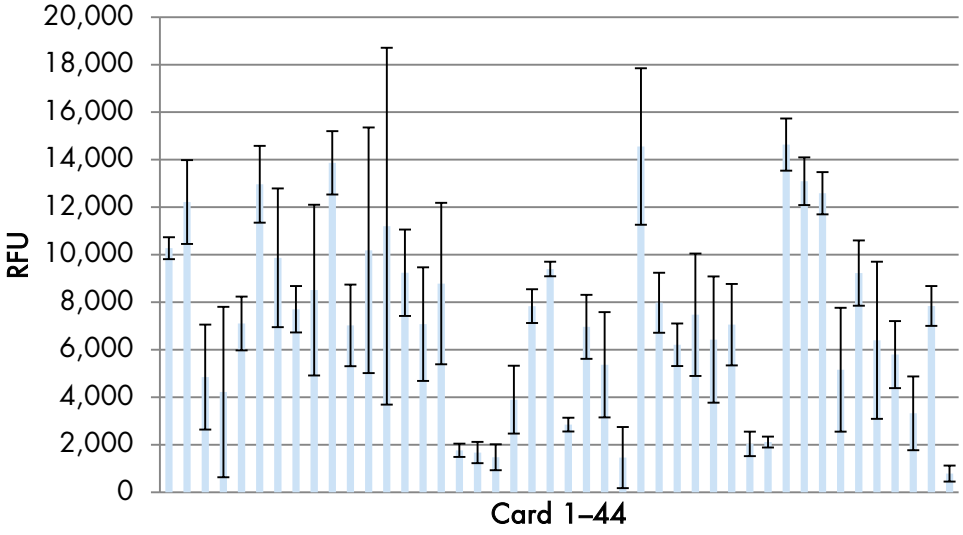


Figure 26. Average peak heights for profiles obtained with buccal cells on EasiCollect cards. Each card was processed in triplicate.

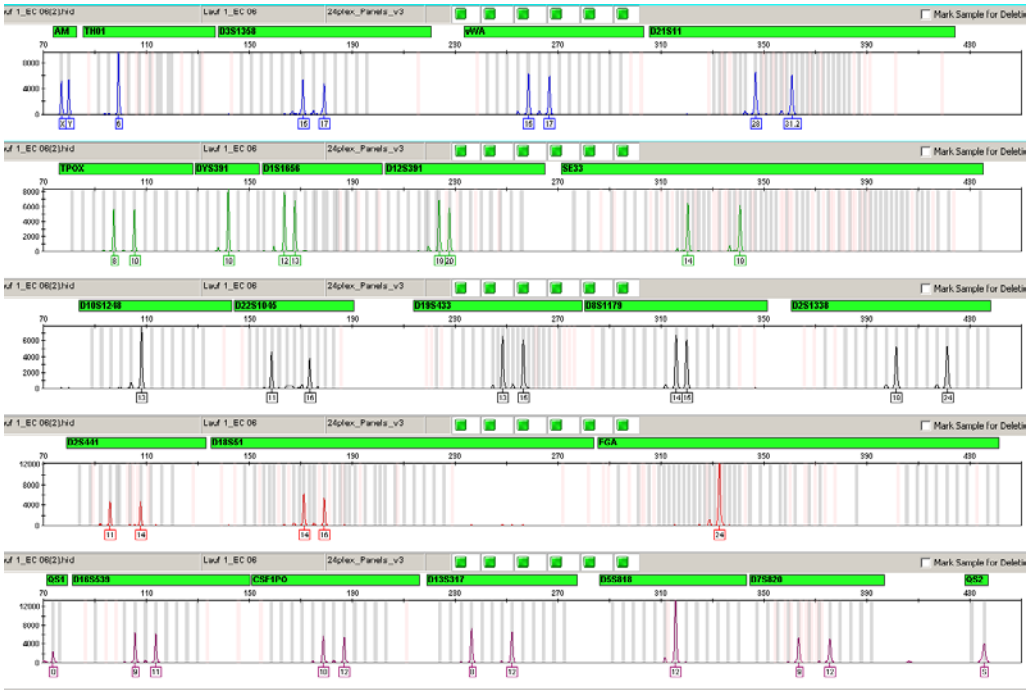


Figure 27. Representative electropherogram for buccal cells on EasiCollect sample using the STAR Q Punch AS instrument for PCR setup.

An identical setup was used to validate Thermo Nucleic-Cards on the STAR Q Punch AS instrument. Here, 127 out of 132 samples gave full profiles at a threshold of 200 RFU. Out of 5 partial profiles, 4 were from the same donor and corresponding cards showed a poor sample transfer during collection. Of the negative samples, 90% did not show background above 50 RFU, and 8% produced background up to 100 RFU, with 2 samples above 100 RFU; no peaks above 200 RFU were observed.

