Validation Report February 2020

Developmental Validation of the Investigator® 26plex QS Kit

The QIAGEN® Investigator 26plex 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 26plex 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 24 polymorphic STR markers recommended by the CODIS (Combined DNA Index System) Core Loci Working Group, the European Network of Forensic Science Institutes (ENFSI), the European DNA Profiling Group (EDNAP), and Chinese National database loci, D1S1656, D2S441, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D22S1045, CSF1PO, FGA, Penta D, Penta E, TH01, TPOX, vWA, 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 26plex 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 26plex QS Kit Handbook*.

Optimal reaction conditions were established, and the effects of variations in those conditions were assessed.

The effect of variations in buffer component concentrations is described using, as an example, $MgCl_2$ concentration (page 2). The robustness of the assay regarding variations in PCR cycling conditions and a range of different thermal cyclers was investigated (pages 3–7). The effects of using increased or decreased PCR cycle numbers were analyzed (page 4). Sensitivity was addressed by amplifying DNA of known concentration in a range typically encountered in forensic casework analysis (page 7).

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 (page 9). Cross-reactivity with non-human DNA (page 13) was also assessed. The reproducibility of the results was verified (page 18).

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 or Applied Biosystems 3500™ XL Genetic Analyzer. The standard conditions specified in the *Investigator 26plex 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 or higher.

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 3.0 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 $MgCl_2$, 1 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 ±17% 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).

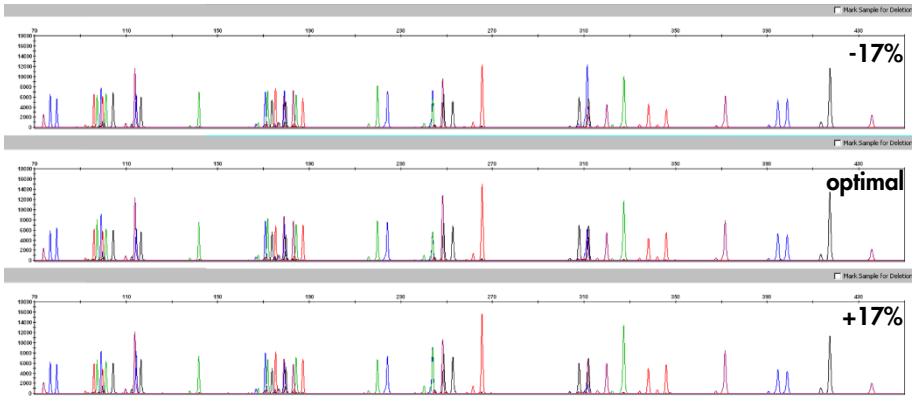


Figure 1. Influence of $MgCl_2$ concentration. Fast Reaction Buffer 3.0 (FRM 3.0) was supplemented with different concentrations of $MgCl_2$ matching the specification of production or 17% higher and lower. Representative electropherograms of sample quadruplicates are shown.

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 26plex QS Kit reaction (30 cycles at 60°C).

Table 1. Standard cycling protocol recommended for all DNA samples

Temperature	Time	Number of cycles
98°C	8 min	–
98°C 60°C 72°C	10 s 55 s 5 s	30 cycles
68°C	2 min	–
60°C	2 min	–
10°C	∞	–

Annealing temperatures between –4°C and +4°C around the optimal annealing temperature of 60°C were applied to the amplification of 500 pg control DNA 9948. PCR was performed on an Biometra TAdvanced 96/96 G 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. However, the average peak height of markers was best for the conditions closest to the actual annealing temperature of 60°C (Figure 2 and Figure 3). No dropouts were observed in the tested range applying a threshold of 100 RFU. No non-specific PCR products were observed. In order to ensure optimal performance of the assay, we strongly recommend regular calibration of thermal cyclers.

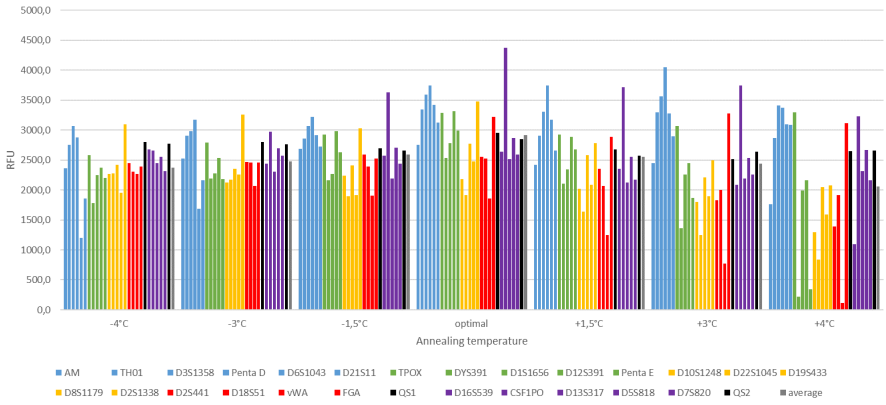


Figure 2. Effect of variations in the PCR annealing temperature. Under standard conditions (30 cycles), 500 pg control DNA 9948 were amplified on an Biometra TAdvanced 96/96 G instrument. Each bar represents an average peak height of triplicates. Bar colors represent the fluorescent dye labels of individual markers, and grey bars show the average of all STR markers.

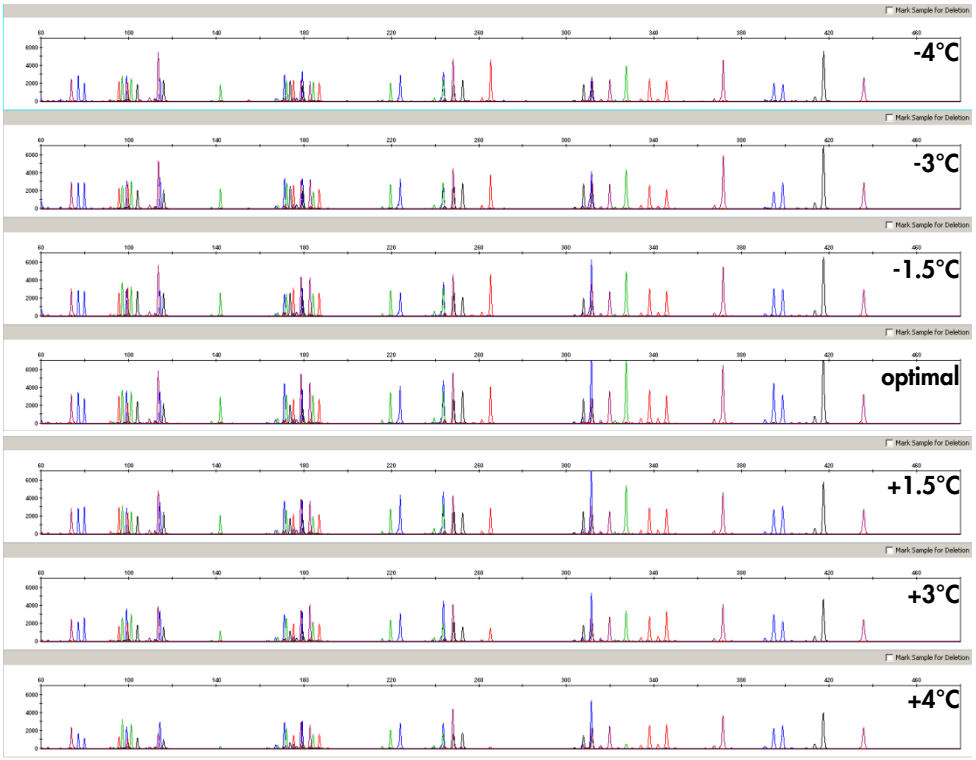


Figure 3. Variations in the PCR annealing temperature. For analysis, 500 pg control DNA 9948 were amplified on an Biometra TAdvanced 96/96 G instrument. Representative electropherograms for reactions using annealing temperatures between –4°C and +4°C are shown. Note that the y-axis was scaled for best 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). 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) provides alternative methods for increasing sensitivity.

