Validation Report October 2015

Developmental validation of the Investigator® ESSplex SE QS Kit

The QIAGEN® Investigator ESSplex SE QS Kit is intended for molecular biology applications in forensic, human identity and paternity testing. The kit is used for multiplex PCR in forensic casework, and was developed specifically for rapid and reliable generation of DNA profiles from blood, buccal swabs and forensic stains.

The performance of the Investigator ESSplex SE QS Kit was evaluated with regards to various sample types and conditions, commonly encountered in forensic and parentage laboratories.

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 European Network of Forensic Science Institutes (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). As a special feature, the Investigator ESSplex SE QS 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 ESSplex SE QS 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, as an example, $MgCl_2$ concentration (page 3). The robustness of the assay, regarding variations in PCR cycling conditions and a range of different thermal cyclers, was investigated (pages 4–10). The effects of using increased or decreased PCR cycle numbers were analyzed (page 8–9). Sensitivity was addressed by amplifying DNA of known concentration, in a range typically encountered in forensic casework analysis (page 13).

Specific issues that can arise during forensic casework were investigated, such as the ability to obtain results from samples that have been subjected to adverse environmental conditions. This was tested using DNA spiked with several types of inhibitors or degraded DNA (pages 16–24). Cross-reactivity with non-human DNA (page 25) was also assessed. The reproducibility of the results was verified (page 33).

The stability of the kit components was validated with regard to repeated freezing and thawing (page 43) and transport (page 43).

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 ESSplex SE QS Kit Handbook* were used for electrophoresis. Unless stated otherwise, a GeneAmp® PCR System 9700 with a Gold-plated Silver 96-Well Block was used for amplification. Data were analyzed using Applied Biosystems GeneMapper® ID-X software, v1.2.

Reaction conditions

Reaction conditions were established for an optimal performance in terms of sensitivity, specificity and reproducibility. This required the optimization of all critical buffer components. The final composition of the Fast Reaction Mix 2.0 is a robust buffer system that tolerates differences in the concentration of individual buffer components, without a decrease in overall amplification performance.

For example, various concentrations of $MgCl_2$, one of the critical buffer components, were added to a standard reaction with 500 pg control DNA 9948 (Figure 1). The assay yielded robust results within a $MgCl_2$ concentration range of ±30% of the optimum.

Under these conditions, amplification of the STR markers was well balanced and no dropout or non-specific amplification occurred. Similar experiments were performed to evaluate optimal concentration for other buffer components, such as the hot-start DNA polymerase, dNTPs and BSA (data not shown).

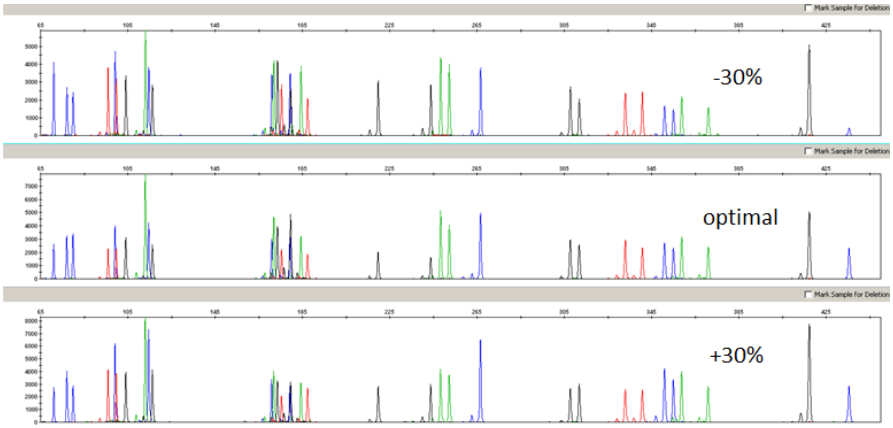


Figure 1. Influence of MgCl2 concentration. Fast Reaction Buffer 2.0 (FRM 2.0) was supplemented with different concentrations of MgCl2 matching the specification of production, or 30% higher or lower. Representative electropherograms of sample quadruplicates are shown.

Effect of PCR annealing temperature variations

Specificity, sensitivity and robustness are critical in forensic casework, all of which 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 ESSplex SE QS 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	55 s	
72°C	5 s	
96°C	10 s	27 cycles
61°C	55 s	
72°C	5 s	
68°C	2 min	
10°C	∞	–

* Hotstart to activate DNA polymerase.

Annealing temperatures between –4°C and +4°C around the optimal annealing temperature of 64°C / 61°C were applied to the amplification of 500 pg control DNA 9948. The annealing temperature of the first 3 cycles and of the following 27 cycles were varied to the same extent. PCR was performed on an Eppendorf® Mastercycler® ep instrument. Reactions using annealing temperatures between -4°C and +4°C resulted in full profiles. Good inter-locus balance was observed for the temperature range of –3°C to +1.5°C (Figure 2 and Figure 3). No dropouts were observed in the tested range applying a threshold of 200 RFU. No non-specific PCR products were observed down to –3°C below the recommended annealing temperature. 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).

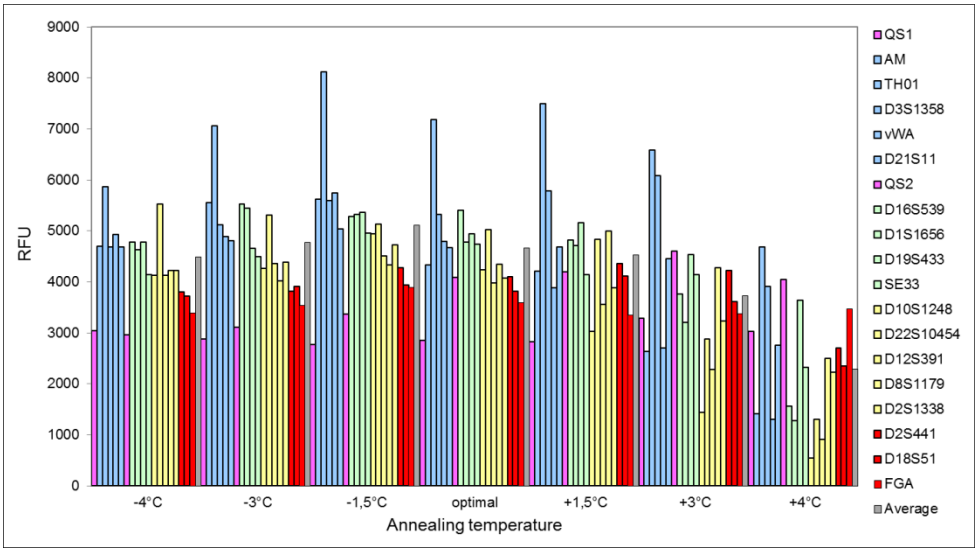


Figure 2. Effect of variations in the PCR annealing temperature. 500 pg control DNA 9948 were amplified on an Eppendorf Mastercycler ep instrument, under standard conditions (30 cycles). Each bar represents an average peak height of triplicates. Bar colors represent the fluorescent dye labels of individual markers, grey bars show the average of all STR markers.

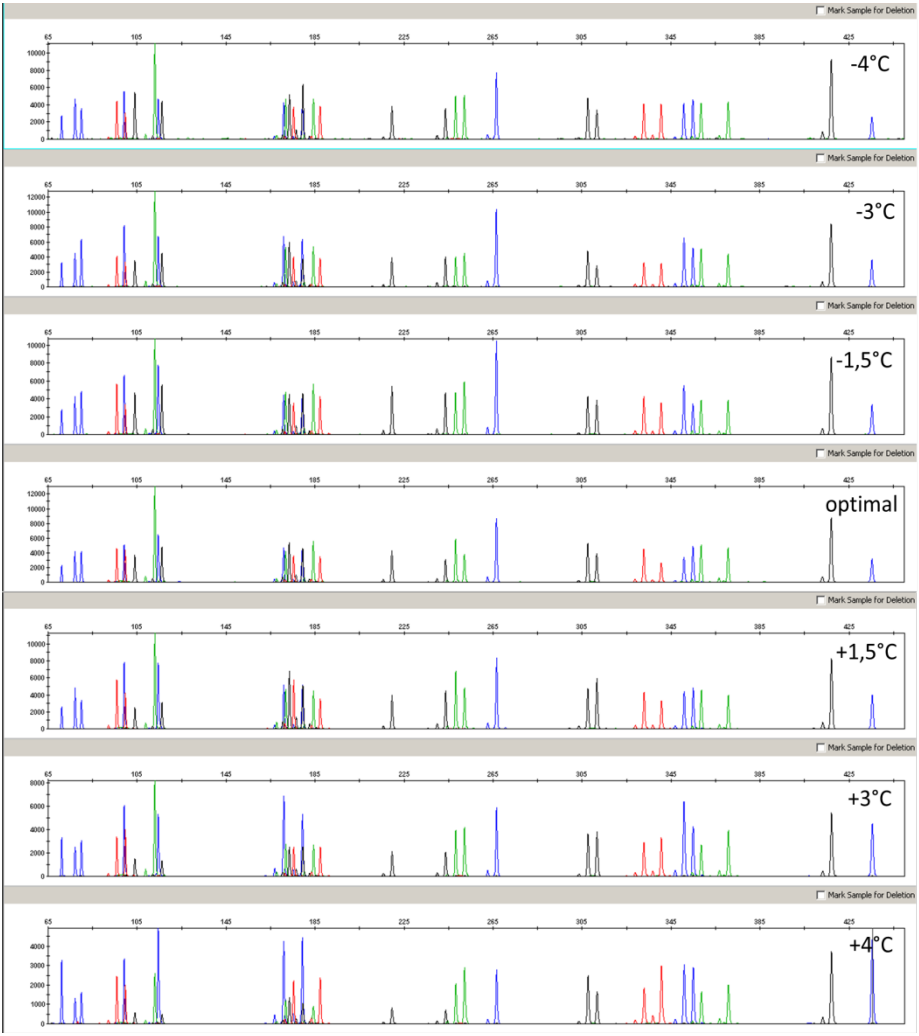


Figure 3. Variations in the PCR annealing temperature. 500 pg control DNA 9948 were amplified on an Eppendorf Mastercycler ep. Representative electropherograms for reactions using annealing temperatures between –4°C and +4°C are shown. Note that the y-axis was scaled for best fit.

Effect of different cycle numbers

PCR cycle numbers can be altered to adapt the reaction conditions to varying DNA template concentrations. Cycle numbers can be either increased to enhance amplification signals when working with low-copy-number DNA, or decreased to speed up the protocol when the DNA sample is abundant (e.g., for database samples).

Cycle numbers were increased to 32 or 34 for reactions containing 32, 16 or 8 pg of control DNA 9948, and the numbers of called alleles, as well as peak heights, were compared to a standard 30-cycle protocol (Figure 4). Here, the cycle numbers of the second cycling block were increased from 27 cycles to 29 or 31, while the first 3 cycles of the standard protocol were not changed. As expected, signal intensities of amplified products increased with higher cycle numbers. However, it should be noted that an increase in overall cycle number to more than 30 will not necessarily result in more information obtained from the low-template-DNA sample. Furthermore, because of stochastic effects, increased peak imbalances or dropouts may in general be observed for low-copy-number samples (with 100 pg or less of template DNA), regardless of any increase in cycle numbers. In this experiment, using a threshold of 50 RFU for allele calling, the number of allelic dropouts due to stochastic effects was not significantly reduced when more PCR cycles were applied (Figure 4). Note that the sensitivity of the capillary electrophoresis instrument, and the setting of the detection threshold, strongly influence the outcome of an assessment of increased cycle numbers. Therefore, such protocol adaptations must be evaluated by individual laboratories using their instrumentation and analysis. Adapting the injection time of the capillary electrophoresis instrument, or using post-PCR purification (e.g., the QIAGEN MinElute® PCR Purification Kit), provide alternative methods for increasing sensitivity.