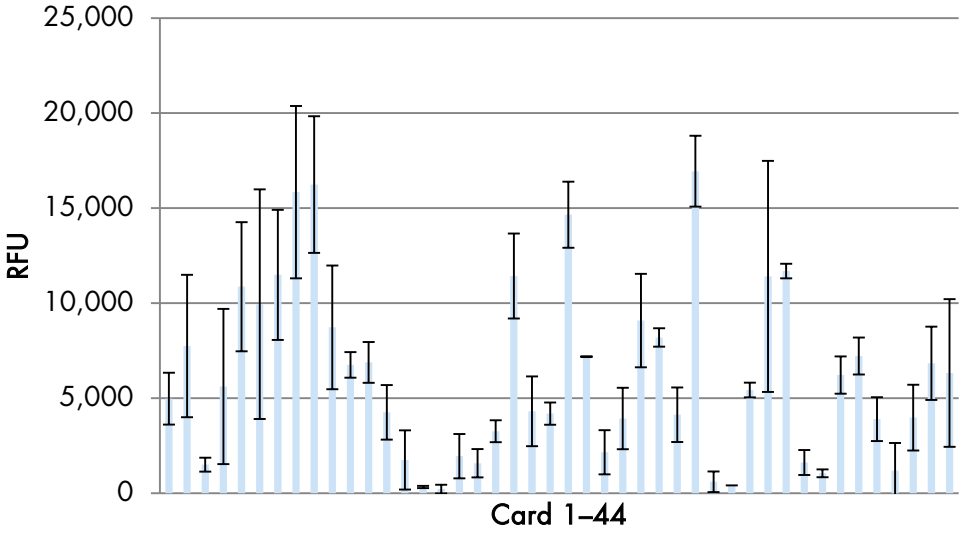


Figure 28. Average peak heights for profiles obtained with buccal cells on Nucleic-Cards. Each card was processed in triplicate.

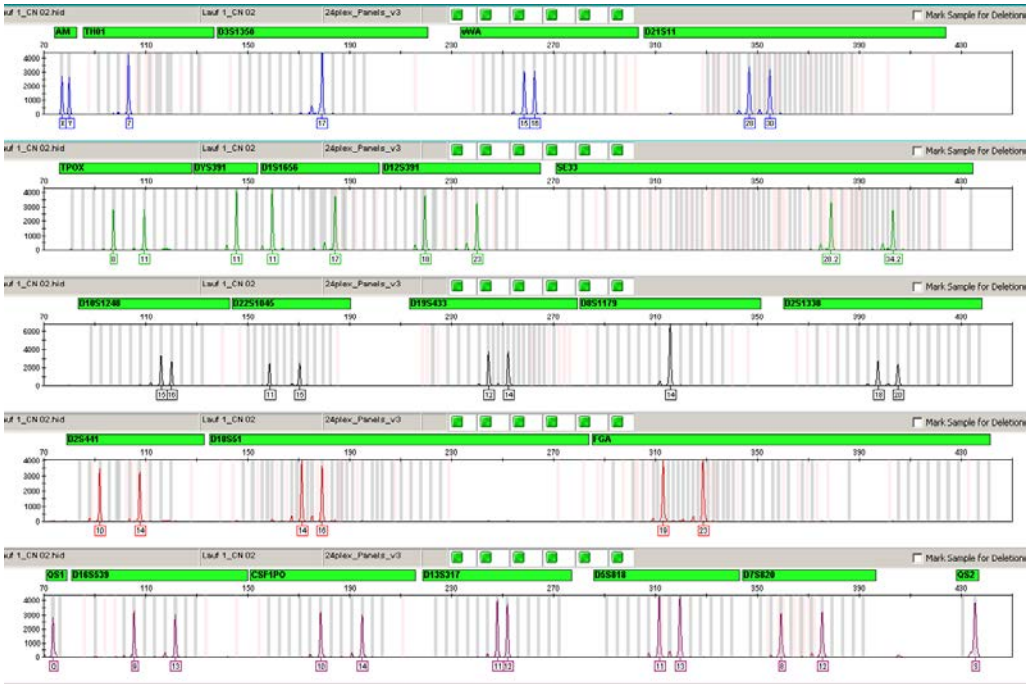


Figure 29. Representative electropherogram for buccal cells on Nucleic-Card sample using the STAR Q Punch AS Instrument for PCR setup.

To test the performance for blood on FTA samples, blood of 36 donors was spotted on Whatman FTA paper. The cards were processed in 5 replicates each. Amplification was done on an

ABI 9700 instrument using 25 cycles. Full profiles were created for all samples at a threshold of 200 RFU.

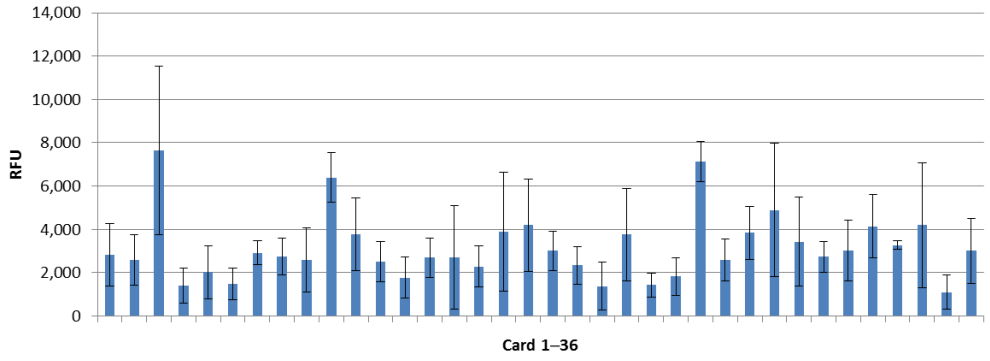


Figure 30. Average peak heights for profiles obtained with blood on FTA cards. Each card was processed in 5 replicates.