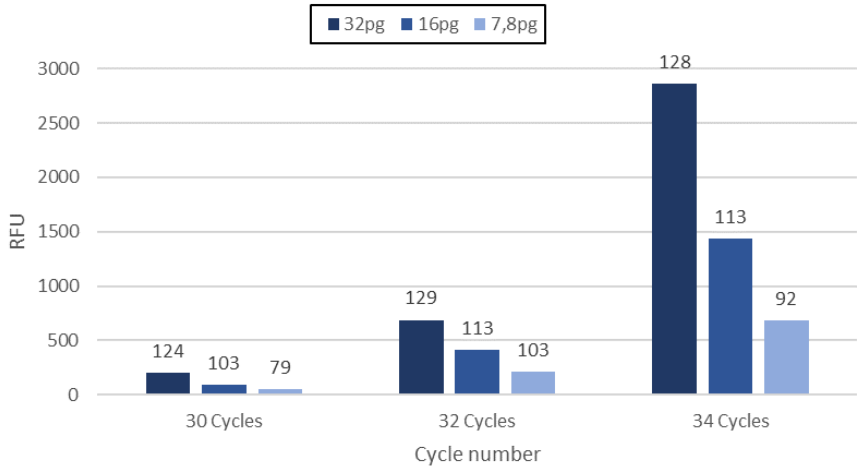


Figure 4. Effect of different cycle numbers on mean peak height and number of called alleles. Different amounts of control DNA 9948 were used as template, as indicated in the figure. Samples were run in triplicates and numbers of detected PCR products (indicated above the bars; 132 PCR products expected) 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). 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 below 24 cycles, 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 24 or more cycles. Hence, the QS signals will provide information about an inhibited PCR or degraded template DNA for 24 or more cycles.

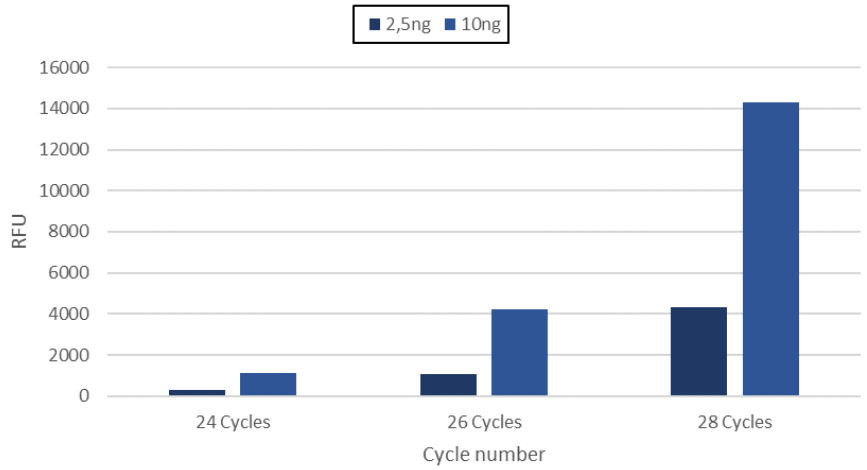


Figure 5. Effect of decreased cycle numbers on mean peak heights. Using a total of 24, 26, or 28 PCR cycles, 2.5 ng or 10 ng of control DNA 9948 were subjected to amplification. Samples were run in triplicates, and average peak heights were calculated.

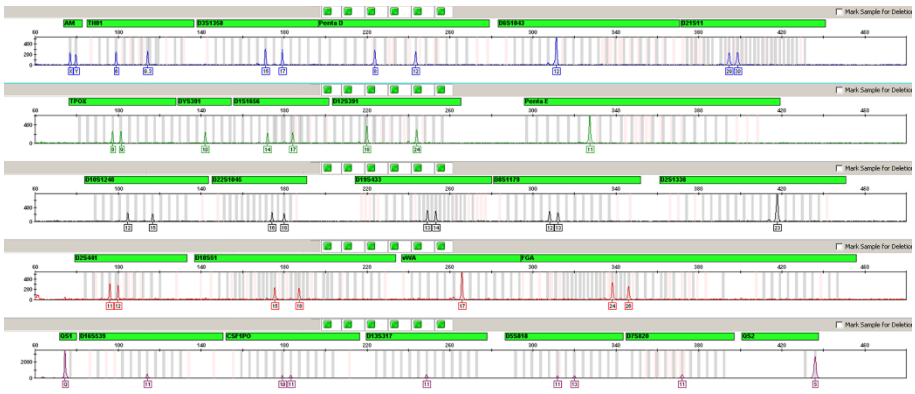


Figure 6. Reduction of PCR cycle numbers with elevated template DNA amounts. Results for amplification of 2.5 ng control DNA 9948, using a total of 24 instead of the standard 30 PCR cycles, are shown. The template amount of the Quality Sensor is optimized for 24 or more cycles, thus, reducing the cycle number does not affect the QS signals detected.

Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator 26plex QS Kit to demonstrate kit robustness independent of the instrument (Figure 7). As a PCR template, 2 ng to 32 pg of control DNA 9948 were used. The reaction took place under standard conditions (30 cycles) and was performed with the following thermal cyclers:

- GeneAmp PCR System 9700 with Silver Block (Applied Biosystems Inc., Foster City, CA, USA)
- GeneAmp PCR System 9700 with 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)
- Bio-Rad C1000 Touch (Bio-Rad Laboratories GmbH, Munich, Germany)

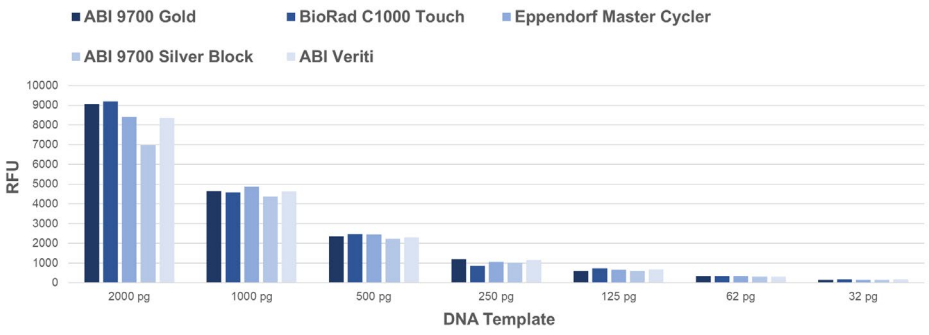


Figure 7. Performance on different PCR thermal cyclers. Different amounts of control DNA 9948 were used, as indicated in the figure. Each sample was run in duplicate. A standard 30-cycle protocol was used. Average peak heights across all markers are shown.

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 were observed on any of the thermal cyclers.

Sensitivity

The Investigator 26plex 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® Pro Kit. In particular, for heavily degraded DNA, the use of increased template amounts may improve results (see page 7).

Control DNA 9948 was serially diluted from 1 ng to 8 pg per reaction. Full profiles (42 PCR products) were consistently obtained at 125 pg, using the standard conditions specified in the *Investigator 26plex 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 which the main peak becomes saturated. Finally, "split peaks" may occur at very high template amounts 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 8 and Figure 9 show examples 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.