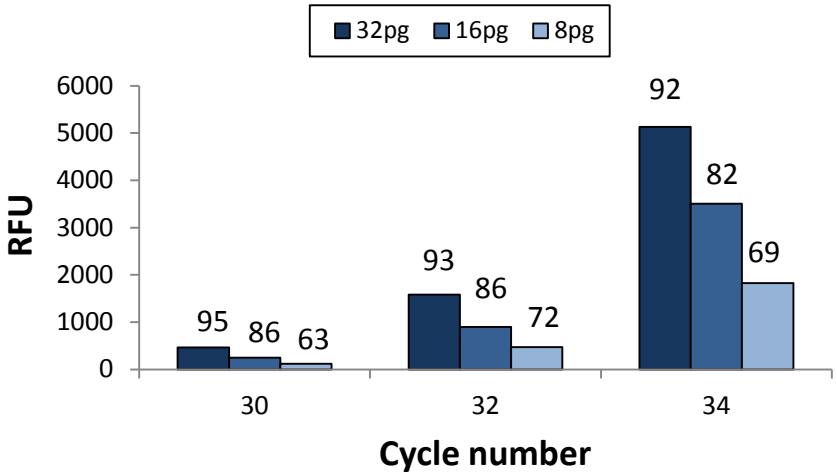


Figure 4. Effect of different cycle numbers on mean peak height and number of called alleles. Different amounts of control DNA 9948 were used as template, as indicated in the figure. Samples were run in triplicates and numbers of detected PCR products (indicated above the bars; 99 PCR products expected) and their peak heights were calculated. 50 RFU was used as a threshold for detection.

In particular, for reference samples such as buccal swabs, where DNA can be extracted in abundance, reduced cycle numbers may be used to streamline the laboratory workflow. Here, cycle numbers were decreased to 24, 26 or 28, for reactions containing 2.5 ng or 10 ng of template DNA (Figure 5). Here, the cycle numbers of the second cycling block were decreased from 27 to 21, 23 or 25, while the first 3 cycles of the standard protocol were not changed. As expected, all reactions resulted in robust amplification and full profiles were obtained using a threshold of 50 RFU for allele calling. However, amplifications with a total of 28 total cycles, using 10 ng template DNA, gave rise to pull up peaks when applying samples to analysis without prior dilution of the PCR product. An example electropherogram for amplification with 24 cycles in total, using 2.5 ng template DNA, is shown in Figure 6, on the following page. Please note, when reducing the cycle number, the peak heights of the Quality Sensor QS1 and QS2 decrease and may drop below the threshold. The template amount of the Quality Sensor is optimized for 30 cycles, thus reducing the cycle number will

reduce the QS signals. Hence, in the case of reduced cycle numbers the QS signals will not give any information about an inhibited PCR or degraded template DNA.

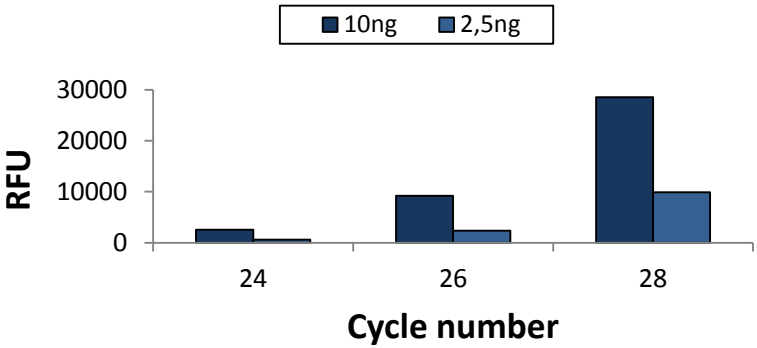


Figure 5. Effect of decreased cycle numbers on mean peak heights. 2.5 ng or 10 ng of control DNA 9948 were subjected to amplification using a total of 24, 26 or 28 PCR cycles. Samples were run in triplicates and average peak heights calculated.

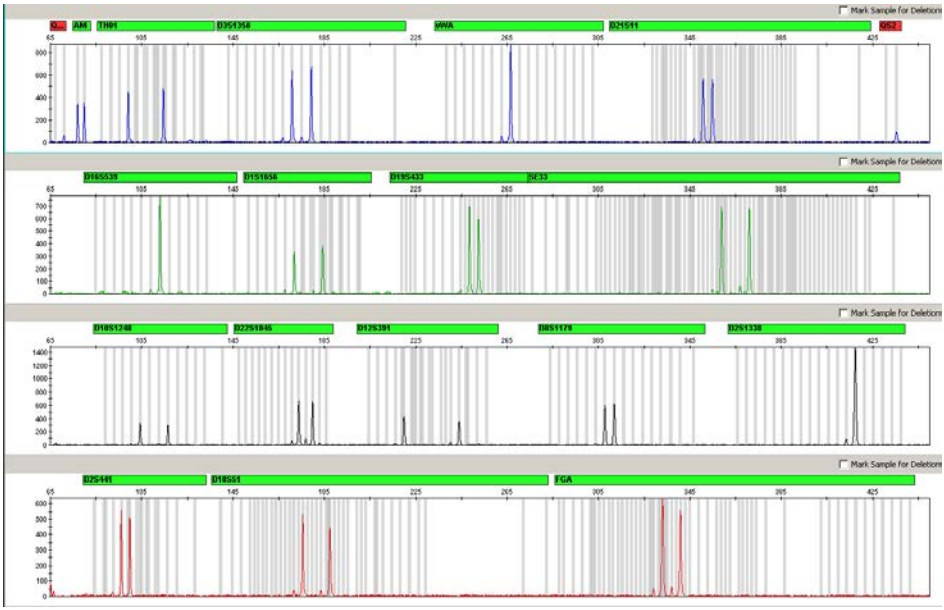


Figure 6. Reduction of PCR cycle numbers with elevated template DNA amounts. Results for amplification of 2.5 ng control DNA 9948, using a total of 24 instead of the standard 30 PCR cycles. The template amount of the Quality Sensor is optimized for 30 cycles, thus reducing the cycle number reduces the QS signals detected.

Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator ESSplex SE QS Kit, to demonstrate kit robustness independent of the instrument (Figure 7). 2 ng to 32 pg of control DNA 9948 were used as a PCR template. The reaction took place under standard conditions (30 cycles) and was performed with the following thermal cyclers.

- 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)
- 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)

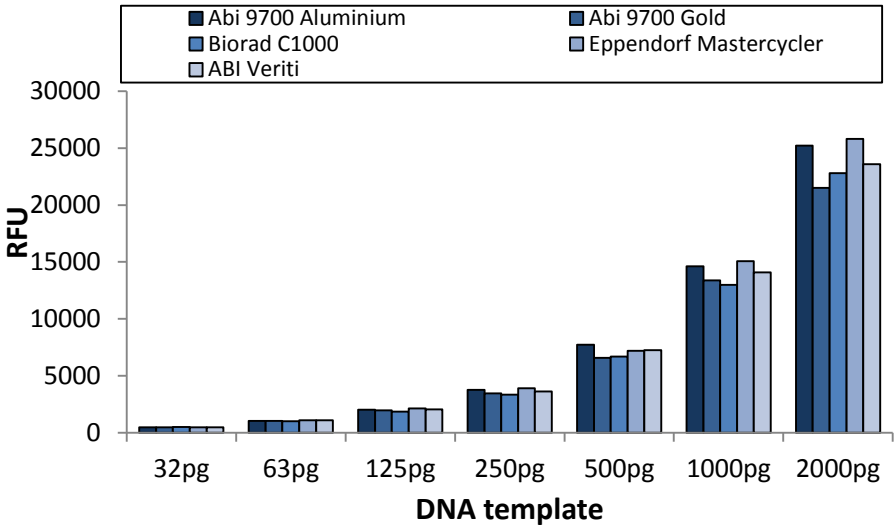


Figure 7. Performance on different PCR thermal cyclers. Different amounts of control DNA 9948 were used as, as indicated in the figure. Each sample was run in duplicate. A standard 30-cycle protocol was used. Average peak heights across all markers are shown.

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 ESSplex SE QS Kit uses a six color setup with fluorescence-labeled primers with the following dyes 6-FAM™, BTG, BTY and BTR 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 ESSplex SE QS Kit Handbook*.

Several Applied Biosystems 3500 Genetic Analyzers and the Applied Biosystems 3130 Genetic Analyzer at different locations were tested, in order to demonstrate the robustness of the Investigator ESSplex SE QS Kit. The data shown in Figure 8 were generated at three different sites using aliquots of the same PCR products. 500 pg of control DNA 9948 and control DNA QHID-1 were each amplified in duplicate. The reaction 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 8). 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.

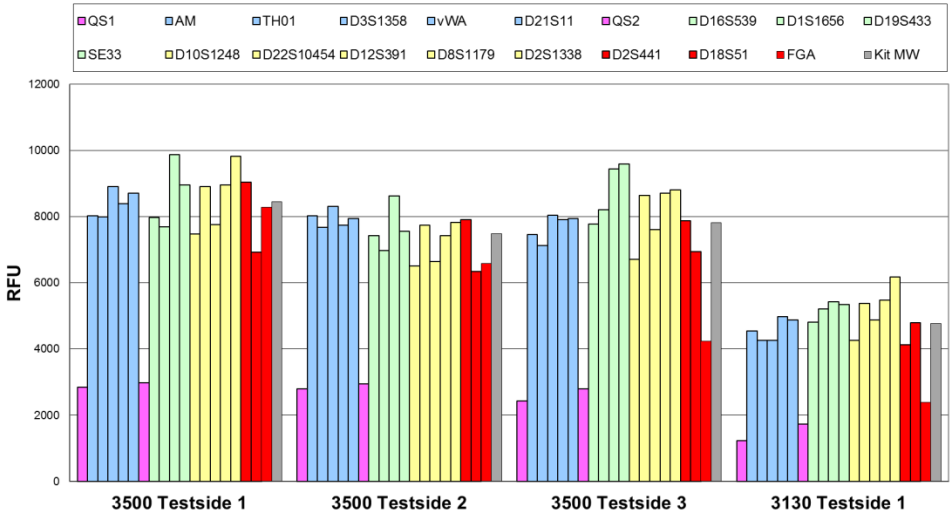


Figure 8. Analysis of PCR products on different Applied Biosystems 3500 Genetic Analyzers. 500 pg of control DNA XY5 and XY13 were amplified on a GeneAmp PCR System 9700, in duplicate. Samples were run according to the *Investigator ESSplex SE QS Kit Handbook*. Aliquots of the same PCR were delivered to three different test sites and analyzed on Applied Biosystems 3500 Genetic Analyzers. Average peak heights for duplicates of both DNAs are shown.

Sensitivity

The Investigator ESSplex SE QS Kit is designed to work robustly over a range of DNA quantities. The recommended amount of input DNA to yield good quality STR profiles is 500 pg, based on real-time PCR quantification of human DNA, for example, using the QIAGEN Investigator Quantiplex™ Kit. In particular, for heavily degraded DNA, the use of increased template amounts may improve results (see page 23).