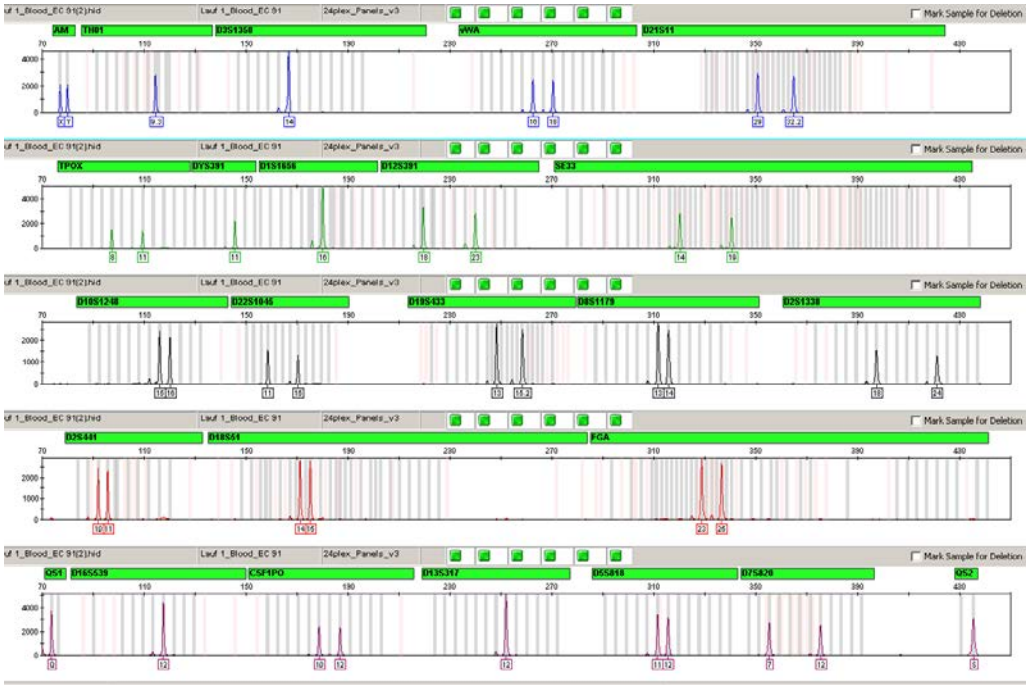


Figure 31. Representative electropherogram for a blood on FTA sample using sample using the STAR Q Punch AS Instrument for PCR setup.

For all sample types analyzed, results obtained using the automated method were comparable to results of manually processed samples from the same donors (data not shown), and variation between samples from different donors was within the expected range. No incidents of sample mix-up or missed samples were observed.

Reproducibility – Automated reaction setup for swabs

STAR Q Swab AS protocol scripts were developed to fully automate both swab pre-treatment and STR assay plate setup. Puritan polyester swabs, Sarstedt cotton swabs and GE Healthcare Omni swabs were tested. Swabs are lysed using 500 µl of Investigator STR GO! Lysis Buffer in a 96 deep well plate on the heated shaker of the instrument. After lysis, 900 µl of water was added to each sample to ensure a liquid level well above the swab for the subsequent pipetting step. For each sample, 2 µl of swab lysate was transferred to the PCR reaction. Amplification was done on an ABI 9700 instrument using 27 cycles.

For each swab type tested, 4 samples were collected from 20 different donors, resulting in 80 swabs in total. Samples were run in checkerboard pattern, alternating with empty wells to test for cross-contamination. Full profiles were obtained for all samples in this study at a threshold of 200 RFU. No sample carryover was observed.

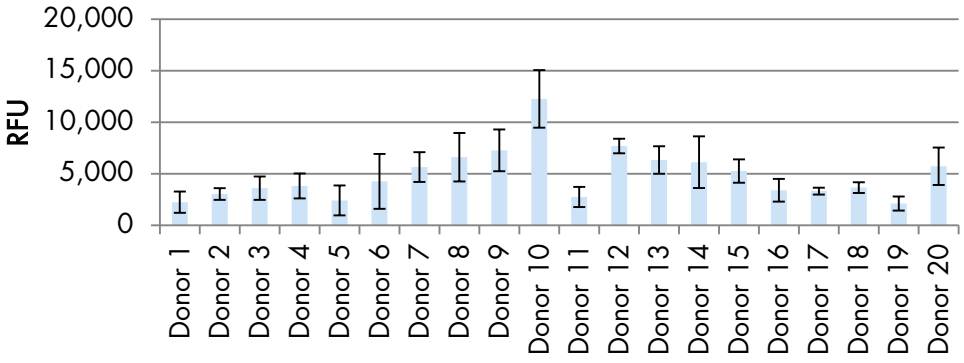


Figure 32. Average peak heights for profiles obtained with Puritan polyester swabs.