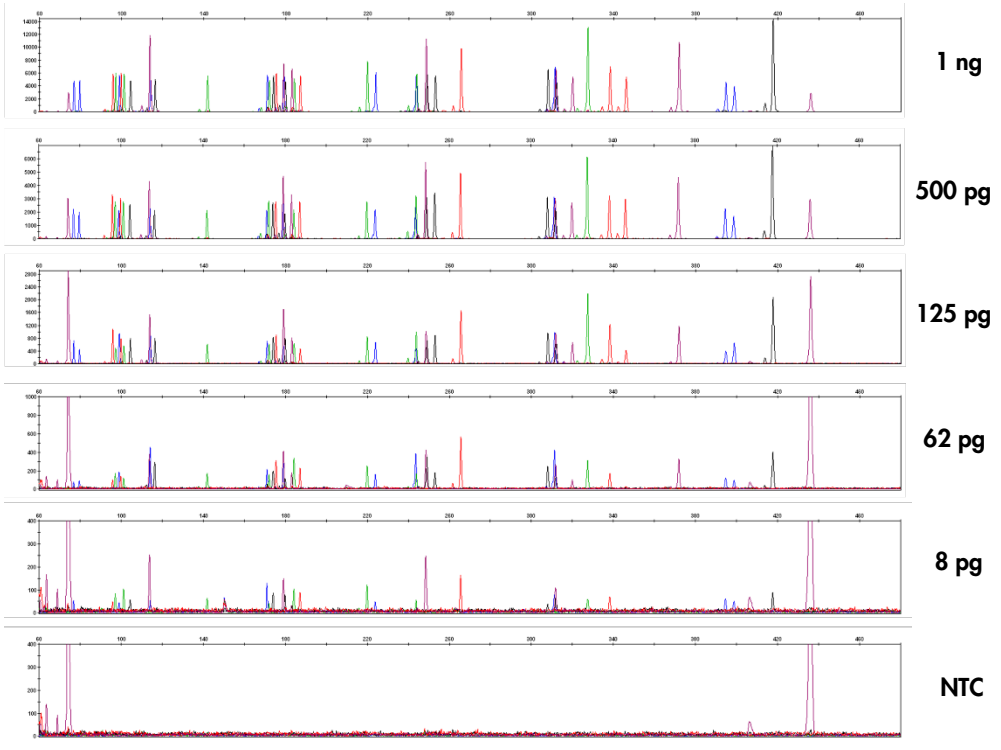


Figure 8. Sensitivity study. Serial dilutions of control DNA 9948 were analyzed. The amounts of DNA indicated were used as template for amplification. The lowest panels show a no-template (negative) control (NTC) to illustrate the expected level of background fluorescence. Y-axis scales were adjusted individually for the highest peak heights of the STR markers. Note: As the Quality Sensor (first and last purple peaks) is amplified with similar amplification efficiency in all experiments, independent of the sample template amounts, the QS peak heights are similar in all experiments.

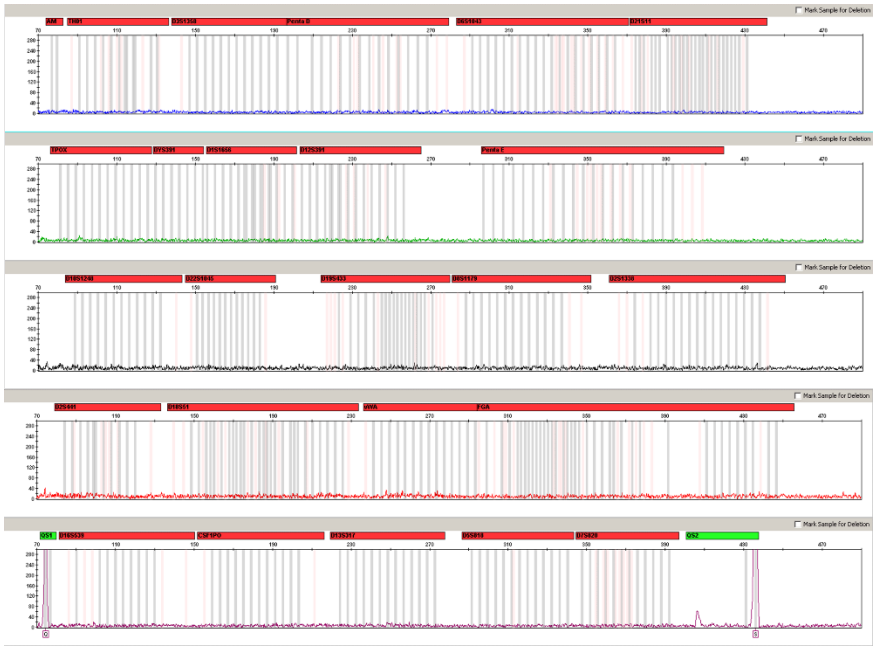


Figure 9. Background fluorescence. Example of a no-template (negative) control to illustrate the expected level of background fluorescence. For correct interpretation of samples having only small amounts of DNA, it is crucial to minimize background fluorescence.

Performance with simulated inhibition

If the DNA extraction from forensic casework samples is done using inappropriate methods, Investigator 26plex 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 Figure 10 to Figure 16. Figure 16 shows an overview of Investigator 26plex QS Kit inhibitor resistance.

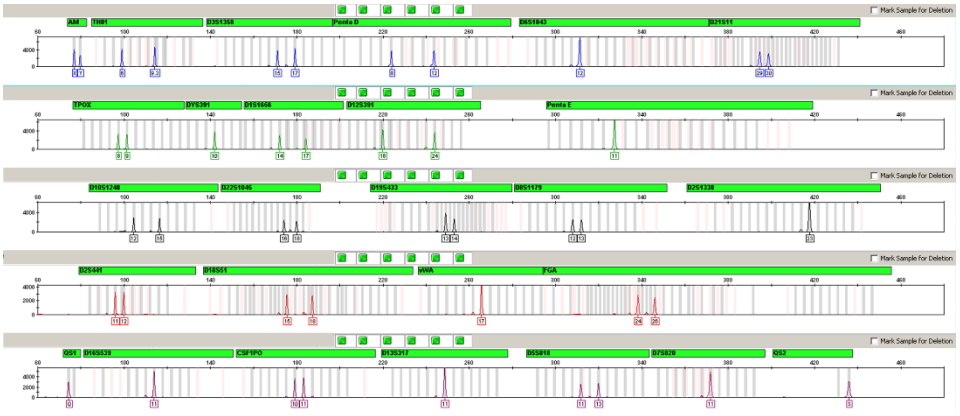


Figure 10. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 500 µM hematin.

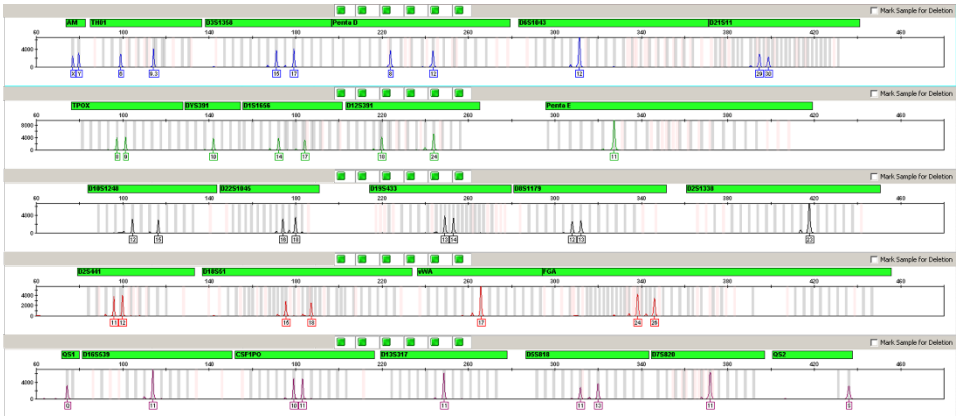


Figure 11. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 100 ng/µl humic acid.

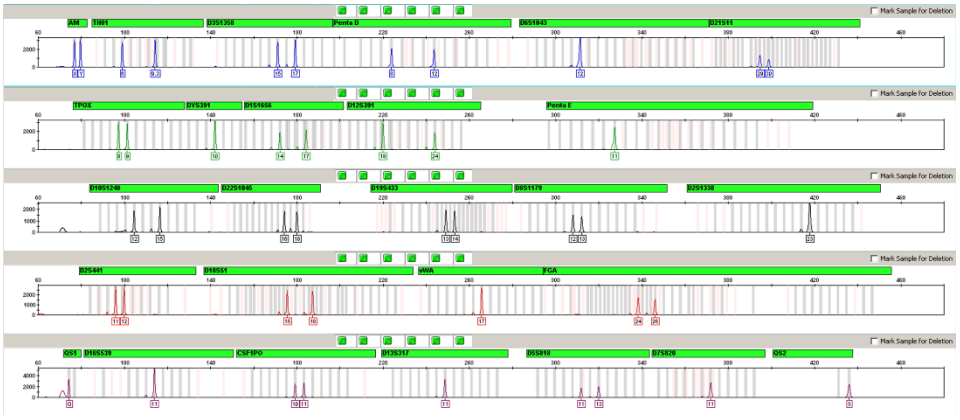


Figure 12. Electropherogram of 500 pg Control DNA 9948 amplified in the presence of 6 mM indigo carmine.