Control DNA 9948 was serially diluted from 500 pg to 8 pg per reaction. Full profiles (31 PCR products) were consistently obtained at 125 pg, using the standard conditions specified in the *Investigator ESSplex SE QS Kit Handbook*. Occasional allele dropouts were found due to stochastic effects when ≤63 pg DNA were used as template. As expected, the number of dropouts increases with decreasing DNA concentration. See "Effect of different cycle

numbers" and Figure 4 for expected numbers of detected alleles with low-copy-number samples.

Issues with very high amounts of DNA

Although DNA template amounts of >1 ng do not cause issues during PCR amplification, these samples can still result in inaccurate or unusable data. 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 DNA concentration can be improved by re-amplifying a sample using less template DNA.

Issues with very low amounts of DNA

Amplification of less than ~100 pg DNA may lead to incomplete profiles, lacking one or more alleles. Furthermore, low allele copy numbers in the PCR can result in an unbalanced amplification of the alleles due to stochastic fluctuation.

These effects can be handled by either performing multiple amplifications of the same sample to create a consensus profile, or by adding the maximum volume of the DNA template to the PCR, in order to get the best possible result from a single reaction. For correct interpretation of samples having only small amounts of DNA, it is crucial to minimize background fluorescence, for example, caused by dye artifacts. Figure 9 and Figure 10 show an example of a no-template amplification. Peak heights of amplification products and the level of background noise depend on the instruments and settings of individual laboratories. We therefore recommend evaluating a suitable threshold for detection of alleles based on results obtained during an internal validation in the laboratory.

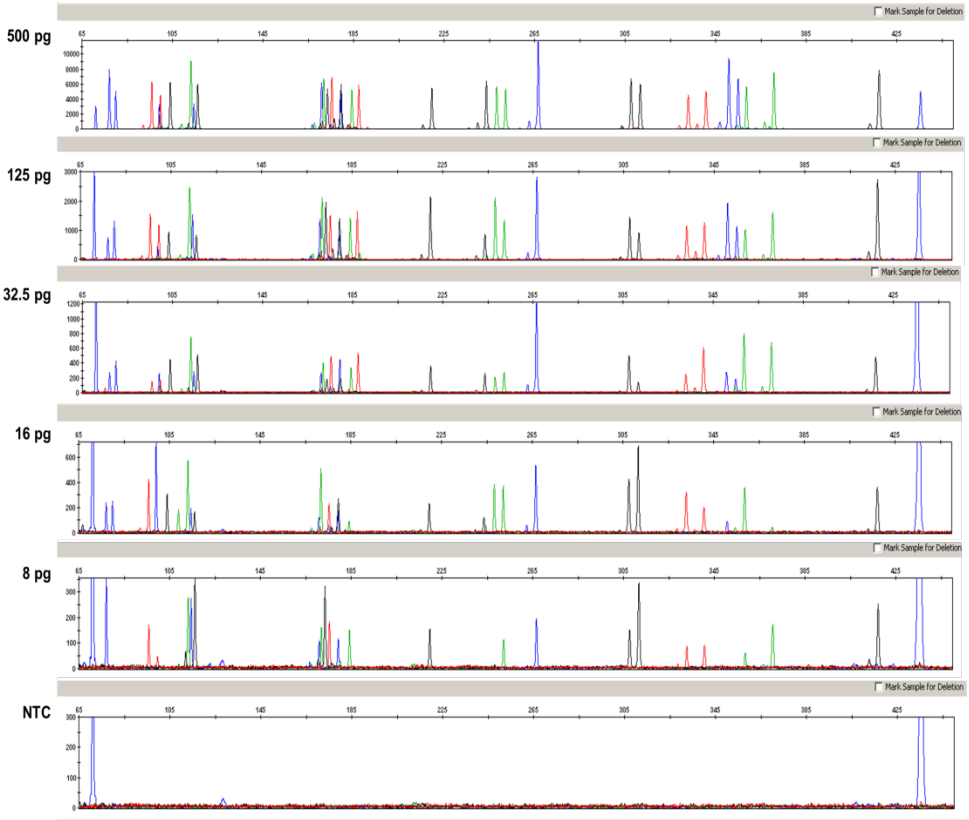


Figure 9. Sensitivity study. Serial dilutions of control DNA 9948 were analyzed. The amounts of DNA indicated were used as template for amplification. The lowest panels show a no-template (negative) control (NTC) to illustrate the expected level of background fluorescence. Y-axis scales were adjusted individually for the highest peak heights of the STR markers. Note: As the Quality Sensor (first and last blue peaks) is amplified with similar amplification efficiency in all experiments independently of the sample template amounts, the QS peak heights are similar in all experiments.

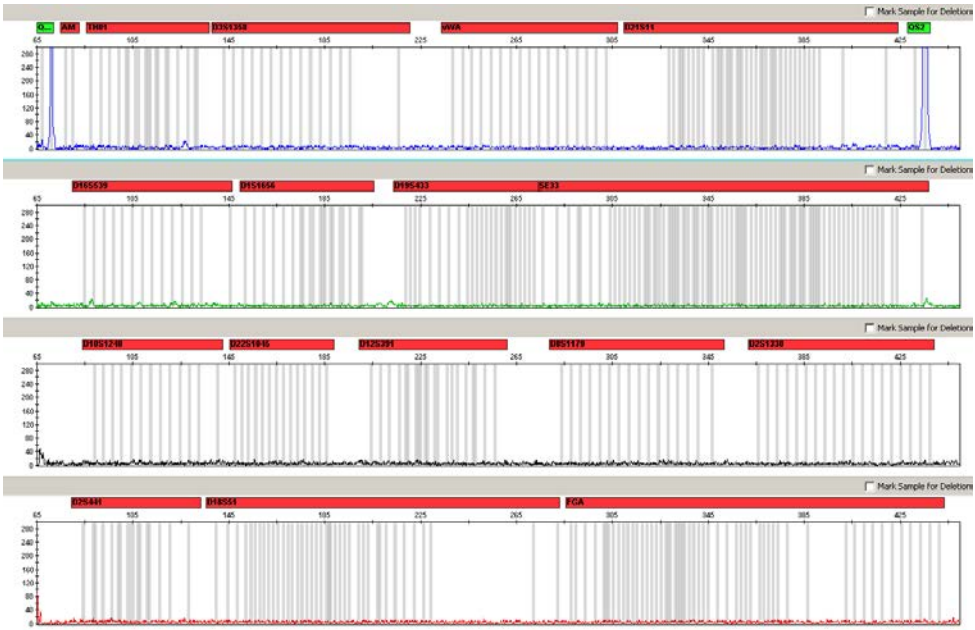


Figure 10. Background fluorescence. Example of a no-template (negative) control to illustrate the expected level of background fluorescence. For correct interpretation of samples having only small amounts of DNA, it is crucial to minimize background fluorescence.

Performance with simulated inhibition

If the DNA extraction from forensic casework samples is done using inappropriate methods, Investigator ESSplex SE QS Kit performance may be compromised, although the reaction chemistry has been optimized for increased inhibitor tolerance. QIAGEN sample preparation technology is recommended for extraction, as it yields pure DNA free from inhibitors.

To test the robustness of the kit in the presence of inhibitors, assays were run in the presence of the following inhibitors, chosen to mimic challenging forensic sample types:

- Humic acid: A principal component of humic substances that has an inhibitory effect on PCR. It may be co-extracted from forensic samples collected from soil.
- Hematin: Formed by the oxidation of heme, the main component of blood. It has been identified as a PCR inhibitor in DNA samples extracted from bloodstains.
- Tannic acid: Typically present in leather. During extraction of casework evidence, tannic acid may be co-extracted with DNA.
- Indigo carmine: The color of blue denim (e.g., jeans).
- Collagen: The main protein compound of many tissues.
- Calcium: Released during lysis of bones.
- Ethanol: Potential carryover of the DNA extraction method.

Results are shown in Figures 11–17. Figure 18 shows an overview of Investigator ESSplex SE QS Kit inhibitor resistance.

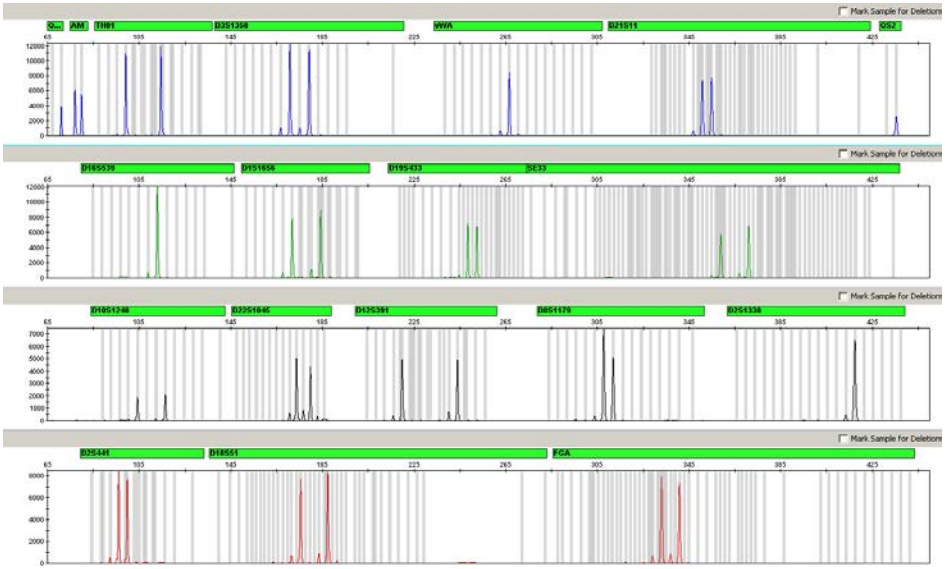


Figure 11. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 500 µM hematin.

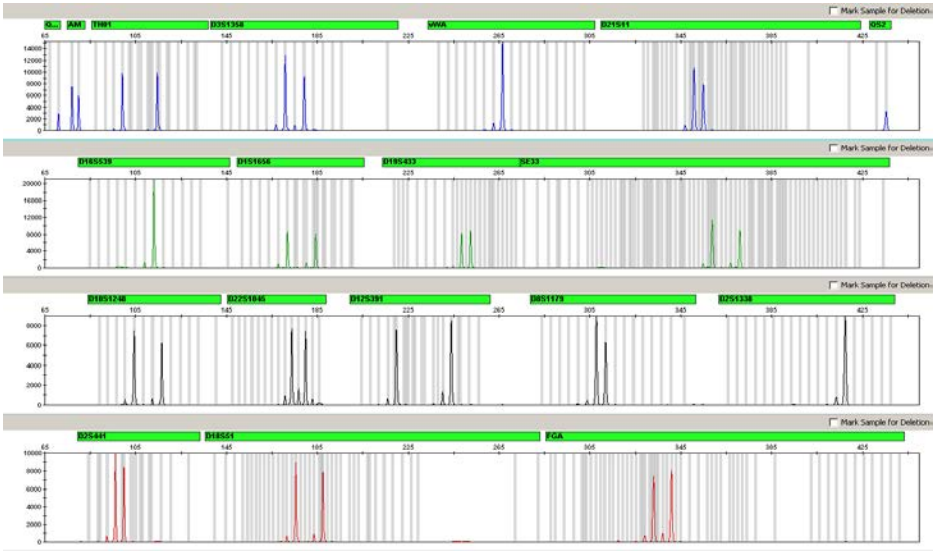


Figure 12. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 100 ng/µl humic acid.