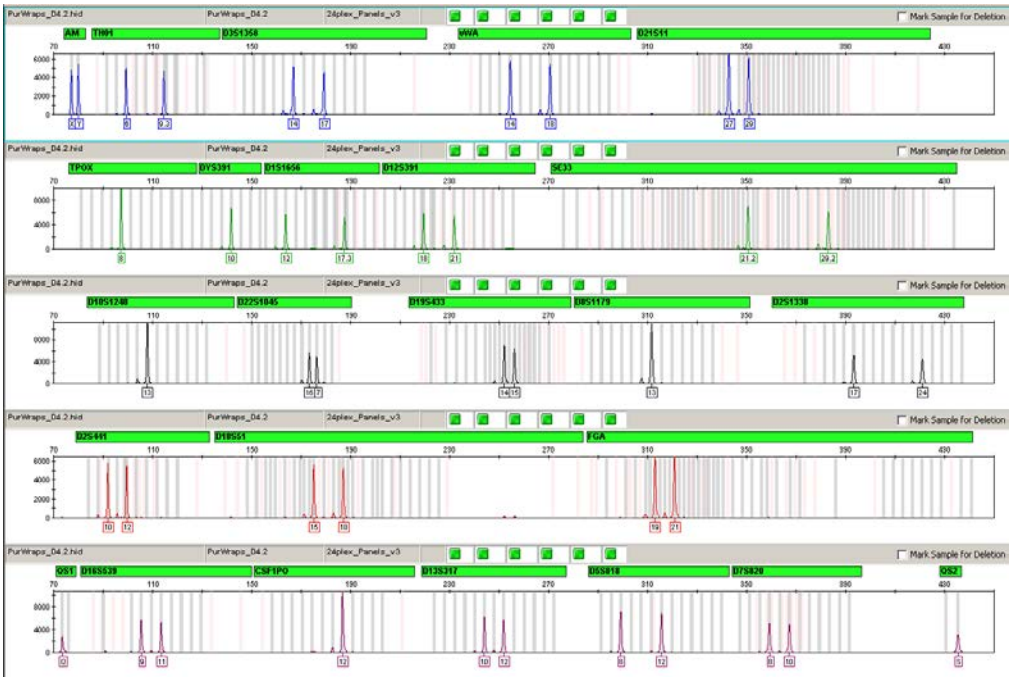


Figure 33. Representative electropherogram for a Puritan polyester swab sample.

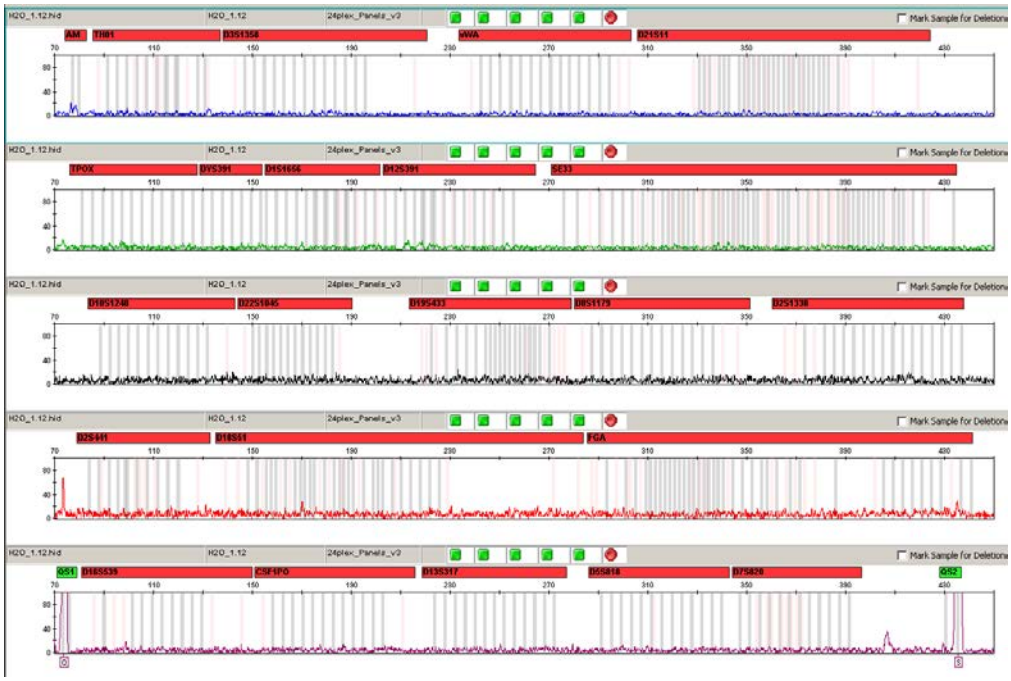


Figure 34. Representative electropherogram for a negative sample.