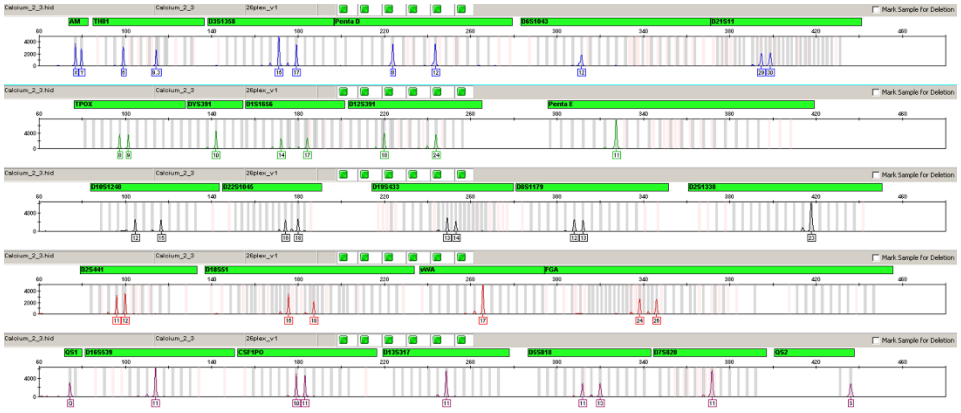


Figure 13. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 2.5 mM calcium.

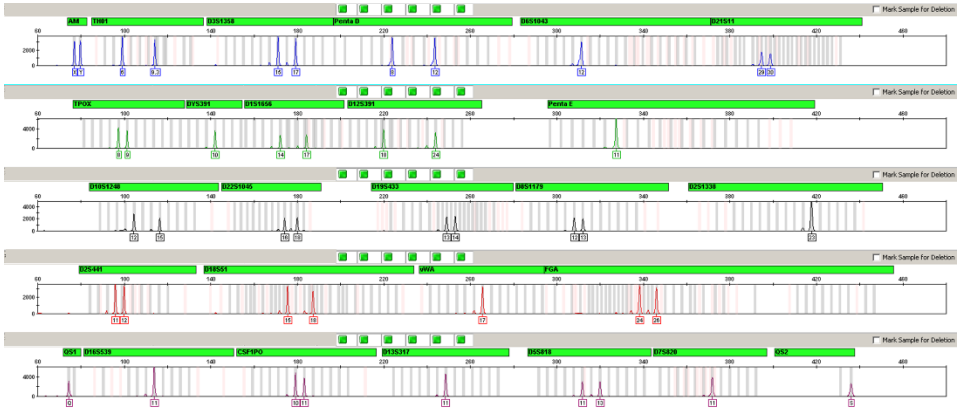


Figure 14. Electropherogram of 500 pg Control DNA 9948 amplified in the presence of 2000 ng/µl tannic acid.

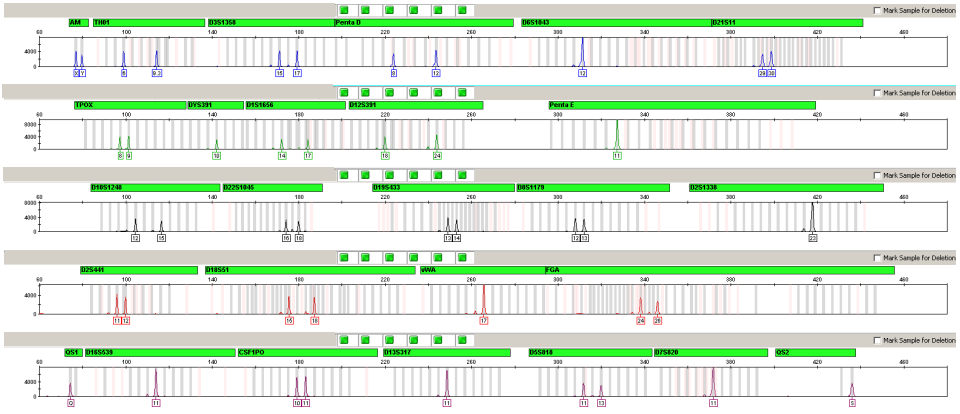


Figure 15. Electropherogram of 500 pg control DNA 9948 amplified in the presence of 0.5% ethanol.

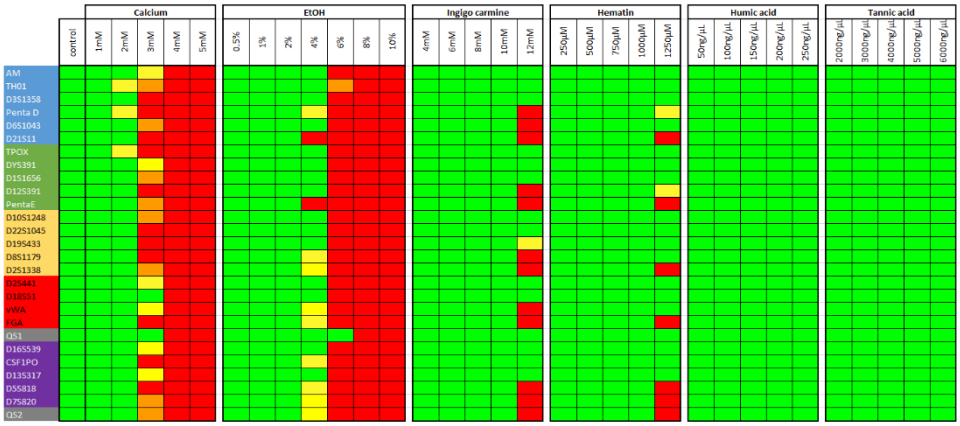


Figure 16. Overview of Investigator 26plex QS inhibitor resistance. The assay was tested for its robustness towards inhibitors (calcium, ethanol, indigo carmine, hematin, humic acid, and tannic acid). As template, 500 pg of control DNA 9948 were used and PCR was performed under standard conditions. As a threshold for allele calling, 50 RFU was used. 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.

Full profiles lacking any PCR artifacts, for example, 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 and hematin analysis).

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.

Stability with degraded DNA

Casework evidence has often been exposed to adverse environmental conditions, like 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. 500 pg DNA were used as template for amplification (all samples in duplicate) (Figure 17). 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, ~70% of expected peaks were detected using a threshold of 50 RFU (Figure 17). Increased amounts of template can be used to improve results for heavily degraded DNA.

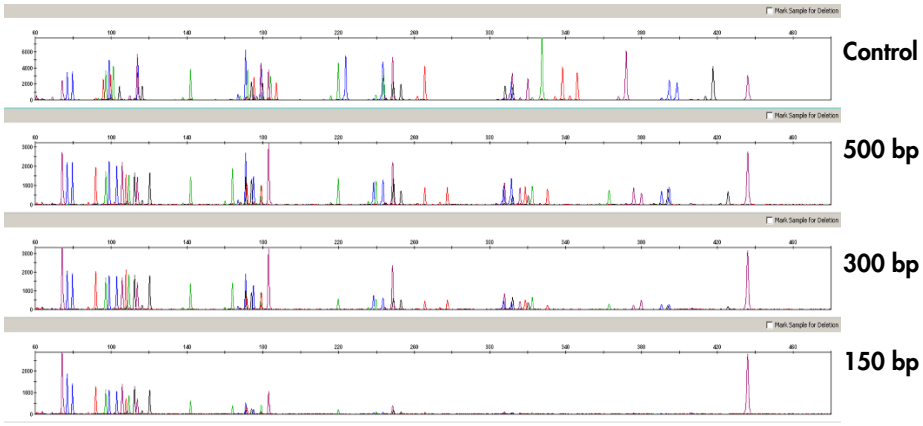


Figure 17. Degraded DNA. Male DNA was sheared to the defined average fragment length indicated. STR analysis of untreated control and degraded DNA samples. 500 pg template DNA was used. Full profiles were obtained down to 300 bp average fragment length. **Note:** The Quality Sensor is not affected by 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 26plex QS Kit species specificity for human DNA, DNA from other species was tested following the standard assay protocol (Figure 18)

Besides common pets and farm animals, some primates were also tested. As expected for primates, amplification of some products is possible. Chimpanzees, 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.