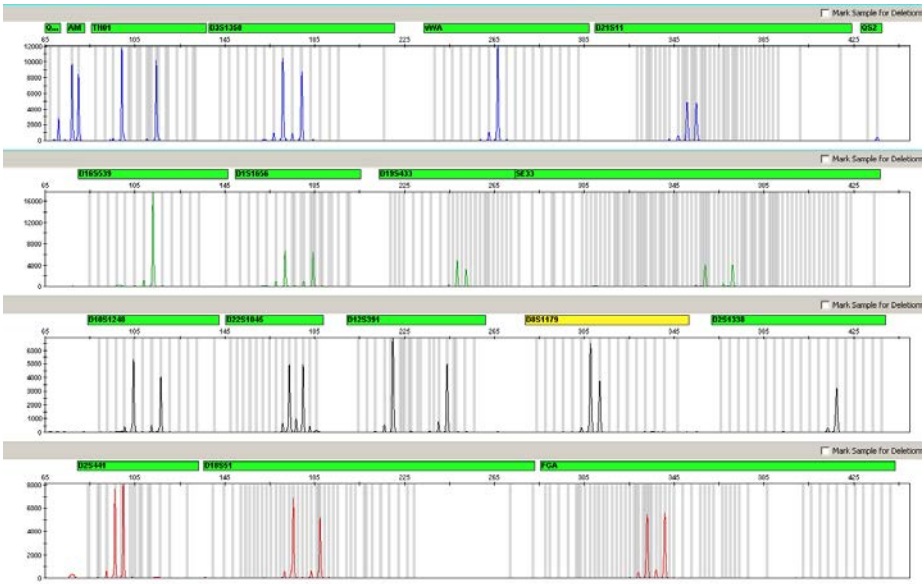


Figure 13. Electropherogram of 500 pg Control DNA 9948 amplified in the presence of 6 mM indigo carmine.

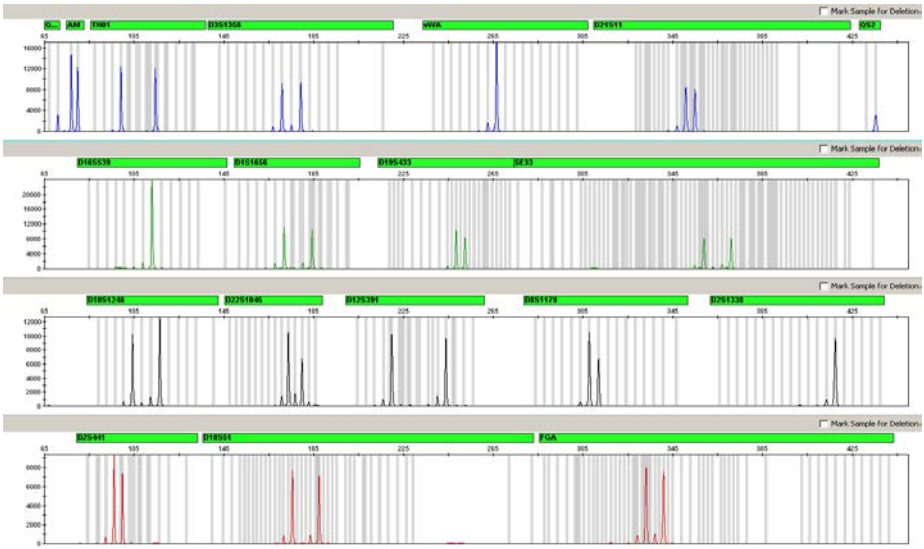


Figure 14. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 150 ng/µl collagen.

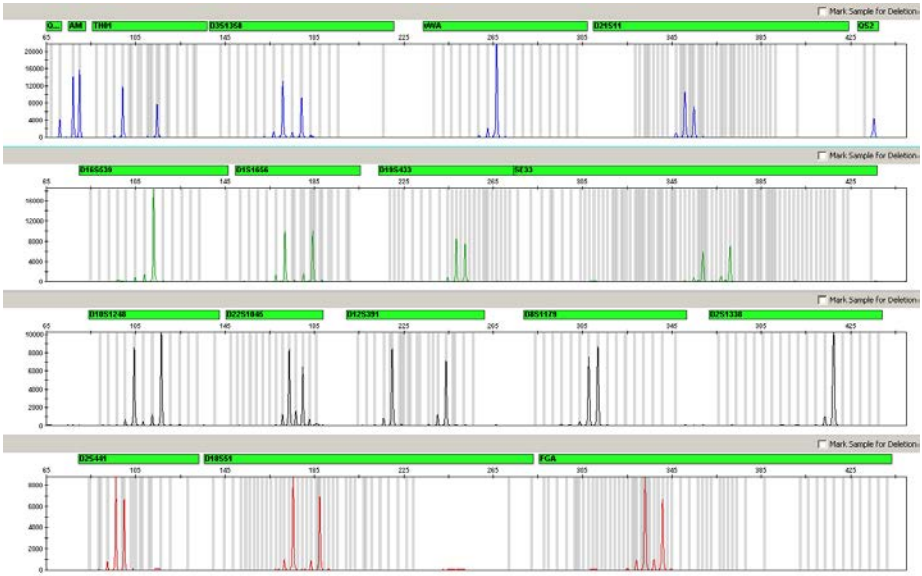


Figure 15. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 2.5 mM calcium.

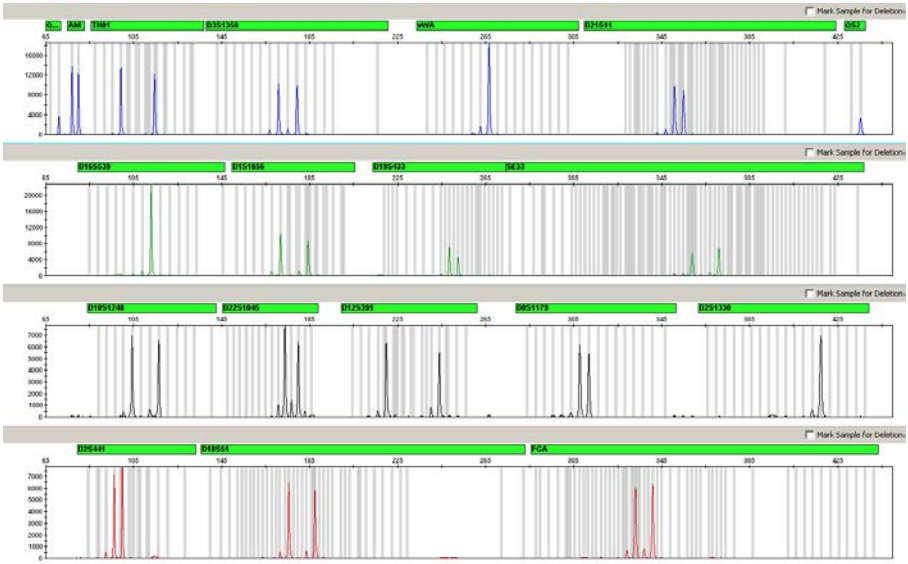


Figure 16. Electropherogram of 500 pg Control DNA 9948 amplified in the presence of 2000 ng/µl tannic acid.

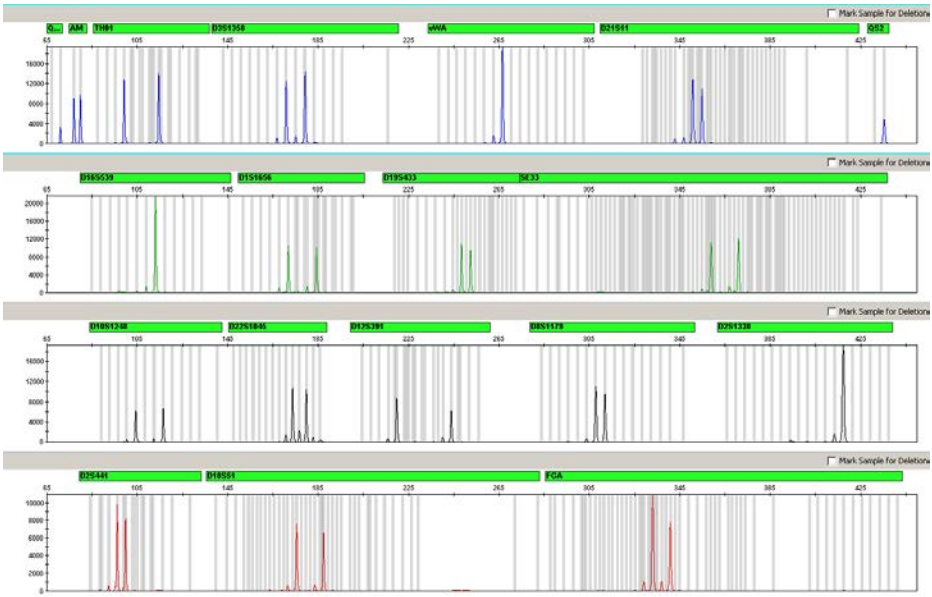


Figure 17. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 0.5% ethanol.

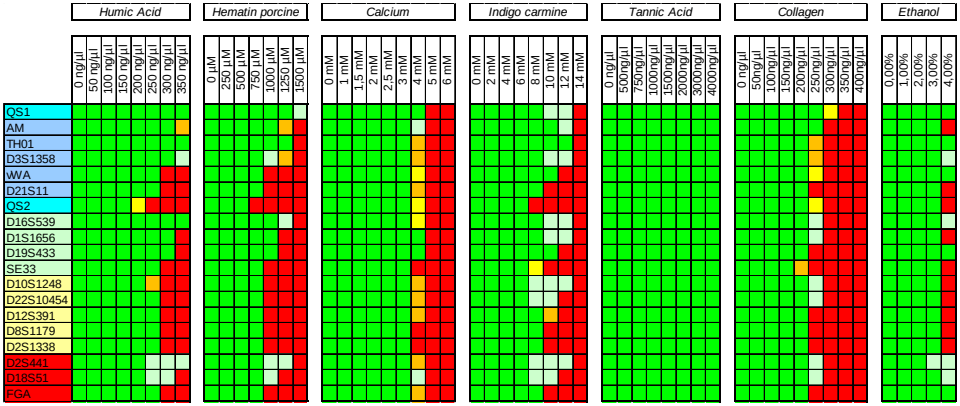


Figure 18. Overview of Investigator ESSplex SE QS inhibitor resistance. The assay was tested for its robustness towards inhibitors (humic acid, hematin, tannic acid, indigo carmine, calcium, collagen and ethanol). 500 pg of control DNA 9948 was used as template and PCR was performed under standard conditions. 50 RFU was used as a threshold for allele calling. Green: Consistently full profile. Yellow: 75% of expected PCR products detected. Orange: 50% of expected PCR products detected. Red: Less than 50% of expected PCR products detected. Light Green: Consistently full profile with split peaks.

Full profiles lacking any PCR artifacts, e.g., split peaks, were obtained over a wide range of inhibitor concentrations. For most inhibitors (e.g., humic acid and hematin), the degree of peak height reduction is correlated to the size of the PCR product and dropouts are first observed for the markers having the largest amplicons. The Quality Sensor QS2 is in general more sensitive to inhibitors, and dropouts of QS2 are observed before the STR markers are affected (compare, for example, the indigo carmine analysis).