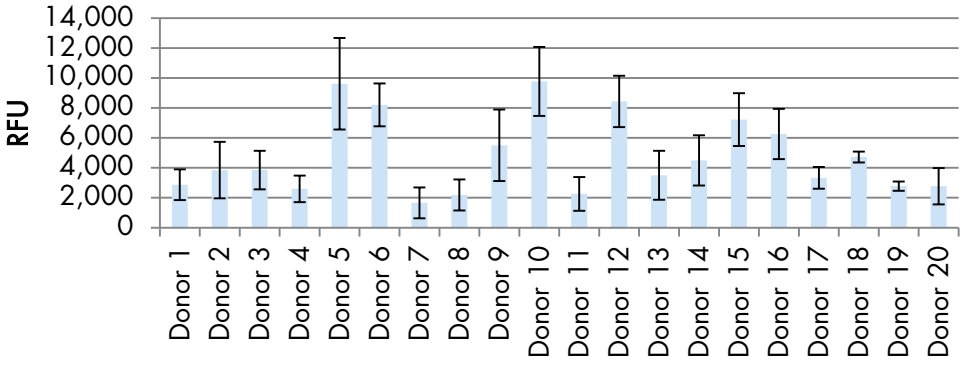


Figure 35. Average peak heights for profiles obtained with Sarstedt cotton swabs.

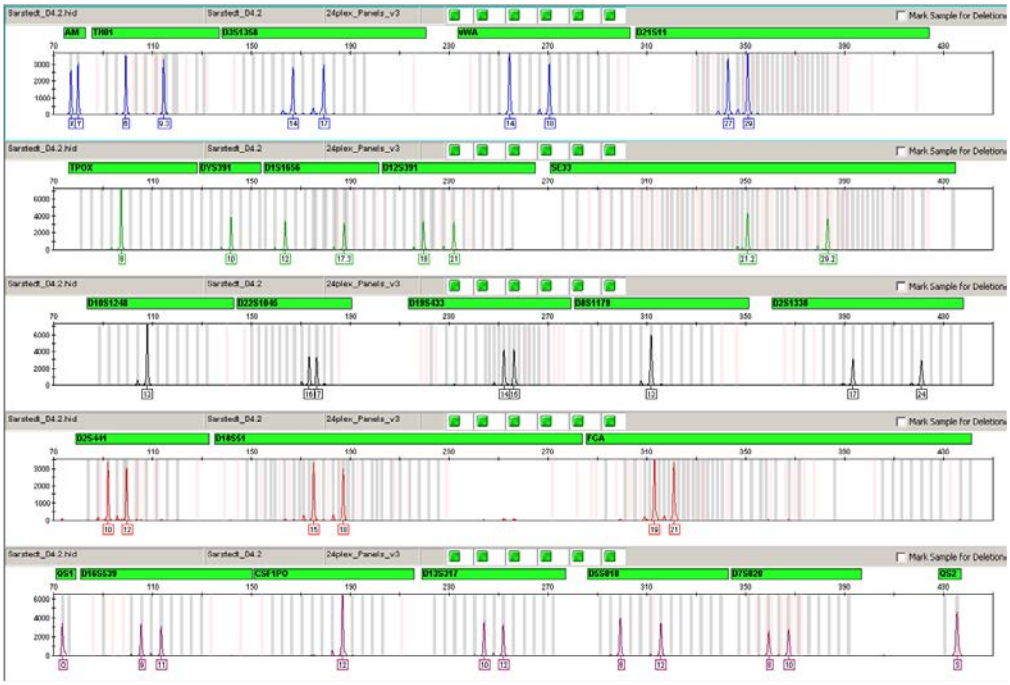


Figure 36. Representative electropherogram for a Sarstedt cotton swab sample.

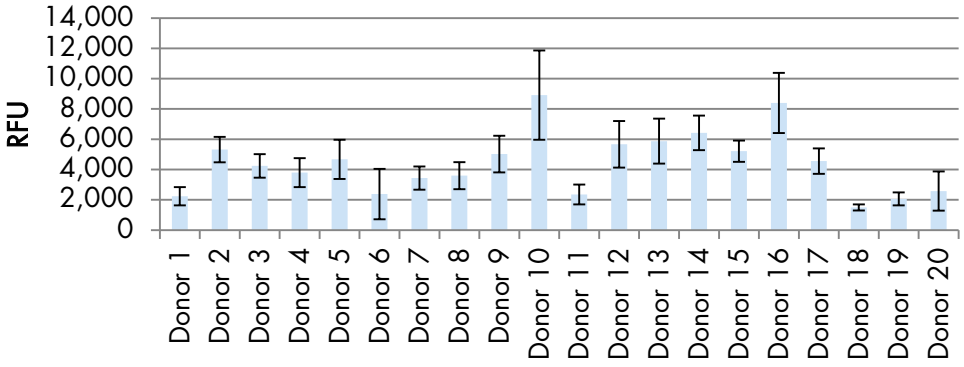


Figure 37. Average peak heights for profiles obtained with GE Healthcare Omni swabs.