Most of the further tested animal DNAs did not show any cross reactivity with the Investigator 26plex QS Kit.

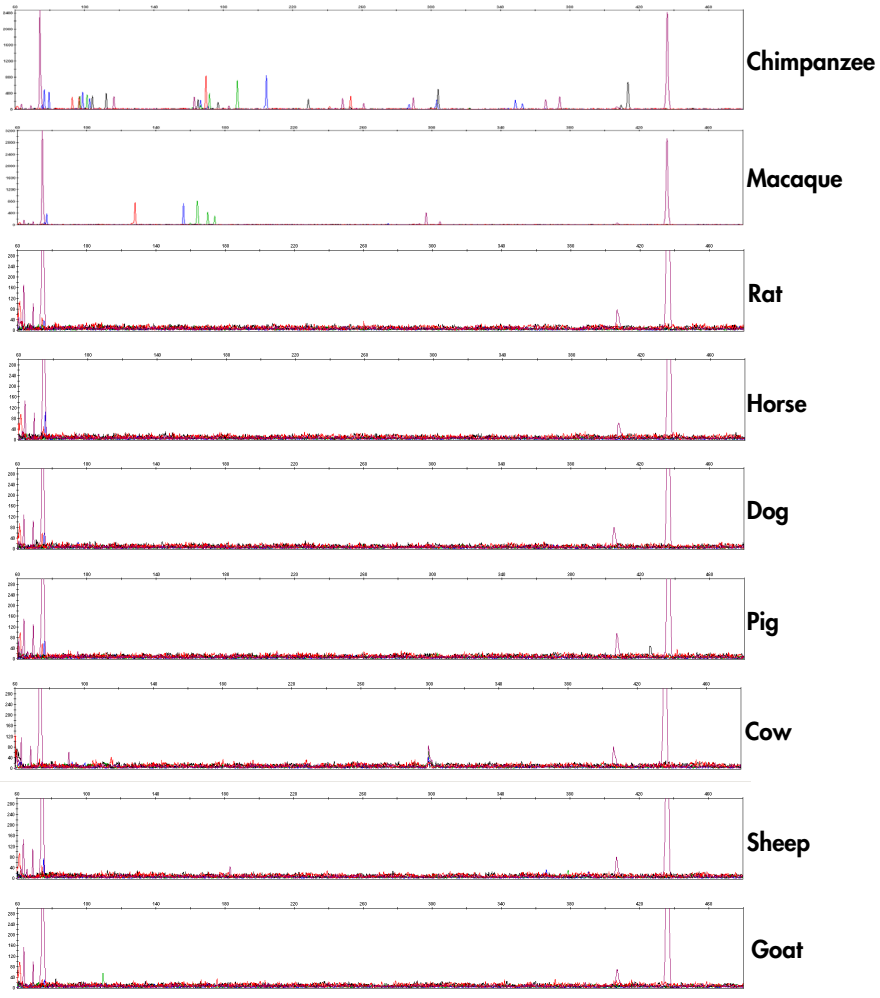


Figure 18. Representative results of the species specificity assessment. As template, 500 pg primate DNA and 2.5 ng DNA from all other species were used.

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 DNA BC2 and control DNA 9948 and in ratios of 1:1, 3:1, 7:1, 10:1, 15:1 (see Table 3 for genotypes of mixed samples). The total amount of mixed DNA used in this study was 500 pg; a 15:1 mixture thus contains 33 pg of the minor component DNA and 467 pg of the major component (Table 2). 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 19. 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 7, for general considerations.

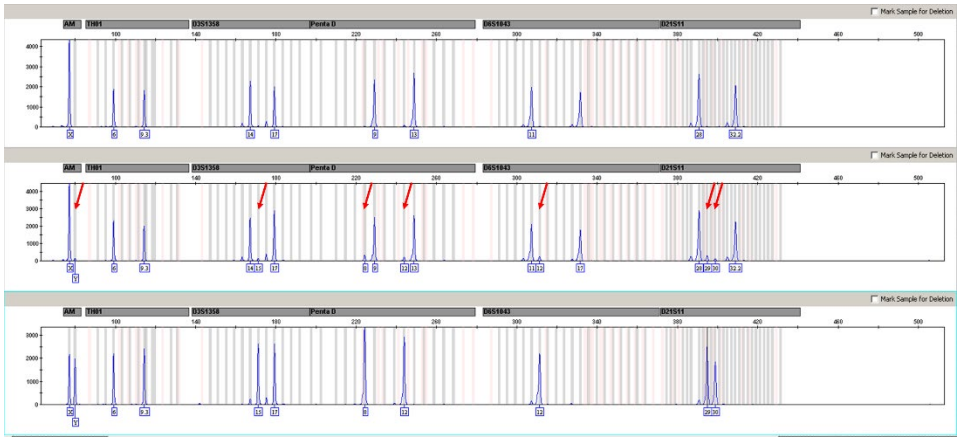
Table 2. Amounts of DNA template in mixtures

Mixture ratio	Major component DNA BC2	Minor component DNA 9948
1:1	250 pg	250 pg
3:1	375 pg	125 pg
7:1	430 pg	70 pg
10:1	450 pg	50 pg
15:1	467 pg	33 pg

Table 3. Genotypes of DNAs used for mixed samples

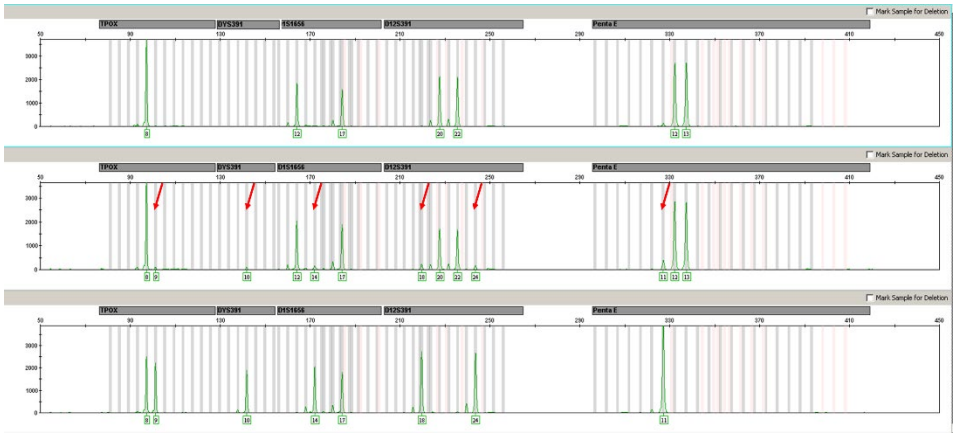
Locus	9948 genotype	BC2 genotype
Amelogenin	X/Y	X/X
CSF1PO	10/11	10/12
DYS391	10/10	-
D1S1656	14/17	12/17
D2S1338	23/23	17/24
D2S441	11/12	14/14
D3S1358	15/17	14/17
D5S818	11/13	11/11
D6S1043	12/12	11/17
D7S820	11/11	8/12
D8S1179	12/13	13/13
D10S1248	12/15	12/14
D12S391	18/24	20/22
D13S317	11/11	8/13
D16S539	11/11	9/11
D18S51	15/18	13/18
D19S433	13/14	13/14
D21S11	29/30	28/32.2
D22S1045	16/18	15/16
FGA	24/26	19/21
Penta D	8/12	9/13
Penta E	11/11	12/13
TH01	6/9.3	6/9.3
TPOX	8/9	8/8
vWA	17/17	16/19