Collagen shows a different mode of interference with PCR amplification by affecting the ability of the polymerase to adenylate the final PCR product. As a consequence, the presence of –A products leads to split peaks that are visible in electropherograms, while the overall amount of amplification product is not significantly reduced (Figure 19). This effect is sequence dependent and therefore markers are affected differently.

Note that the highest inhibitor concentrations used in this study will be found only rarely in casework samples, even if inappropriate sample extraction methods have been applied.

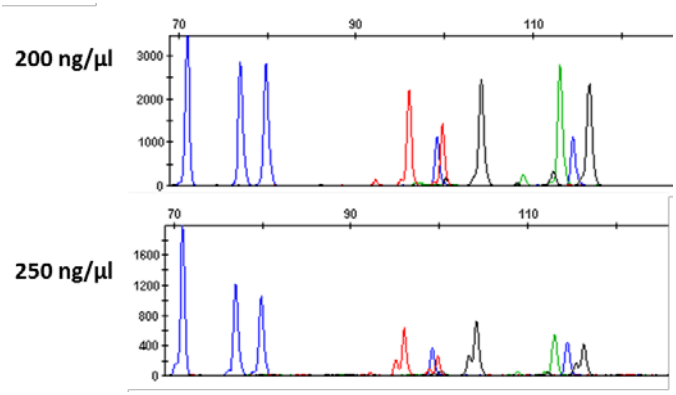


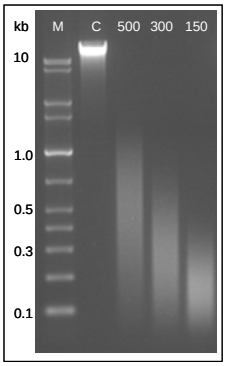
Figure 19. Effect of high levels of collagen. 500 pg of control DNA 9948 was amplified in the presence of 200 ng or 250 ng/µl collagen. Arrows indicate PCR products showing split peaks caused by partial adenylation.

Stability with degraded DNA

Casework evidence has often been exposed to adverse environmental conditions, for example, UV light, humidity or microbial growth. Human DNA extracted from these samples may therefore show varying degrees of degradation.

Artificially degraded male genomic DNA was used for validation. DNA was sheared using Adaptive Focused Acoustics™ (Covaris system, Covaris Inc., Woburn, MA, USA) to average fragment lengths of 500 bp, 300 bp or 150 bp according to the manufacturer's instructions. Fragment lengths were verified on an agarose gel and 500 pg DNA were used as template for amplification (all samples in duplicate) (Figure 20A). Full profiles were obtained with DNA of 300 bp and 500 bp average length. As expected, loci with larger amplicon sizes become affected as DNA degradation progresses. At an average fragment length of 150 bp, 65 out of 99 expected peaks (66%) were detected using a threshold of 50 RFU (Figure 20B). Increased amounts of template (up to 2 ng), can be used to improve results for heavily degraded DNA.

A



B

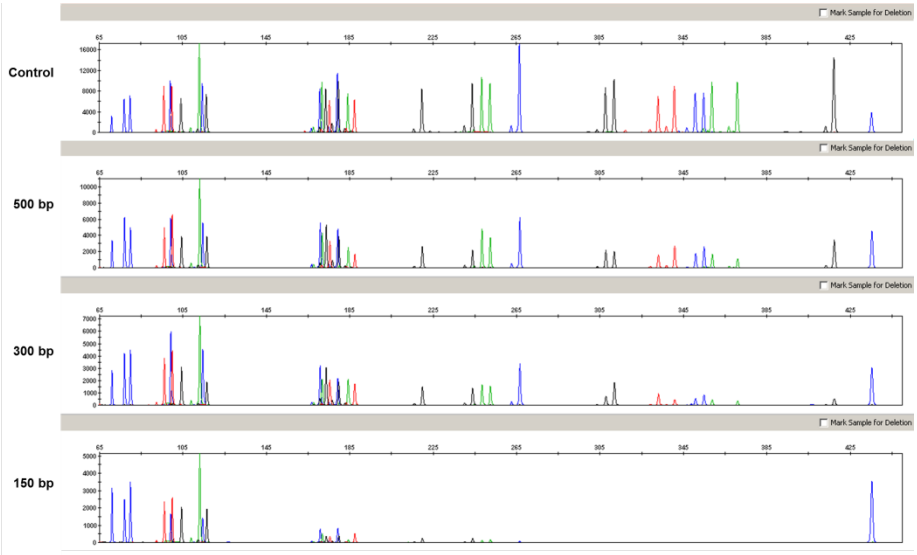


Figure 20. Degraded DNA. Male DNA was sheared to the defined average fragment length indicated. **A.** Agarose gel analysis of degraded DNA. **M**: DNA size standard markers, **C**: Untreated DNA. 500, 300, 150: average fragment lengths in base pairs. **B.** STR analysis of untreated control and degraded DNA samples. 500 pg template DNA was used. Full profiles were obtained down to 300 bp average fragment length. Note: The Quality Sensor is not affected by the degraded DNA and was amplified with equal peak heights.

Species specificity

Non-human DNA can be present in forensic casework samples. It is critical that assays show no cross-reactivity between species. To verify Investigator ESSplex SE QS Kit species specificity for human DNA, DNA from other species was tested following the standard assay protocol (Figure 21, on the following page).

Besides common pets and farm animals, some primates were also tested. As expected for primates, amplification of some products is possible. Chimpanzese, bonobos, orangutans and gorillas give 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, one allele call for D10S12486 allele 13 and further off-ladder peaks in the FAM, BTG and BTR panel. Pig DNA gives one peak in the green channel at 305 bp at about 50 RFU. Goat DNA gives one peak in the green channel at 250bp at about 50 RFU.

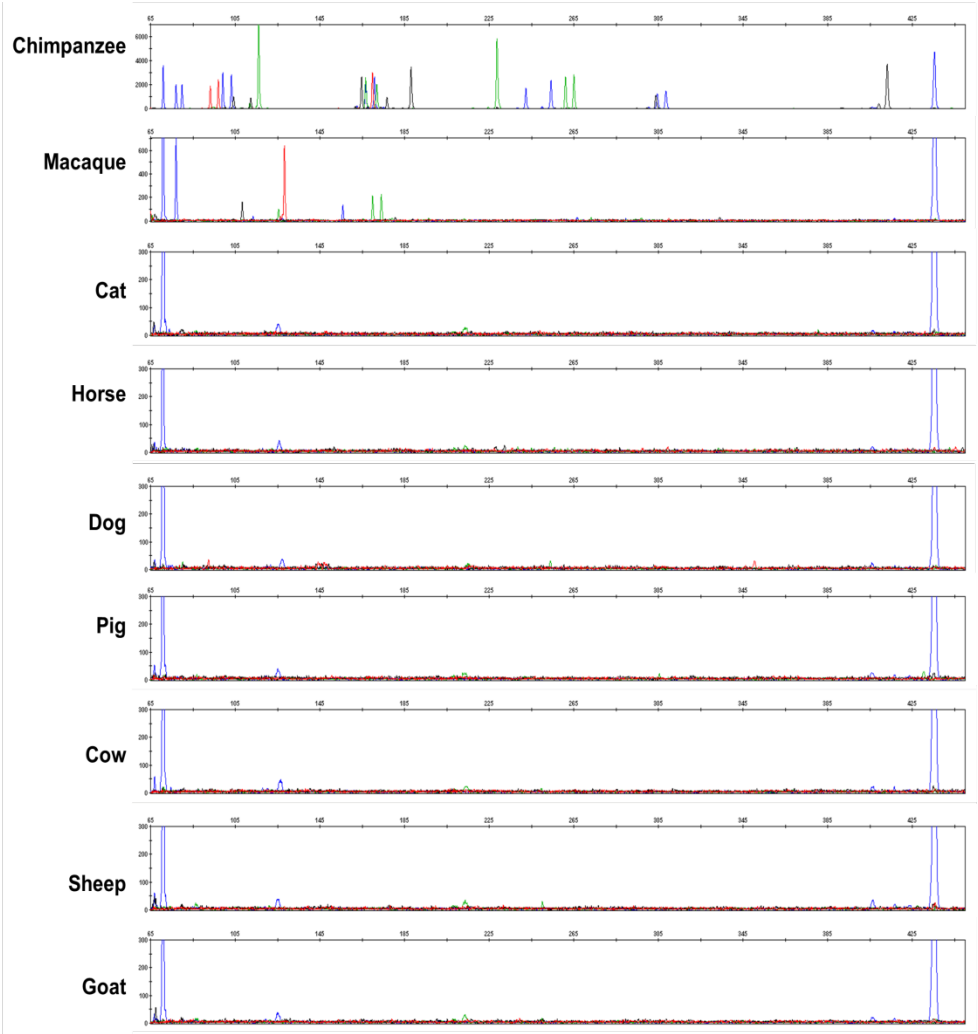


Figure 21. Representative results of the species specificity assessment. 500 pg primate DNA and 2.5 ng DNA from all other species was used as template.

Mixture studies

Evidence samples are frequently composed of more than one individual's DNA. For correct interpretation of results from mixtures, it is important to know the limit of the minor contributing component that still can be resolved.

Samples were created by mixing control DNA 9948 and XX107 in ratios of 1:1, 3:1, 7:1, 10:1, 15:1 and vice versa (see Table 2 for genotypes of mixed samples). The total amount of mixed DNA used in this study was 500 pg; a 15:1 mixture thus contains 31 pg of the minor component DNA and 469 pg of the major component (Table 1). The limit of detection of the minor component was determined by analyzing non-overlapping alleles of both DNAs. All expected alleles were found for minor components of 3:1 and 7:1 mixtures. 10:1 and 15:1 typically resulted in partial profiles of the minor component. An example for 1:10 mixtures is shown in Figure 22. Since these contain ≤50 pg of the minor component, the results are in concordance with the sensitivity for single-source samples reported here. In order to increase the sensitivity for the minor component, higher overall DNA amounts may be used if the amount of available DNA is not limited. See "Sensitivity", page 13, for general considerations.

