

# QIAGEN Validation Report

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## Developmental validation of the Investigator<sup>®</sup> ESSplex Plus Kit

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

The performance of the Investigator ESSplex Plus Kit was evaluated with regard 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 (SWGDM) (2). The assay makes use of well-established methodologies for forensic DNA analysis. It co-amplifies the 15 polymorphic STR markers recommended by the ENFSI and the European DNA Profiling Group (EDNAP) as the new European Standard Set (ESS) of loci (D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045, FGA [FIBRA], TH01 [TC11], and vWA), and the gender-specific Amelogenin (3). These genetic loci have been characterized in numerous studies by other laboratories (4–6). Optimal reaction conditions were established, and the effects of variations in those conditions were assessed. The kit was tested in-house and at independent external forensic laboratories.

The effect of variations in buffer component concentrations is described using the example of the MgCl<sub>2</sub> concentration (page 2). Variations in PCR cycling conditions and a range of thermal cyclers and genetic analyzers were used to demonstrate the robustness of the assay (pages 4–8). The effects of using increased or decreased PCR cycle numbers were analyzed (page 6). Sensitivity was addressed by amplifying DNA of known concentration in a range typically encountered in forensic casework analysis (page 11).



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 13 and 17). Cross-reactivity with non-human DNA (page 18) was also assessed. The reproducibility of the results was verified (page 25).

## Results of developmental validation

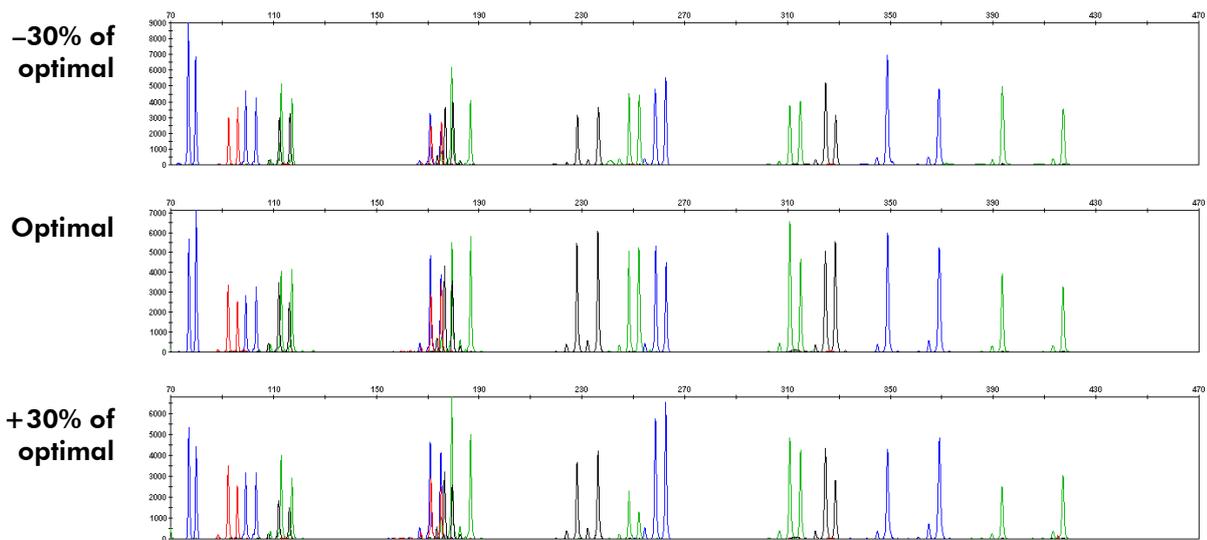
The validation study was performed at the QIAGEN R&D department. All of the electropherograms shown were generated on an Applied Biosystems® 3500 Genetic Analyzer. The standard conditions specified in the Investigator ESSplex Plus Handbook were used for the electrophoresis. Unless stated otherwise, a GeneAmp® PCR System 9700 with Gold-plated Silver 96-Well Block was used for amplification. Data were analyzed using the QIAGEN Investigator IDproof Software, v2.0 or Applied Biosystems GeneMapper® ID-X Software, v1.2.