A



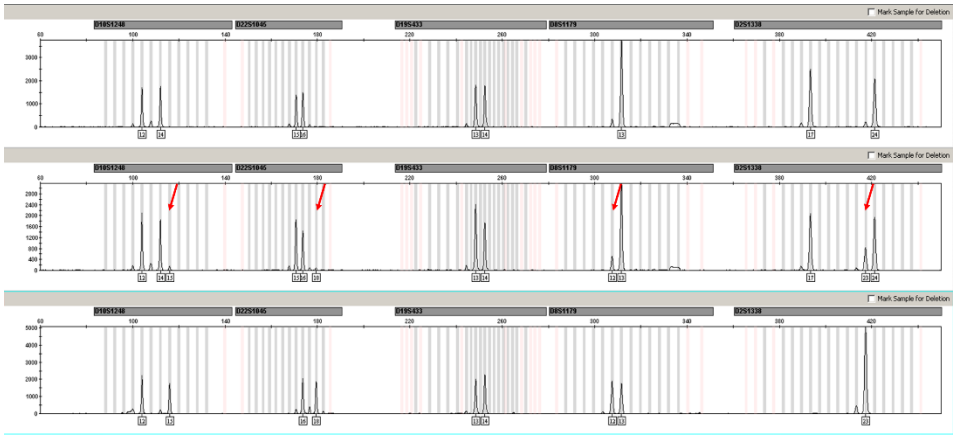
A. Expected bp positions of uncovered minor component peaks are indicated by red arrows. **Amelogenin**: Homozygote + heterozygote, two overlapping alleles, the non-overlapping minor component peak can be assigned. **TH01**: Heterozygote + heterozygote, two overlapping alleles, minor component peaks cannot be assigned. **D3S1358**: Heterozygote + heterozygote, one overlapping allele, one minor component can be assigned. **Penta D**: Heterozygote + heterozygote, no overlapping alleles, both minor component peaks are overlapping with stutter position. **D6S1043**: Heterozygote + homozygote, minor component peaks can be assigned. **D21S11**: Heterozygote + heterozygote, no overlapping alleles, minor component can be assigned.

B



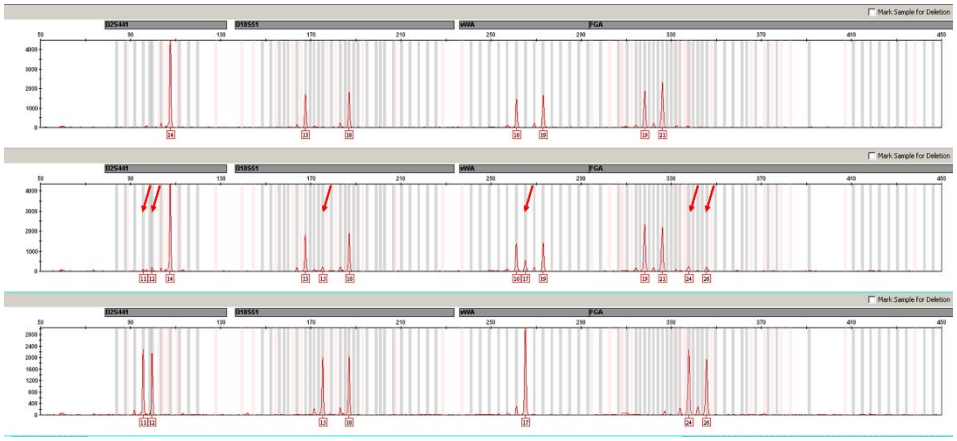
B. Expected bp positions of uncovered minor component peaks are indicated by red arrows. **TPOX:** Homozygote + heterozygote, one overlapping allele, one minor component can be assigned. **DYS391:** Null-Allele + Homozygote, the non-overlapping minor component peak can be assigned. **D1S1656:** Heterozygote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

C



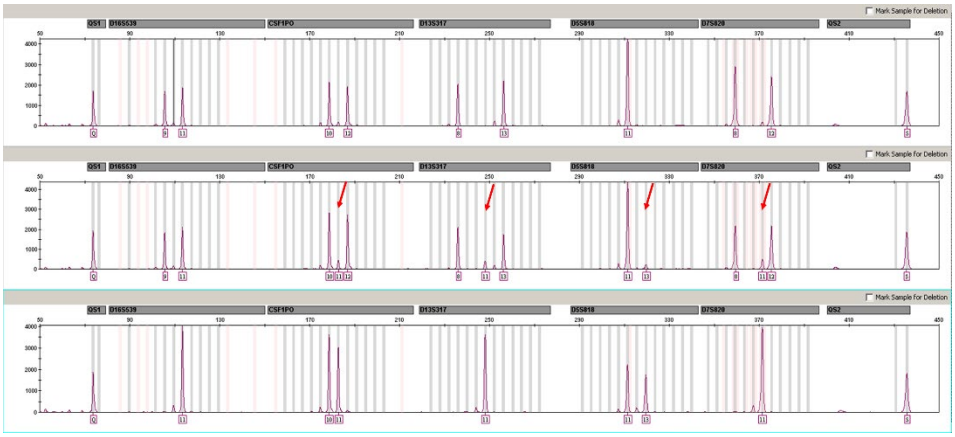
C. Expected bp positions of uncovered minor component peaks are indicated by red arrows. **D10S1248:** Heterozygote + heterozygote, one overlapping allele, one non overlapping minor component peaks can be assigned. **D22S1045:** Heterozygote + heterozygote, one overlapping allele, one non-overlapping minor component peak can be assigned. **D19S433:** Heterozygote + heterozygote, two overlapping alleles, minor component alleles are covered by major component alleles. **D8S1179:** Homozygote + heterozygote, one overlapping allele, one minor allele is overlapping with stutter position, major component completely covers other minor component. **D2S1338:** Heterozygote + homozygote, one minor allele is overlapping with stutter position.

D



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

E



E. Expected bp positions of uncovered minor component peaks are indicated by red arrows. **D16S539:** Heterozygote + homozygote, one overlapping allele, major component completely covers other minor component. **CSF1PO:** Heterozygote + heterozygote, one overlapping allele, one minor component peak can be assigned and is overlapping with stutter position. **D13S317:** Heterozygote + homozygote, no overlapping alleles, the one minor component peak can be assigned. **D5S818:** Homozygote + heterozygote, one overlapping alleles, the one minor component peak can be assigned. **D7S820:** Heterozygote + homozygote, no overlapping allele, the minor component peak can be assigned and is overlapping with stutter position. QS1 and QS2 are internal PCR controls and are amplified with equal peak heights in all experiments.

Figure 19. Results of the mixture analysis. Figures 19A–19E show 1:10 mixtures of DNA BC 2 and control DNA 9948 (middle panel 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 26plex QS Kit are highly conserved and adopted from other QIAGEN kits, including, the Investigator 24plex QS, Investigator ESSplex SE QS, or Investigator IDplex Plus Kits. 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. Primers for D6S1043, D21S11, Penta D, Penta E, and FGA were newly designed using sophisticated bioinformatics software to ensure high specificity and sensitivity, as well as an optimal annealing temperature (T_m) matching the T_m range of existing primers. To demonstrate the concordance for the newly designed primers of the Investigator 26plex QS Kit, an in-house study was run on 94 samples. The Investigator 26plex QS Kit result for the markers D6S1043, Penta D, Penta E, D21S11, and FGA is concordant to kits, such as VeriFiler™ Plus PCR Amplification Kit and PowerPlex® 21 System. The primers for the remaining markers are identical to Investigator 24plex QS Kit. For Investigator 24plex QS Kit, the concordance was demonstrated and the NIST compared the results of the Investigator 24plex QS Kit to the NIST final data set with 656 unrelated individuals (NIST U.S. population set [650 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 discordant result for D7S820 (1 sample out of 656 samples) was detected. The Investigator 24plex QS Kit result for D7S820 is concordant to kits, such as, Identifiler®, PowerPlex Fusion, and Profiler Plus®, but it is discordant to other kits, like PowerPlex 16, AmpFℓSTR® MiniFiler™ and IDplex Plus. Out of 29,520 alleles compared, 1 discordant call was observed, given a 0.003% discordance. In conclusion, there was a concordance of 99.997% between the Investigator 24plex QS Kit and the NIST final data set.