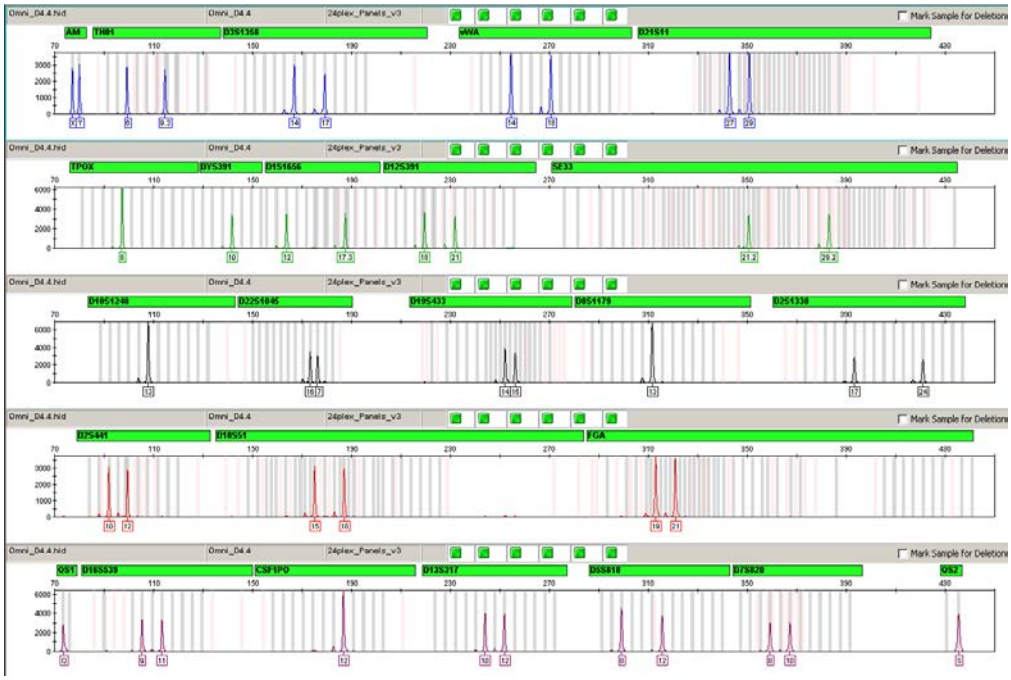


Figure 38. Representative electropherogram for a GE Healthcare Omni swab sample.

Stability – Improved HotStart function

Usually PCR reactions were setup in a straightforward manner, without any interruptions in between the PCR pipetting step and the start of the thermocycler run. When using an instrument for the automated PCR setup, it can take several hours to pipet one or more reaction plates. To avoid the deleterious effects of mispriming at room temperatures the Investigator 24plex GO! Kit contains an improved HotStart function. Based on a novel, antibody-mediated hot-start mechanism, the kit affords enhanced specificity and efficiency of the multiplex PCR reaction. The

added convenience of extreme stability at room temperature, without a need for any cooling, makes it ideal for handling of high-throughput samples and automated workflows.

For buccal swab samples, the kit affords an extreme stability for up to 16 hours incubation at 20°C prior to PCR cycling. The improved HotStart function allows enhanced specificity and efficiency for more than 16 hours incubation at room temperature in between the PCR pipetting step and the start of the thermocycler, without a need for any cooling. As demonstrated, the overall kit performance was not compromised, no unspecific PCR products were amplified and no precipitates were observed (Figure 39).