### 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 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 XY13 (Figure 1). The assay yielded robust results within an  $MgCl_2$  concentration range of  $\pm 30\%$  of the optimum.

Under these conditions, amplification of the markers was well balanced and no dropout or nonspecific 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).



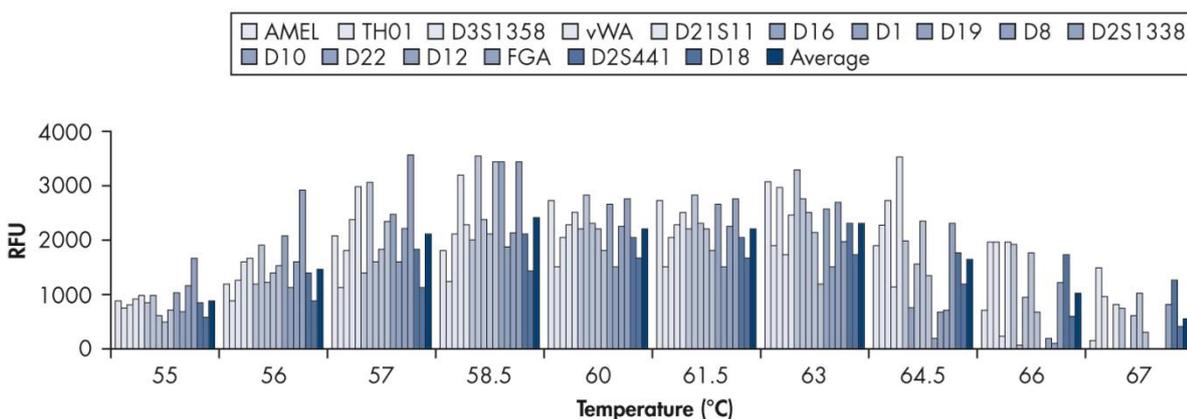
**Figure 1. Influence of MgCl<sub>2</sub> concentration on the amplification of 500 pg control DNA XY13.** Fast Reaction Buffer was supplemented with different concentrations of MgCl<sub>2</sub> matching the specification of production, or 30% higher or lower. Representative electropherograms of sample duplicates are shown.