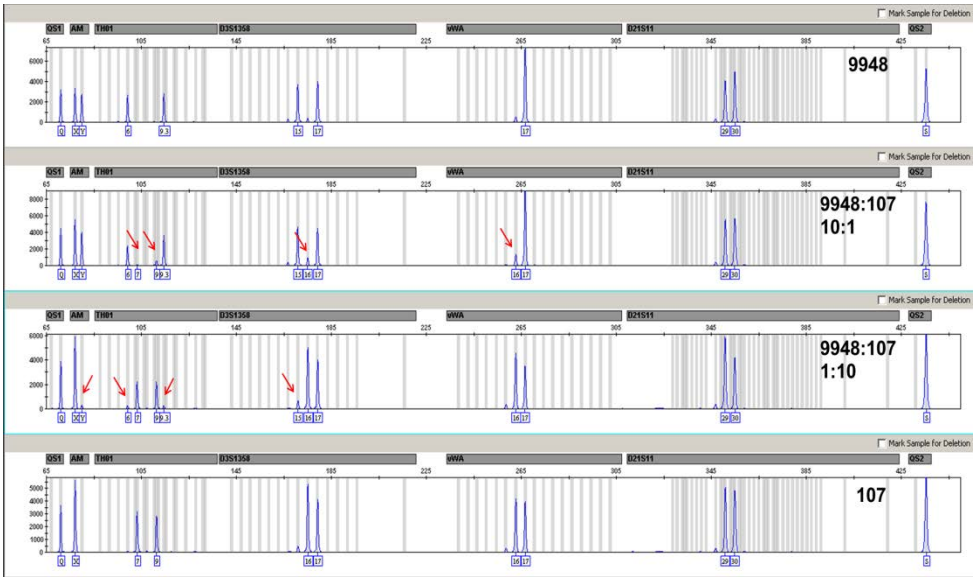
Table 1. Amounts of DNA template in mixtures

Mixture ratio	Major component	Minor component
1:1	250 pg	250 pg
3:1	375 pg	125 pg
7:1	429 pg	71 pg
10:1	450 pg	50 pg
15:1	467 pg	33 pg

Table 2. Genotypes of DNA used for mixed samples

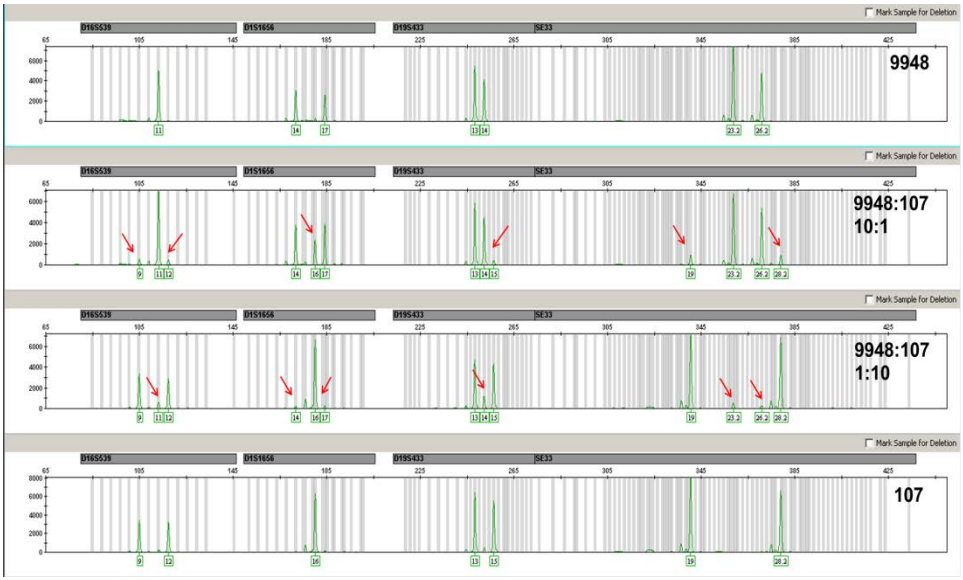
Locus	9948 genotype	107 genotype
Amelogenin	X/Y	X/X
D1S1656	14/17	16/16
D2S441	11/12	14/14
D2S1338	23/23	19/21
D3S1358	15/17	16/17
D8S1179	12/13	12/13
D10S1248	12/15	13/16
D12S391	18/24	21/23
D16S539	11/11	9/12
D18S51	15/18	19/21
D19S433	13/14	13/15
D21S11	29/30	29/30
D22S1045	16/18	14/14
FGA	24/26	21/22
SE33	23.2/26.2	19/28.2
THO1	6/9.3	7/9
vWA	17/17	16/17

A



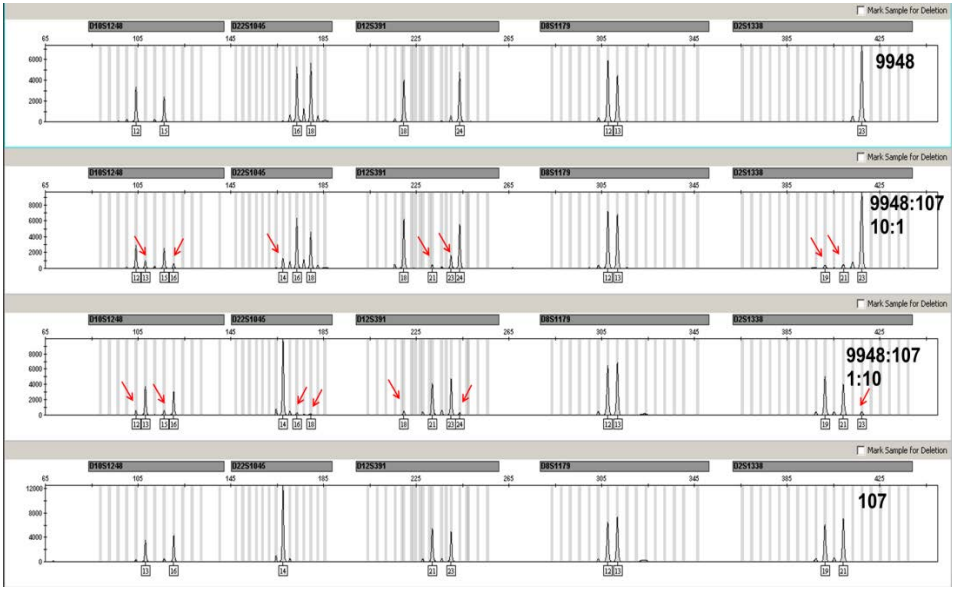
A. Expected bp positions of uncovered minor component peaks are indicated by red arrows. Amelogenin: Heterozygote + homozygote, two overlapping alleles, the non-overlapping minor component peak can be assigned. TH01: Heterozygote + heterozygote, no overlapping alleles, three minor component peaks can be assigned, one minor component peak is overlapping with stutter position. D3S1358: Heterozygote + heterozygote, two overlapping alleles, two minor component peaks are overlapping with stutter positions. vWA: Homozygote + heterozygote, two overlapping alleles, one minor component peak is overlapping with stutter position. D21S11: Heterozygote + heterozygote, four overlapping alleles, major component completely covers minor component.

B



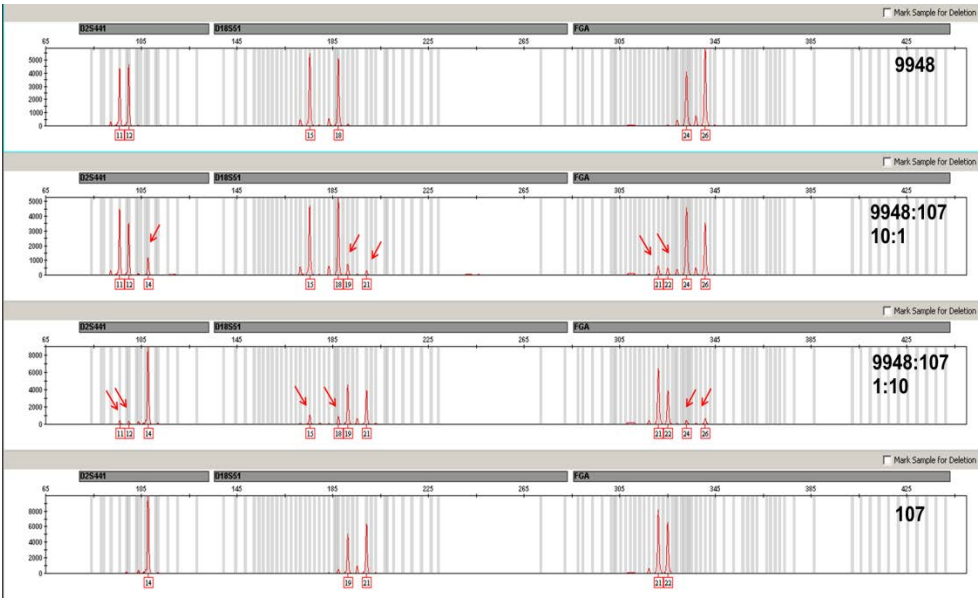
B. Expected bp positions of uncovered minor component peaks are indicated by red arrows. TPOX: Heterozygote + heterozygote, four overlapping alleles, major component completely covers minor component. DYS391: Homozygote + Null-Allele, the non-overlapping minor component peak can be assigned. D1S1656: Heterozygote + homozygote, no overlapping alleles, one minor component peak is overlapping with stutter position, two non-overlapping minor component peaks can be assigned. D12S391: Heterozygote + heterozygote, no overlapping alleles, three minor component peaks can be assigned, one minor component peak is overlapping with stutter position. SE33: Heterozygote + heterozygote, no overlapping alleles, all non-overlapping minor component peaks can be assigned.

C



C. Expected bp positions of uncovered minor component peaks are indicated by red arrows. D10S1248: Heterozygote + heterozygote, no overlapping alleles, two non-overlapping minor component peaks can be assigned, two minor component peaks are overlapping with stutter position. D22S1045: Heterozygote + homozygote, no overlapping alleles, all non-overlapping minor component peaks can be assigned. D19S433: Heterozygote + heterozygote, two overlapping alleles, one minor component peak is overlapping with stutter position, one non-overlapping minor component peak can be assigned. D8S1179: Heterozygote + heterozygote, 4 overlapping alleles, major component completely covers minor component. D2S1338: Homozygote + heterozygote, no overlapping allele, the three non-overlapping minor component peaks can be assigned.

D



D. Expected bp positions of uncovered minor component peaks are indicated by red arrows. D2S441: Heterozygote + homozygote, no overlapping allele, the three non-overlapping minor component peaks can be assigned. D18S51: Heterozygote + heterozygote, no overlapping allele, three minor component peaks can be assigned, one minor component peak is overlapping with stutter position. FGA: Heterozygote + heterozygote, no overlapping alleles, all minor component peaks can be assigned.

Figure 22. Results of the mixture analysis. Figures A–D show 1:10 mixtures of control DNA 9948 and XX107 (middle panels of each figure) and the corresponding single source samples as a reference (upper and lower panel of each figure). Red arrows indicate positions where a peak of the minor component DNA that does not overlap with a main peak, is to be expected. A brief description of the mixture situation given for each individual marker is given below the figures.

Reproducibility: Concordance test

The majority of primer sequences of the Investigator ESSplex SE QS Kit is highly conserved and is adopted from previously released QIAGEN kits, like the Investigator ESSplex SE Plus Kit and the 24plex QS Kit. These primers showed a high level of concordance in corresponding concordance studies of the National Institute of Standards and Technology (NIST), and furthermore showed reliable results at various laboratory sites to date. To demonstrate the concordance of the Investigator ESSplex SE QS Kit the NIST institute compared the results of the new kit to the NIST final data set with 660 unrelated individuals (NIST U.S. population set [654 samples] and SRM 2391c [6 samples]). A full concordance was assessed with NIST SRM 2391c certified values. Out of the total data set no null alleles were observed. One Peak High Ration imbalance result for Th01 (1 sample out of 660 samples) was detected. Out of 22,440 alleles compared, no discordant call was observed, given a 0.00% discordance.

In conclusion, the concordance between the Investigator ESSplex SE QS Kit and the NIST final data set is accounted to 100%.

Peak height ratios of heterozygous markers (Figure 23) and stutter peak heights (Figure 24) were analyzed for the NIST data set with 660 unrelated individuals.