For Investigator 26plex QS Kit, peak height ratios of heterozygous markers (Figure 20) were analyzed with 50 samples, and stutter peak heights (Figure 21) were analyzed for 94 samples.

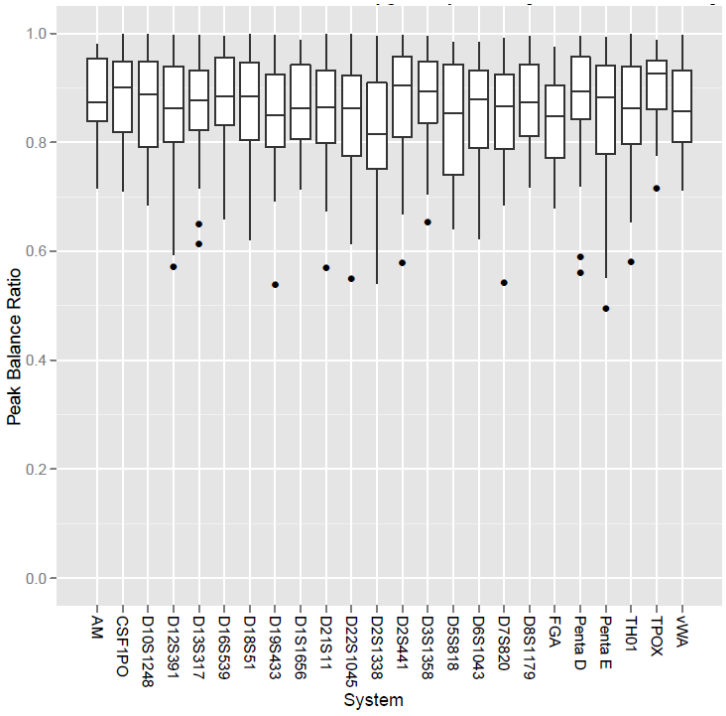
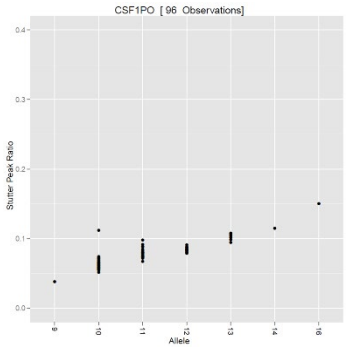
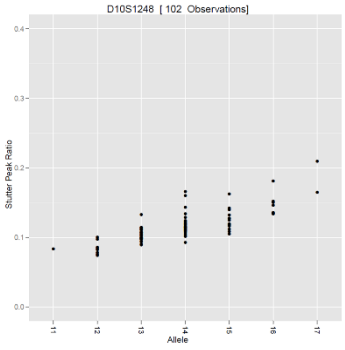
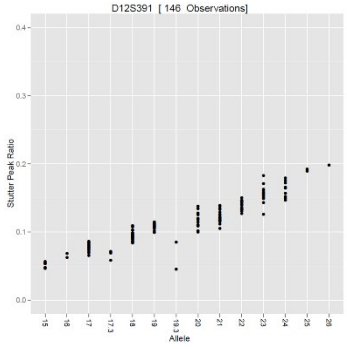
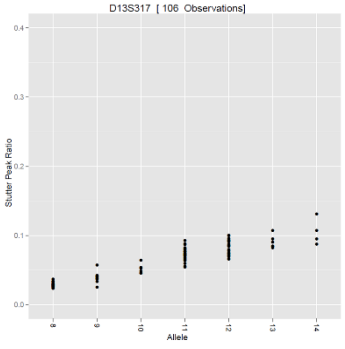


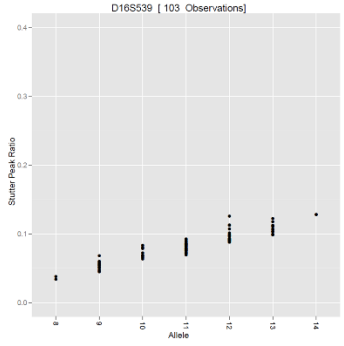
Figure 20. Box plot analysis of intra-locus peak height ratios. Values refer to the lower of the 2 peaks in a heterozygous sample. Boxes represent the middle 50% (interquartile range, [IQR]) of data, lines inside the boxes the median. Whiskers show data within 1.5 IQR, dots represent outliers.