## Effect of PCR annealing temperature variations

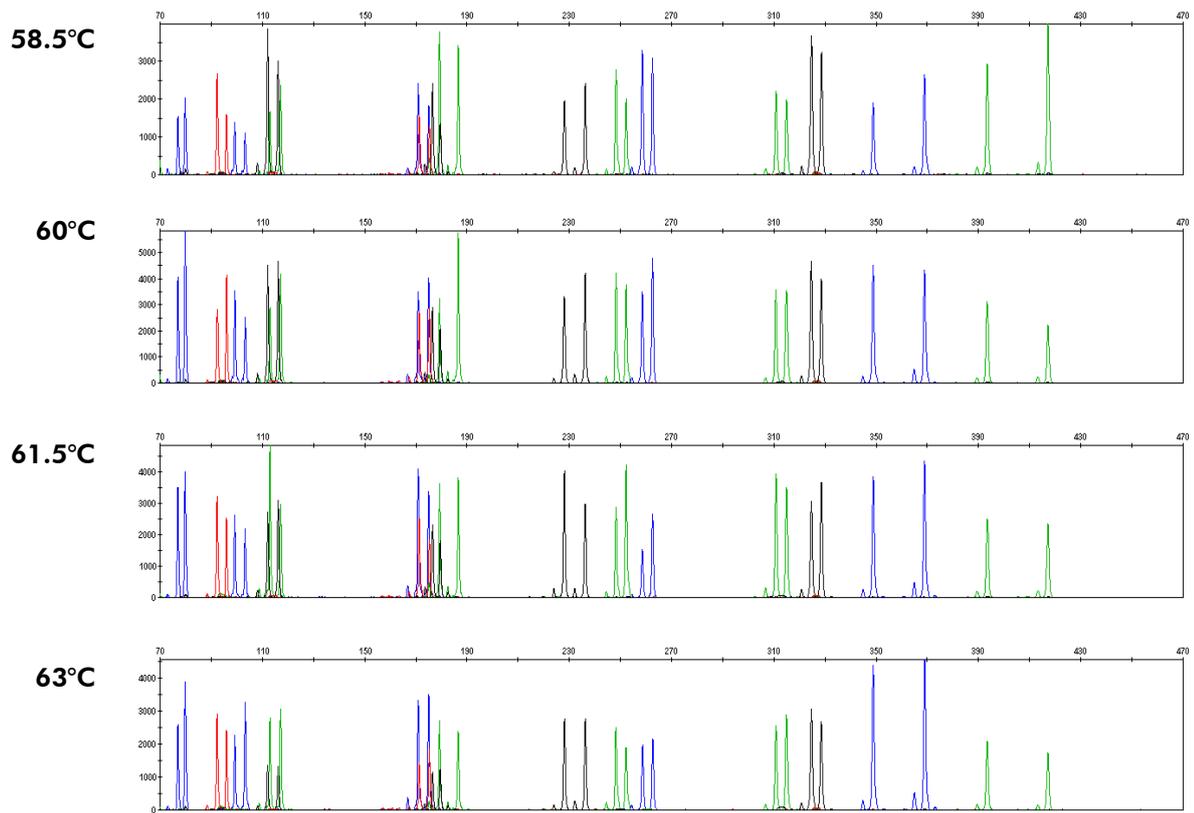
Specificity, sensitivity, and robustness are critical in forensic casework, and all are affected by the annealing temperature ( $T_m$ ). Since the actual  $T_m$  may vary depending on cyclor conditions, the assay was validated in a range surrounding the optimal  $T_m$  of the Investigator ESSplex Plus reaction (61°C).

Annealing temperatures between 55°C and 67°C were applied to the amplification of 500 pg Control DNA XY13 and PCR was performed on an Eppendorf® Mastercycler® ep instrument. Reactions using annealing temperatures between 55°C and 64.5°C resulted in full profiles. Similar overall peak heights were observed for the temperature range 58.5–63°C. However, the peak balance between markers was best for the conditions closest to the actual annealing temperature of 61°C (Figure 2 and Figure 3). The first dropouts were observed at 66°C. No nonspecific PCR products were observed. In order to ensure optimal performance of the assay, we strongly recommend regular calibration of thermal cyclers.

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



**Figure 2. Effect of variations in the PCR annealing temperature.** Each bar represents an average peak height of duplicates. Bar colors represent the individual markers; the darkest blue bars show the average of all markers.

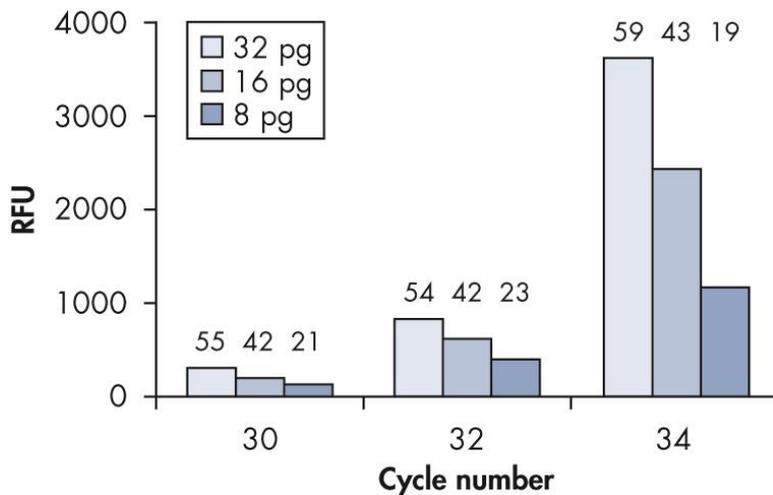


**Figure 3. Variations in the PCR annealing temperature.** Representative electropherograms for reactions using annealing temperatures of 58.5–63°C are shown. **Note:** The x-axis was scaled for best fit.