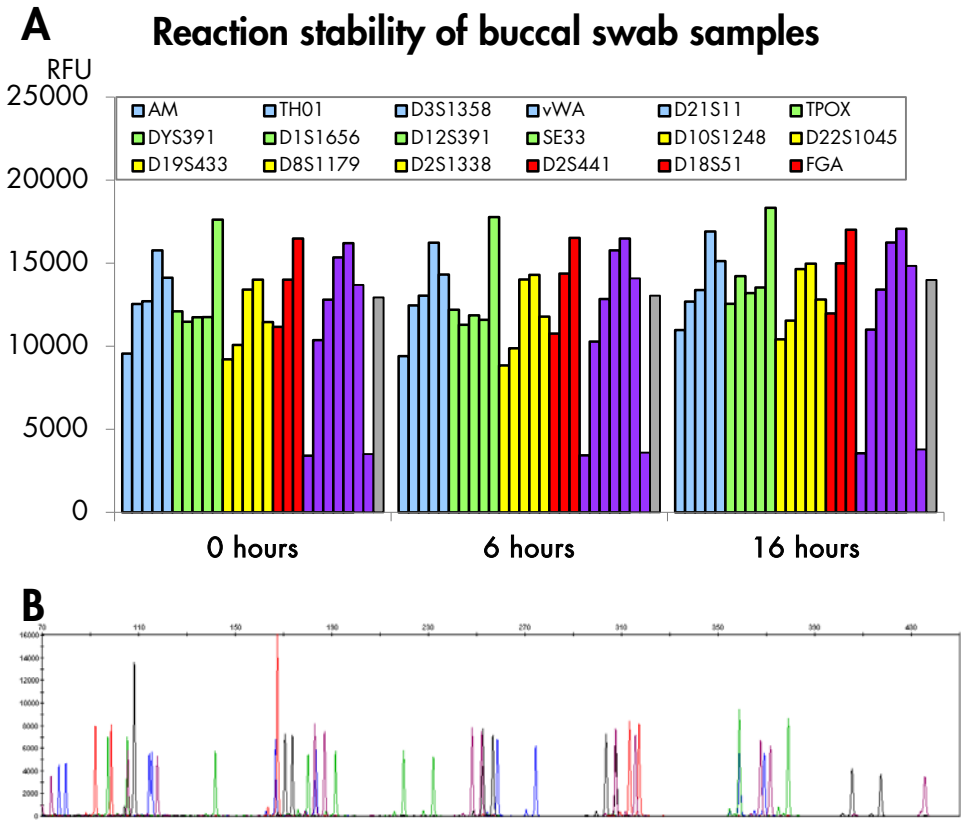


Figure 39. Reaction stability of buccal swab samples. PCR master mix containing a buccal swab sample was incubated at 20°C for 0, 6 and 16 hours in between the PCR pipetting step and the start of the thermocycler. Average peak heights of triplicates from 3 different donors are shown for each marker. (A) Bar colors represent the fluorescent dye labels of individual markers, grey bars show the average of all STR markers. (B) Exemplary electropherogram for a buccal swab sample, incubated for 16 h at 20°C before cycling, to demonstrate the improved HotStart function of Investigator 24plex GO! Kit.

In comparison, the latency time of a master mix containing blood or buccal samples on FTA cards should not exceed more than two hours at room temperature prior to PCR cycling. After one hour incubation, the FTA punches start to dissolve in the master mix and to build an insoluble precipitate. The improved HotStart function allows enhanced specificity and efficiency for more than two hours incubation at 20°C in between the PCR pipetting step and the start of the thermocycler, but the dispersed FTA punches impair the further handling steps and make a longer incubation impossible. Punches from FTA samples incubated for two hours at room temperature gave full profiles with good heterozygote balance (Figure 40). Here, the PCR products for capillary electrophoresis were taken off the supernatant after a short centrifugation step.

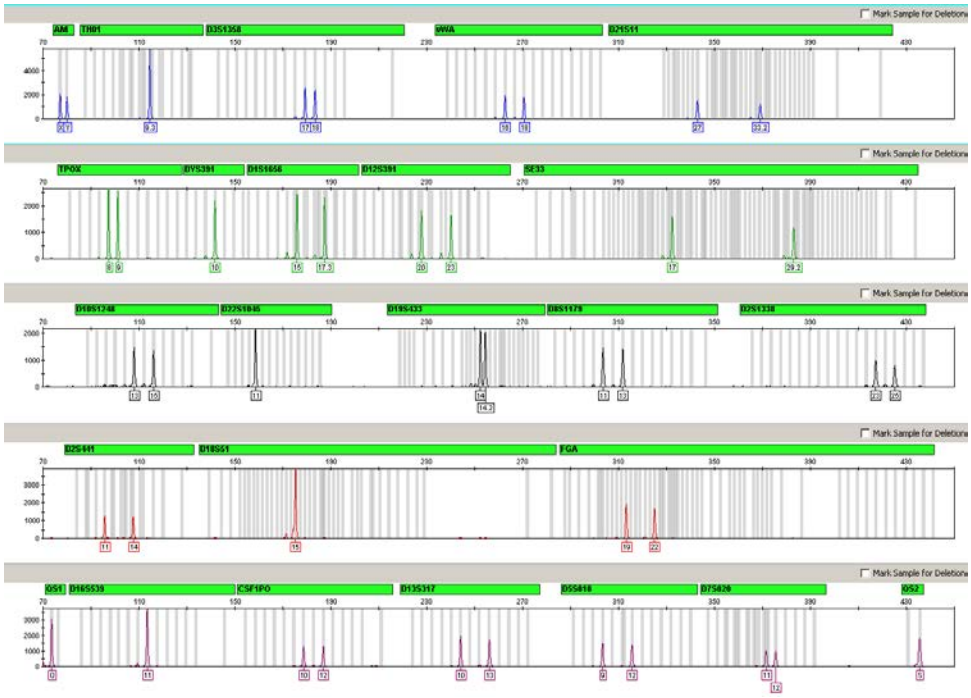


Figure 40. Reaction stability of FTA card samples after 2 hours incubation of the master mix at room temperature. PCR master mix containing a blood on FTA card sample was incubated for 2 hours at 20°C in between the PCR pipetting step and the start of the thermocycler.

Stability – 20 freeze / thaw cycles

In a forensic lab, the maximum number of reactions of a kit may not be used up in a single day. Therefore, the Investigator 24plex GO! Kit components were tested to prove that they would yield stable results after multiple rounds of freeze / thawing. Regardless of these results, we do not recommend repeated freezing and thawing of the kit contents.