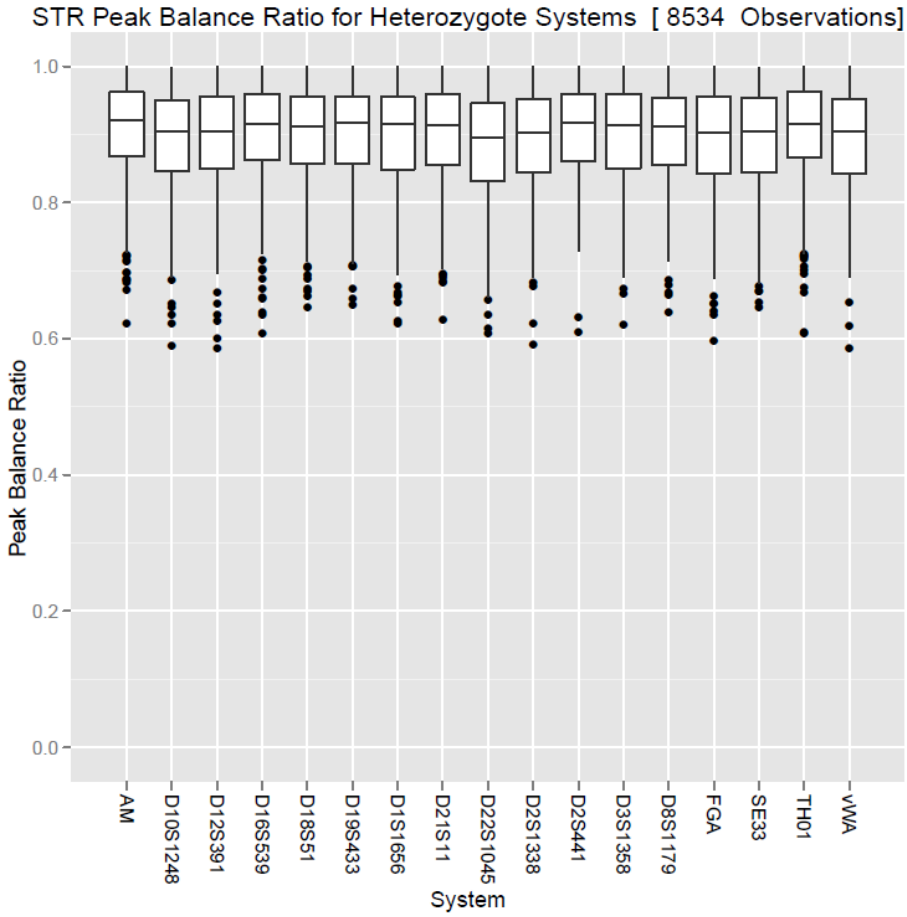


Figure 23. Box plot analysis of intra-locus peak height ratios. Values refer to the lower of the 2 peaks in a heterozygous sample. Boxes represent the middle 50% (interquartile range, [IQR]) of data, lines inside the boxes the median. Whiskers show data within 1.5 IQR, dots represent outliers.

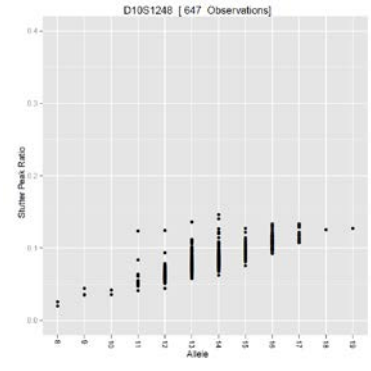
Table 3. Heterozygote peak height ratios. Observed intralocus balance for 660 direct PCR samples from different donors.

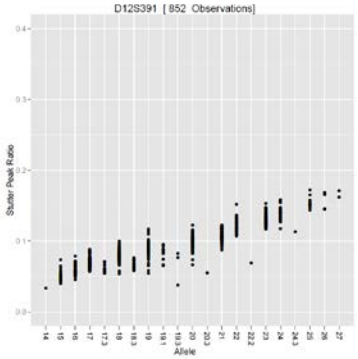
System	Observations	Mean (%)	Min (%)	Max (%)
AM	628	88.9	62.3	100.0
D10S1248	499	88.9	58.9	99.8
D12S391	503	89.4	58.6	100.0
D16S539	545	90.2	60.7	100.0
D18S51	457	89.9	64.7	100.0
D19S433	567	90.1	65.0	100.0
D1S1656	552	89.8	62.3	100.0
D21S11	440	90.0	62.7	100.0
D22S1045	501	88.3	60.7	100.0
D2S1338	489	89.0	59.1	100.0
D2S441	443	90.3	60.9	100.0
D3S1358	464	89.8	62.1	99.9
D8S1179	510	89.7	63.9	100.0
FGA	467	89.1	59.6	99.9
SE33	489	89.3	64.6	99.8
TH01	529	90.5	60.7	100.0
vWA	499	89.2	58.5	99.9

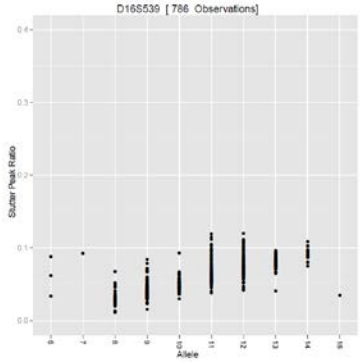
In addition, stutter peak heights were analyzed for all 660 PCR samples. Stutter peak heights are characteristic for each marker and the number of repeat motifs of an allele (Table 4). TH01 in general shows the lowest stutter ratio of all STR markers of the Investigator ESSplex SE QS Kit. Smaller alleles display lower stutter levels than longer alleles of the same marker (Figure 24). 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.

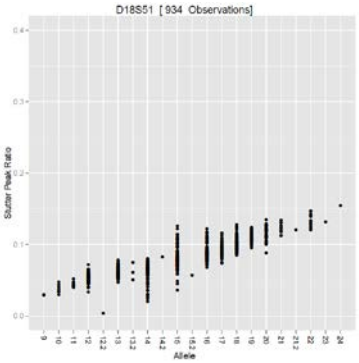
Table 4. Peak height ratios of forward stutters. Peak heights of forward stutters compared to main peaks were analyzed for 660 DNA samples from different donors.

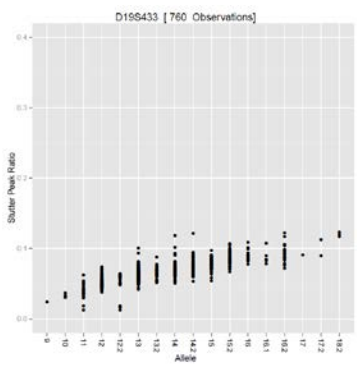
System	Observations	Stutter Mean (%)	Stutter Min (%)	Stutter Max (%)
D10S1248	647	8.3	2.0	14.6
D12S391	852	8.9	3.4	17.2
D16S539	786	6.0	1.1	12.0
D18S51	934	8.1	0.4	15.5
D19S433	760	6.6	1.3	12.3
D1S1656	968	8.7	2.1	17.7
D21S11	944	7.1	2.2	13.8
D22S1045	746	8.0	0.9	18.7
D2S1338	896	7.9	1.5	13.5
D2S441	875	4.9	0.1	12.4
D3S1358	691	8.5	1.5	15.0
D8S1179	759	6.7	2.4	12.5
FGA	840	7.8	3.1	13.0
SE33	976	9.2	2.0	15.0
TH01	845	2.0	0.6	6.6
vWA	787	6.7	0.5	12.9