## Effect of different cycle numbers

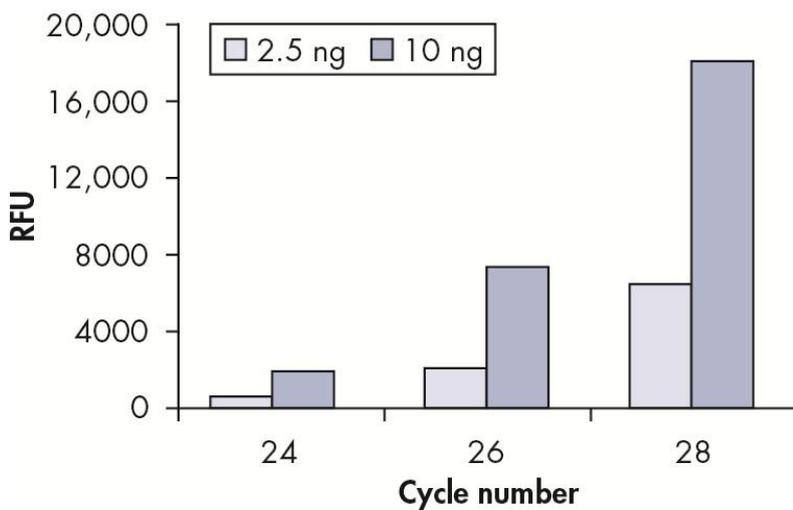
Altering the number of PCR cycles can be used to adapt the reaction conditions to varying DNA template concentrations. Cycle numbers can be either increased to enhance amplification signals when working with low-copy-number DNA, or decreased to speed up the protocol when the DNA sample is abundant (e.g.,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

Cycle numbers were increased to 32 or 34 for reactions containing 32, 16, or 8 pg of Control DNA XY13, 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 increase with higher cycle numbers. However, it should be noted that an increase in cycle number to more than 30 will not necessarily result in more information obtained from the low template DNA sample. Furthermore, due to 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 threshold of detection strongly influence the outcome of an assessment of the use 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) are alternative methods for increasing sensitivity.