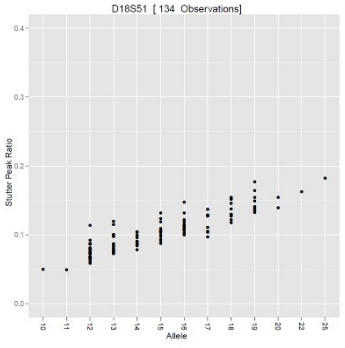

CSF1PO [96 Observations]
Stutter Peak Ratio
Allele

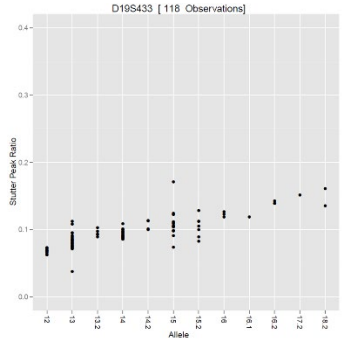

D10S1248 [102 Observations]
Stutter Peak Ratio
Allele

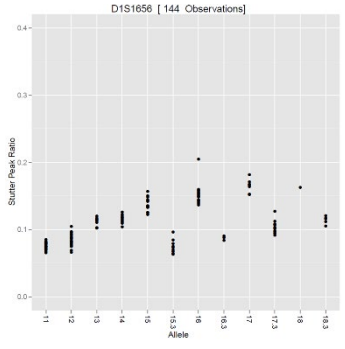

D12S391 [146 Observations]
Stutter Peak Ratio
Allele

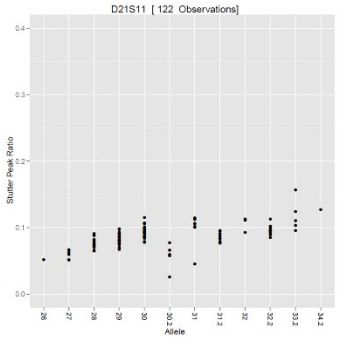

D13S317 [106 Observations]
Stutter Peak Ratio
Allele

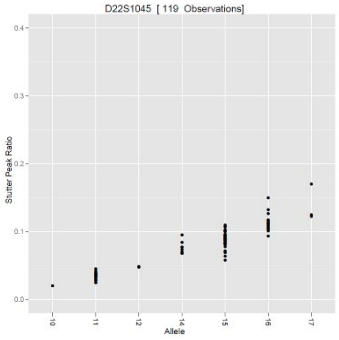

D16S539 [103 Observations]
Stutter Peak Ratio
Allele

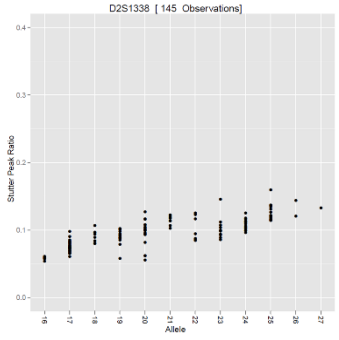

D18S51 [134 Observations]
Stutter Peak Ratio
Allele

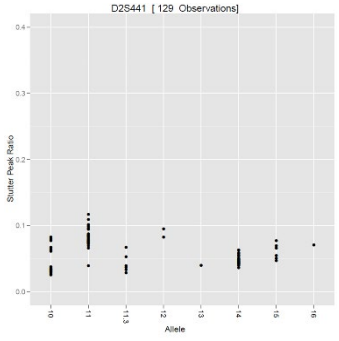

D19S433 [118 Observations]
Stutter Peak Ratio
Allele

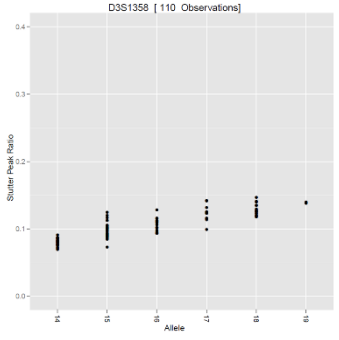

D1S1656 [144 Observations]
Stutter Peak Ratio
Allele

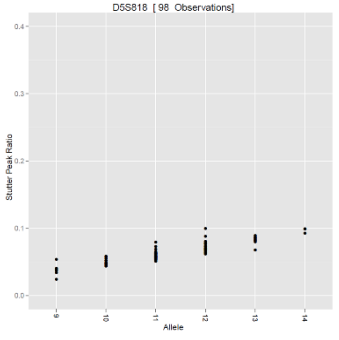

D21S11 [122 Observations]
Stutter Peak Ratio
Allele

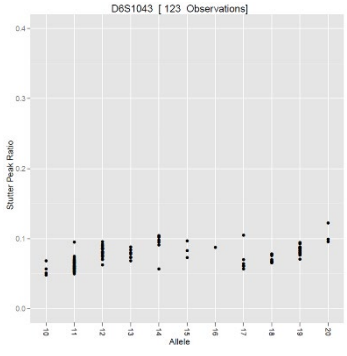

D22S1045 [119 Observations]
Stutter Peak Ratio
Allele

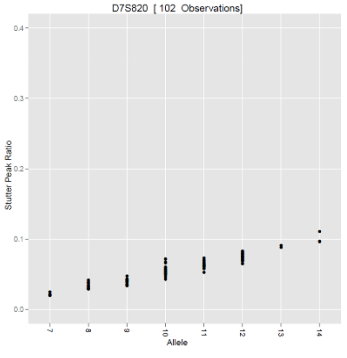

D2S1338 [145 Observations]
Stutter Peak Ratio
Allele


D2S441 [129 Observations]
Stutter Peak Ratio
Allele


D3S1358 [110 Observations]
Stutter Peak Ratio
Allele


D5S818 [98 Observations]
Stutter Peak Ratio
Allele


D6S1043 [123 Observations]
Stutter Peak Ratio
Allele


D7S820 [102 Observations]
Stutter Peak Ratio
Allele

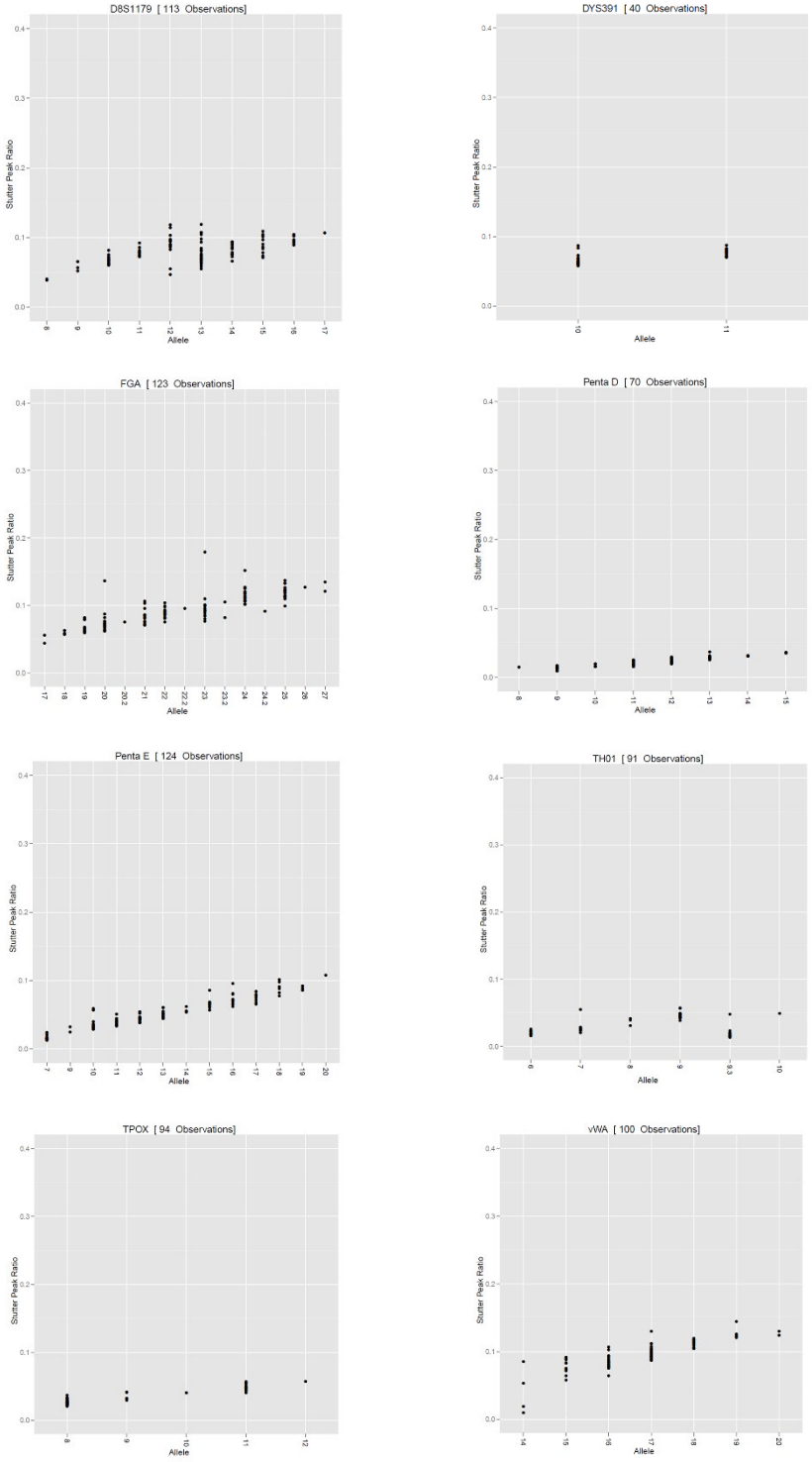


Figure 21. Stutter peak height ratios. Peak heights of forward stutters compared to main peaks were analyzed for 94 DNA samples from different donors.

Stability – Improved hot-start function

Usually, PCR runs were set up 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 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 26plex QS Kit contains an improved hot-start function. The kit affords enhanced specificity and efficiency of the multiplex PCR reaction (Figure 22). The added convenience of stability for up to 3 hours at room temperature, without a need for any cooling, makes it ideal for handling of high-throughput samples and automated workflows.

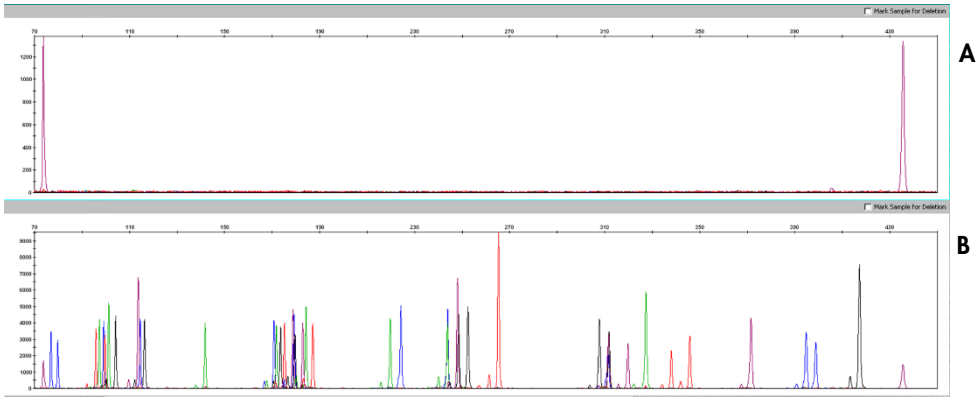


Figure 22. Reaction stability for 3 hours incubation at room temperature. PCR samples were incubated at 20°C for 3 hours in between the PCR pipetting step and the start of the thermocycler. **A:** Negative control. **B:** Results of 500 pg DNA 9948 as PCR template.

As demonstrated, after 3 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.

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- 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.
- 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 26plex QS Kit (100)	Primer mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder 26plex QS, and RNase-free water	382615
Investigator 26plex QS Kit (400)	Primer mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder 26plex QS, and Rnase-free water	382617

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