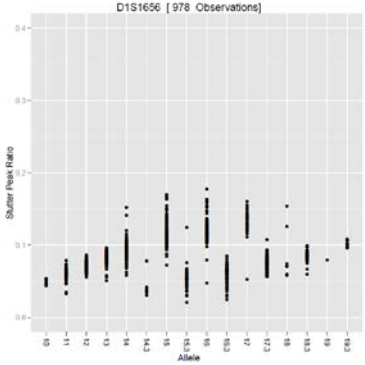

D10S1248 [647 Observations]
Stutter Peak Ratio
Allele

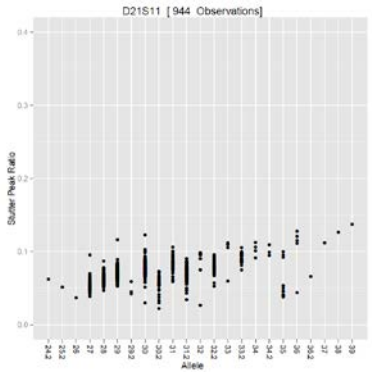

D12S391 [852 Observations]
Stutter Peak Ratio
Allele

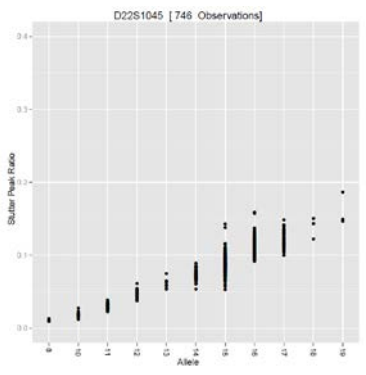

D16S539 [786 Observations]
Stutter Peak Ratio
Allele

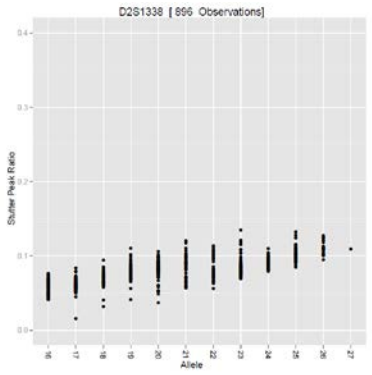

D18S51 [934 Observations]
Stutter Peak Ratio
Allele

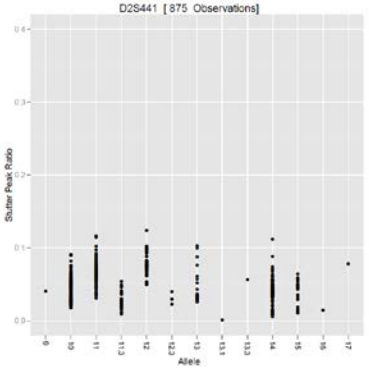

D19S433 [760 Observations]
Stutter Peak Ratio
Allele

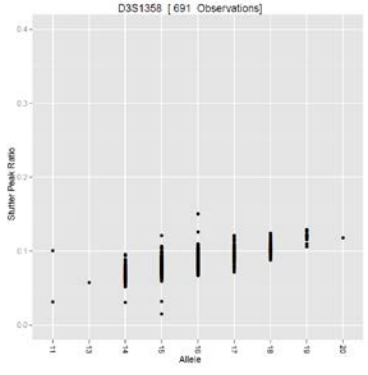

D1S1656 [978 Observations]
Stutter Peak Ratio
Allele

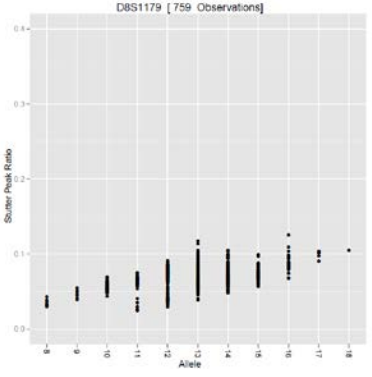

D21S11 [944 Observations]
Stutter Peak Ratio
Allele


D22S1045 [746 Observations]
Stutter Peak Ratio
Allele


D2S1338 [896 Observations]
Stutter Peak Ratio
Allele


D2S441 [875 Observations]
Stutter Peak Ratio
Allele


D3S1358 [691 Observations]
Stutter Peak Ratio
Allele


D8S1179 [759 Observations]
Stutter Peak Ratio
Allele

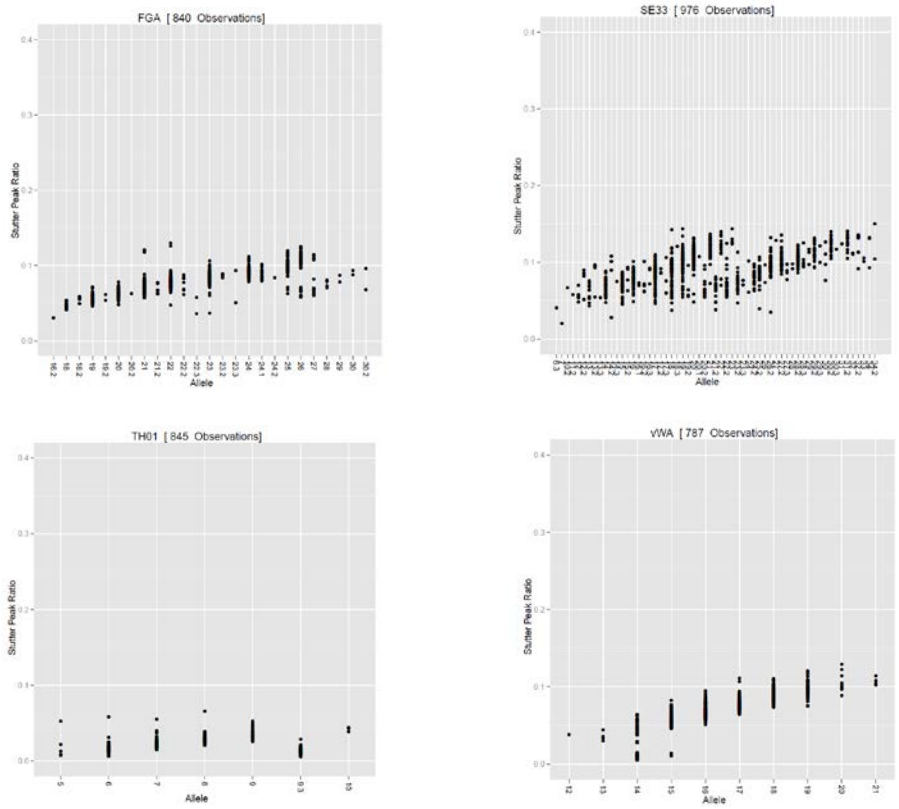


Figure 24. Stutter peak height ratios. Peak heights of forward stutters compared to main peaks were analyzed for 60 DNA samples from different donors.

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 of backward stutters. Peak heights of backward stutters compared to main peaks were analyzed for 660 samples from different donors.

System	Observations	Stutter Mean (%)	Stutter Min (%)	Stutter Max (%)
D10S1248	310	0.6	0.2	2.4
D12S391	301	0.7	0.1	2.4
D16S539	655	1.3	0.3	5.3
D18S51	644	1.3	0.4	4.9
D19S433	107	1.1	0.1	4.4
D1S1656	789	1.6	0.3	7.4
D21S11	788	1.2	0.2	4.0
D22S1045	730	5.2	0.3	11.6
D2S1338	74	1.1	0.1	3.5
D2S441	522	1.3	0.2	4.1
D3S1358	436	1.2	0.3	5.7
D8S1179	563	1.1	0.3	3.6
FGA	574	1.0	0.3	3.8
SE33	625	1.3	0.3	4.9
TH01	67	0.4	0.1	1.8
vWA	358	0.7	0.1	5.2

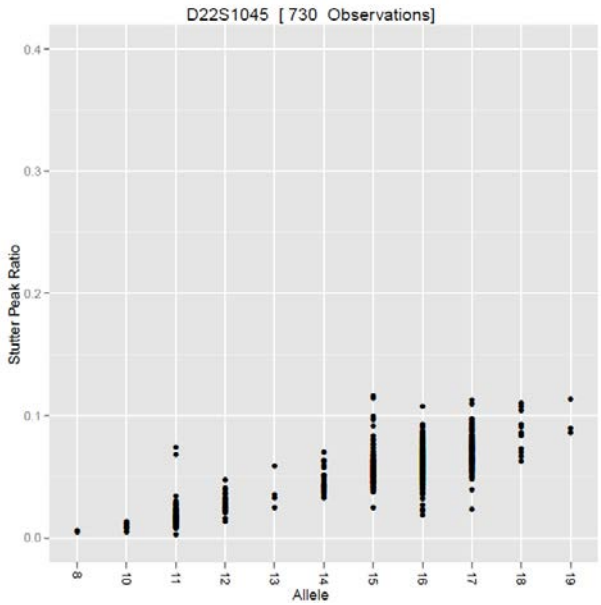


Figure 25. Peak height ratios of backward stutters of marker D22S1045. Peak heights of backward stutters compared to main peaks were analyzed.

Stability – Improved hot-start function

Usually PCR runs were setup in a straightforward manner, without any interruptions between the PCR pipetting step and the start of the thermocycler run. When using an instrument for 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 ESSplex SE QS Kit contains an improved hot-start function. Based on a novel, antibody-mediated hot-start mechanism, the kit affords enhanced specificity and efficiency of the multiplex PCR reaction (Figure 26). The added convenience of extreme stability for up to 16 hours at room temperature, without a need for any cooling, makes it ideal for handling of high-throughput samples and automated workflows (Figure 27).

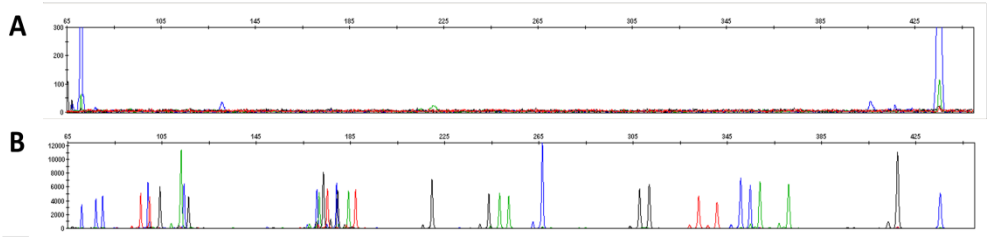


Figure 26. Reaction stability for 6 hours incubation at room temperature. PCR samples were incubated at 20°C for 6 hours in between the PCR pipetting step and the start of the thermocycler. **A.** Negative control. **B.** Results of 500 pg DNA 9948 as PCR template.

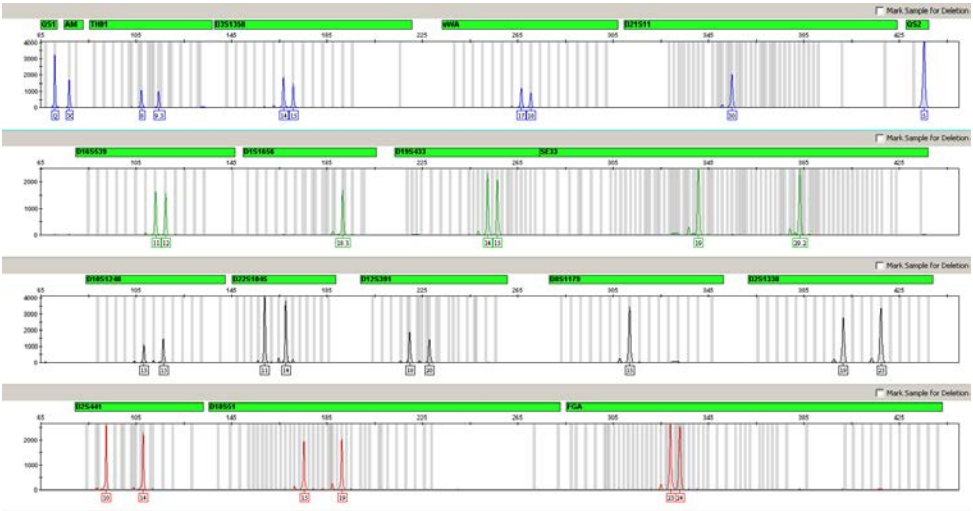


Figure 27. Reaction stability for 16 hours incubation at room temperature. PCR samples were incubated at 20°C for 16 hours in between the PCR pipetting step and the start of the thermocycler. 500 pg DNA 9948 were used as template.

As demonstrated, after 6 or even 16 hours of storing the PCR Master Mix at room temperature, before the PCR cycle, the overall kit performance was not compromised; no unspecific PCR products were amplified.

Stability – 20x 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 ESSplex SE QS Kit components were tested to prove that they would yield stable results after multiple rounds of freezing and thawing. Regardless of these results, we do not recommend repeated freezing and thawing of the kit contents.

Figure 28 shows the electropherograms obtained by amplifying 500 pg control DNA 9948 with fresh kit components (no freeze/thaw) and with kit components stressed by 20 rounds of freezing and thawing (20 x freeze/thaw). 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.

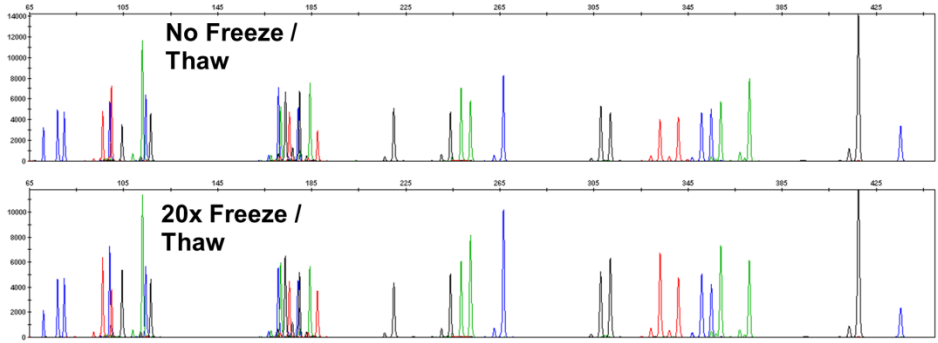


Figure 28. Results of a simulated freeze/thaw stability test of kit components.

Stability – Simulated shipment conditions on dry ice

Investigator ESSplex SE QS Kits are shipped on dry ice. To assess the performance of the kit after this type of 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 500 pg control DNA 9948.

The results indicate that the kit performance before and after storage on dry ice is comparable (Figure 29).

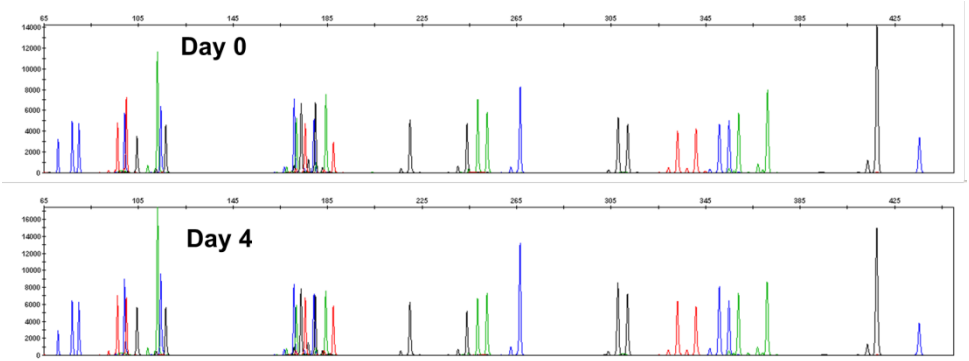


Figure 29. Effect of prolonged storage of Investigator ESSplex SE QS Kit components on dry ice.

References

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