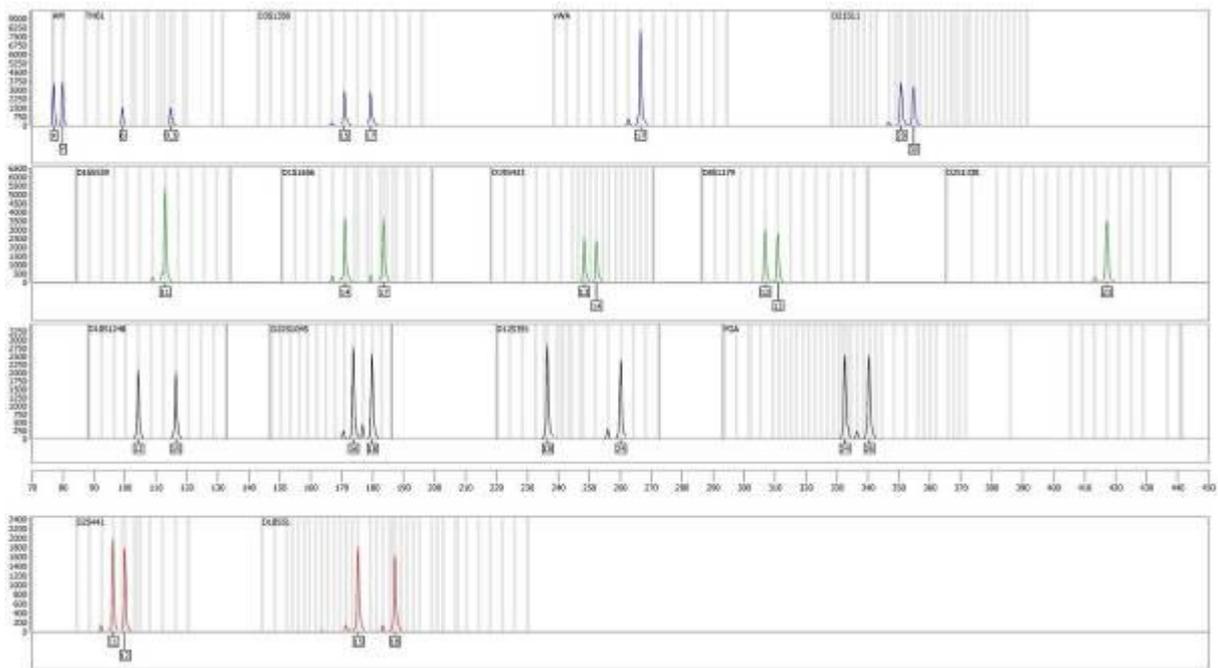


**Figure 4. Effect of different cycle numbers on mean peak height and number of called alleles.** Samples were run in duplicates and the numbers of called alleles (indicated above the bars; 64 alleles expected) and their peak heights were calculated. 50 RFU was used as a threshold for allele calling.

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). All reactions resulted in robust amplification and full profiles were obtained using a threshold of 50 RFU for allele calling. However, 28 cycle amplifications of 10 ng DNA gave rise to pull-up peaks when applying samples to analysis without prior dilution of the PCR product. An example electropherogram for a 24 cycle amplification of 10 ng template DNA is shown in Figure 6.



**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 24, 26, or 28 PCR cycles. Samples were run in duplicate and average peak heights were calculated.