Figure 41 shows the electropherograms obtained by amplifying blood on FTA sample with fresh kit components (no freeze / thawing) and with kit components stressed by 20 rounds of freezing and thawing (20 x freeze / thaw cycles). The overall kit performance was not compromised under the chosen conditions. Comparable peak heights were obtained before and after 20 rounds of freezing and thawing.

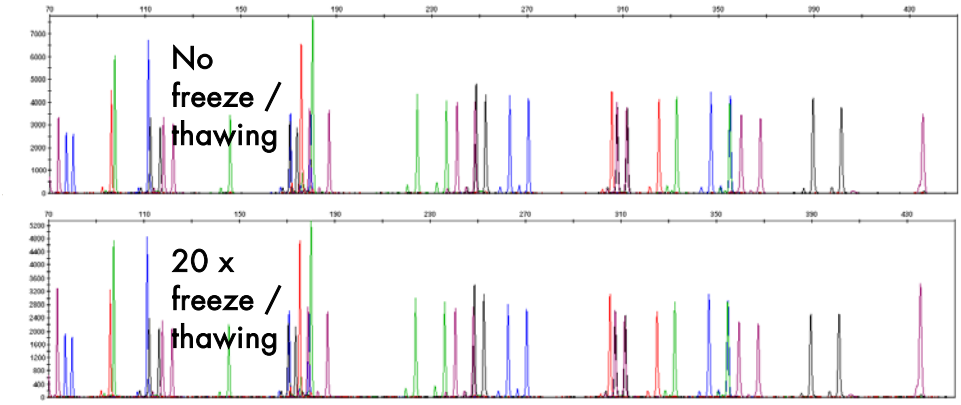


Figure 41. Results of a simulated freeze / thawing stability test of kit components.

Stability – Simulated shipment condition on dry ice

Investigator 24plex GO! Kits are shipped on dry ice. To assess the performance of the kit after such transportation, the components were stored on dry ice and at –20°C for 5 days.

Kits were stored for 16 hours on dry ice, and then transferred to –20°C for 8 hours. This cycle was repeated for 5 days. Each day, components from these kits were used to amplify blood on FTA sample.

The results indicate that the performance before and after storage on dry ice is comparable (Figure 42).

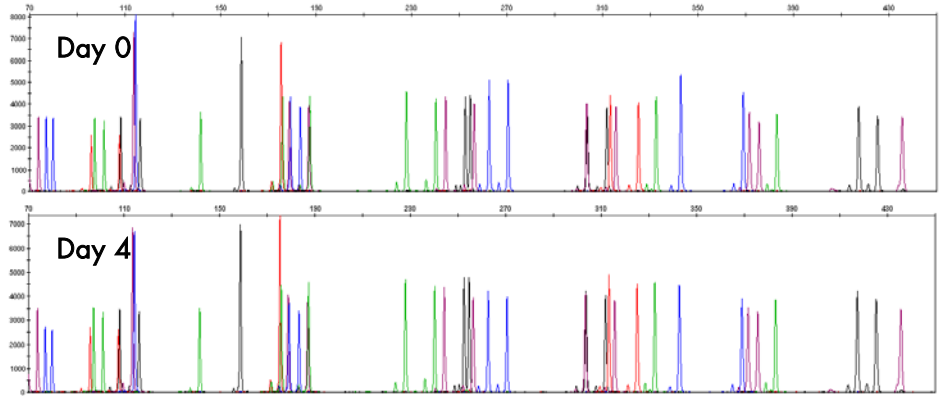


Figure 42. Effect of prolonged storage of Investigator 24plex GO! Kit components on dry ice.

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.

For a complete list of references, contact QIAGEN Technical Services.

Cited References

1. ENFSI Standing Committee for Quality and Competence (QCC). (2006) Validation and Implementation of (New) Methods. Ref. Code: QCC-VAL-001, Issue No. 001, November 2006. http://www.enfsi.eu/get_doc.php?uid=144.
2. Forensic Science Communications. (2004) Revised Validation Guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM), 6 (3), July 2004. www.cstl.nist.gov/strbase/validation/SWGDAM_Validation.doc.
3. Gill, P., et al. (2006) The evolution of DNA databases-Recommendations for new European STR loci. Forensic Sci. Int. **156**, 242.
4. Phillips, C., et al. (2011) Analysis of global variability in 15 established and 5 new European Standard Set (ESS) STRs using the CEPH human genome diversity panel. Forensic Sci. Int. Genet. **5**, 155.
5. Butler, J.M., Shen, Y., and McCord, B.R., (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. J. Forensic Sci. **48**, 1054.

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

Ordering Information

Product	Contents	Cat. no.
Investigator 24plex GO! Kit (200)	Primer mix, Fast Reaction Mix including *Taq* DNA Polymerase, Control DNA, allelic ladder 24plex, DNA size standard 550 (BTO) and Nuclease-free water	382426
Investigator 24plex GO! Kit (1000)	Primer mix, Fast Reaction Mix including *Taq* DNA Polymerase, Control DNA, allelic ladder 24plex, DNA size standard 550 (BTO) and Nuclease-free water	382428

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.