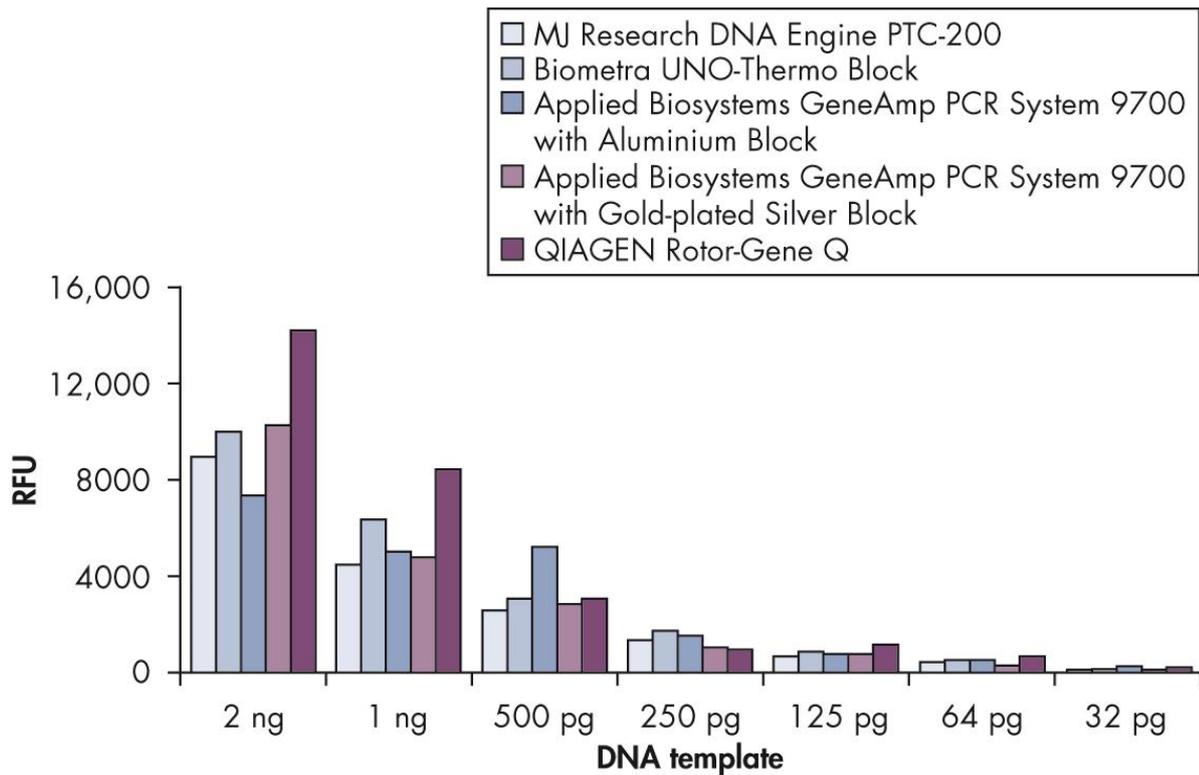


**Figure 6. Reduction of PCR cycle numbers with elevated template DNA amounts.** Results for the amplification of 10 ng Control DNA 9948 using 24 instead of standard 30 PCR cycles. Data were analyzed using QIAGEN Investigator IDproof Software.

## Effect of different cycler types

Several PCR thermal cyclers were tested with the Investigator ESSplex Plus Kit to demonstrate kit robustness independent of the instrument (Figure 7). 2 ng to 32 pg Control DNA XY5 was 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)
- MJ Research DNA Engine® PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany)
- UNO-Thermo Block (Biometra Biometra biomedizinische Analytik GmbH, Göttingen, Germany)
- QIAGEN Rotor-Gene® Q



**Figure 7. Performance on different PCR thermal cyclers.** DNA XY5 was used as template in the indicated amounts. Each sample was run in duplicate. A standard 30-cycle protocol was used. Average peak heights across all markers are shown.

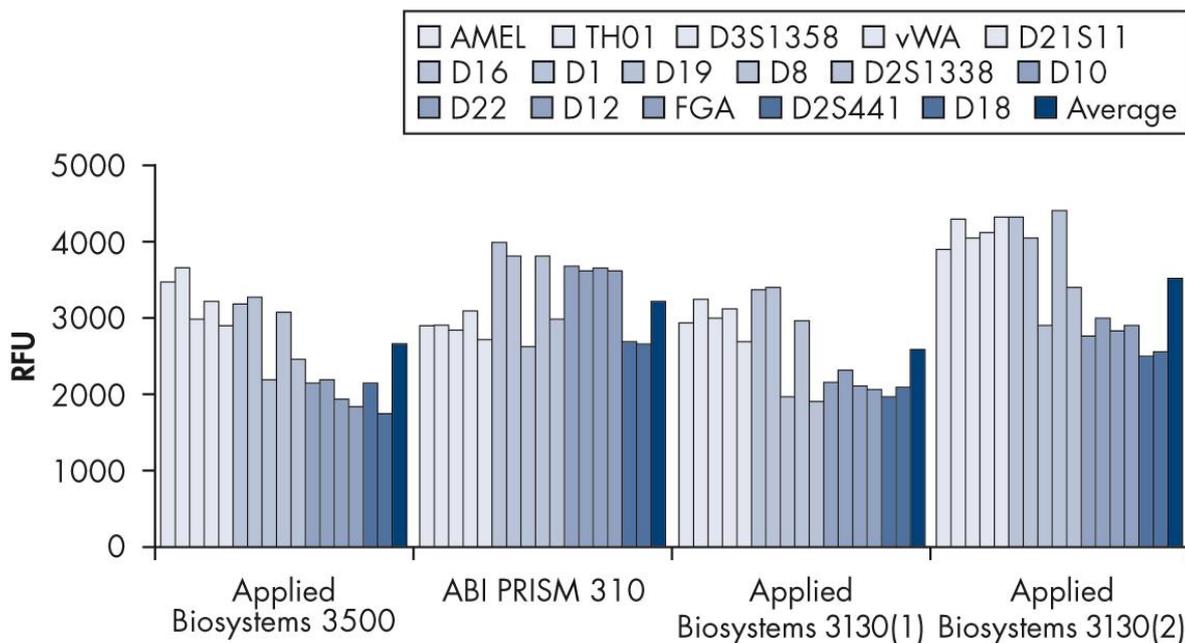
Comparable mean peak heights were obtained for all of the tested PCR cyclers. No significant differences in intra- and inter-locus balance or nonspecific amplification were observed on any of the thermal cyclers. In the case of the GeneAmp PCR System 9700, it is essential to use the “Standard mode” in order to achieve optimal results. Do not use the “9600 emulation mode”.

## Effect of different genetic analyzers

Four genetic analyzers were tested with the Investigator ESSplex Plus Kit in order to demonstrate its robustness. 500 pg Control DNA XY5 and Control DNA XY13 were each amplified in duplicate. The reaction took place under standard conditions and the PCR products were tested on all four genetic analyzers in parallel:

- Applied Biosystems 3500™ Genetic Analyzer
- Applied Biosystems 3130 Genetic Analyzer (2 independent instruments)
- ABI PRISM® 310 Genetic Analyzer

Similar overall peak heights and a comparable balance of peaks between individual markers of the same color channel, as well as between different channels, were observed (Figure 8). Variations reflected differences in sensitivity between individual instruments, e.g., 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 genetic analyzers.** 500 pg Control DNA XY5 and XY13 was amplified on a GeneAmp PCR System 9700 in duplicate. Samples were run according to the *Investigator ESSplex Plus Handbook*. Average peak heights for duplicates of both DNAs are shown.

## Sensitivity

The Investigator ESSplex Plus 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, e.g., using the QIAGEN Investigator Quantiplex Kit. In particular, for heavily degraded DNA, the use of increased template amounts may improve results (see page 17).

Control DNA XY5 was serially diluted from 2 ng to 8 pg per reaction. Full profiles (30 PCR products) were consistently obtained at 125 pg using the standard conditions specified in the *Investigator ESSplex Plus Handbook*. Occasional allele dropouts were found due to stochastic effects when  $\leq 63$  pg DNA was 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  $>1$  ng do not cause issues during PCR amplification, those 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.

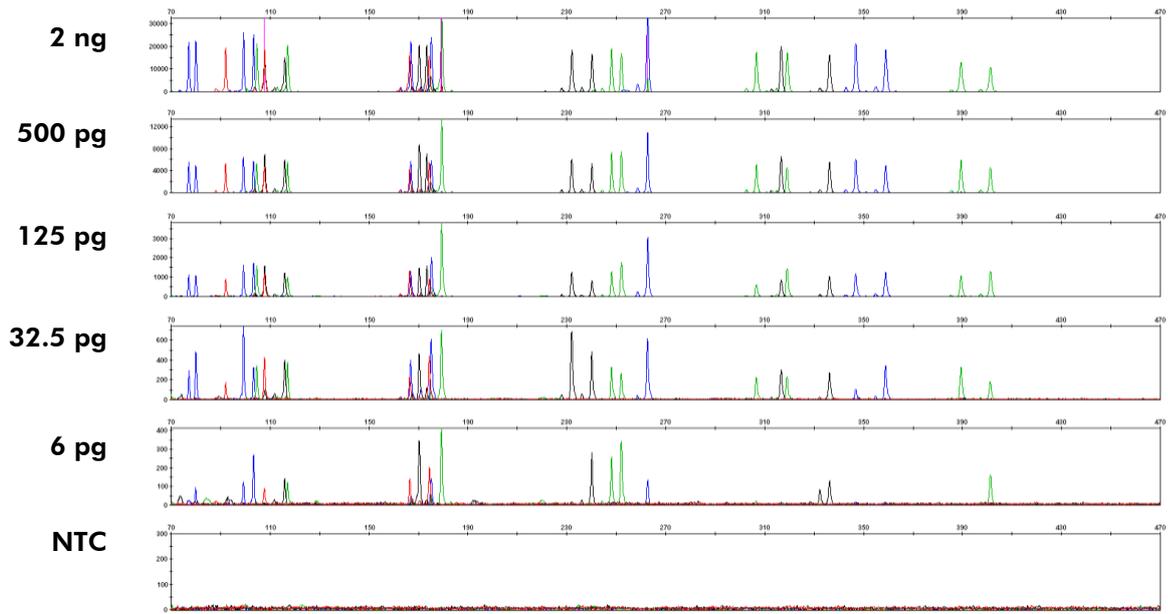
“Off-scale” peaks are often accompanied by “pull-up” peaks. Furthermore, higher stutter-peak height ratios may be observed as the signal from the main peak gets saturated. Finally, “split peaks” may occur as a result of incomplete +A nucleotide addition.

Poor STR profiles resulting from high DNA concentration can be improved by reamplifying a sample using less template DNA.

### Issues with very low amounts of DNA

Amplification of less than  $\sim 100$  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 with only small amounts of DNA, it is crucial to minimize background fluorescence, e.g., caused by dye artifacts. Figure 9 shows 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 XY5 were analyzed. The amounts of DNA indicated were used as template for amplification. **Note:** Y-axis scales were adjusted for best fit. The lowest panel shows a no-template (negative) control to illustrate the expected level of background fluorescence.

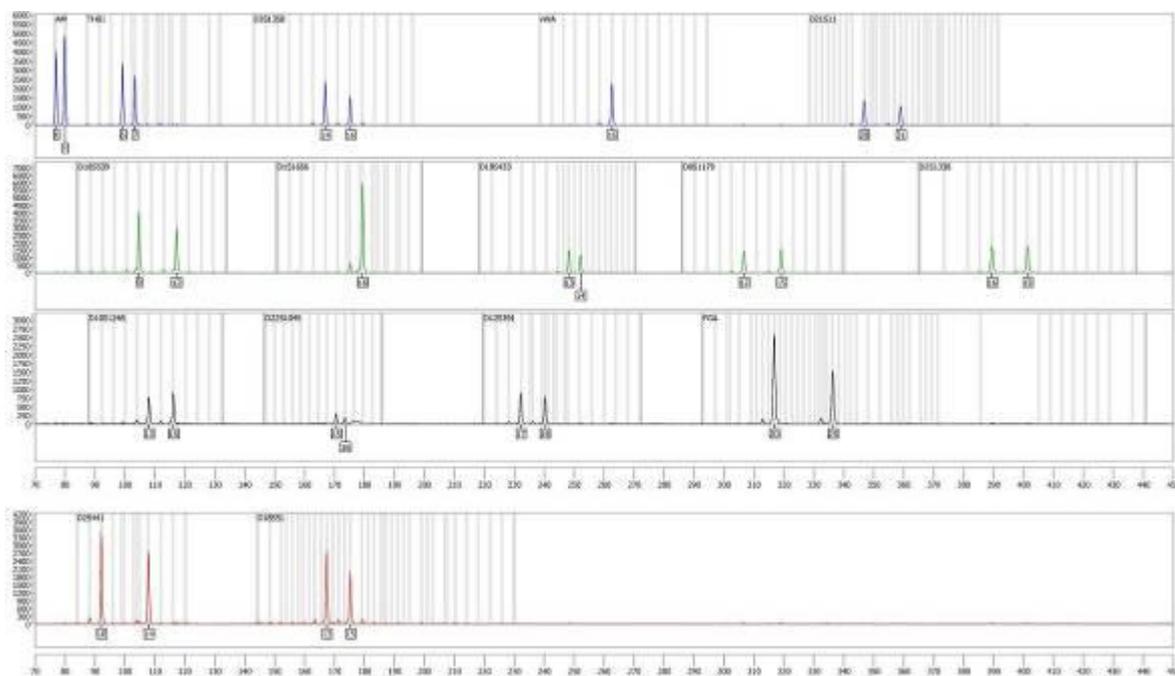
## Performance with simulated inhibition

If the DNA extraction from forensic casework samples is done using inappropriate methods, Investigator ESSplex Plus assay 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.

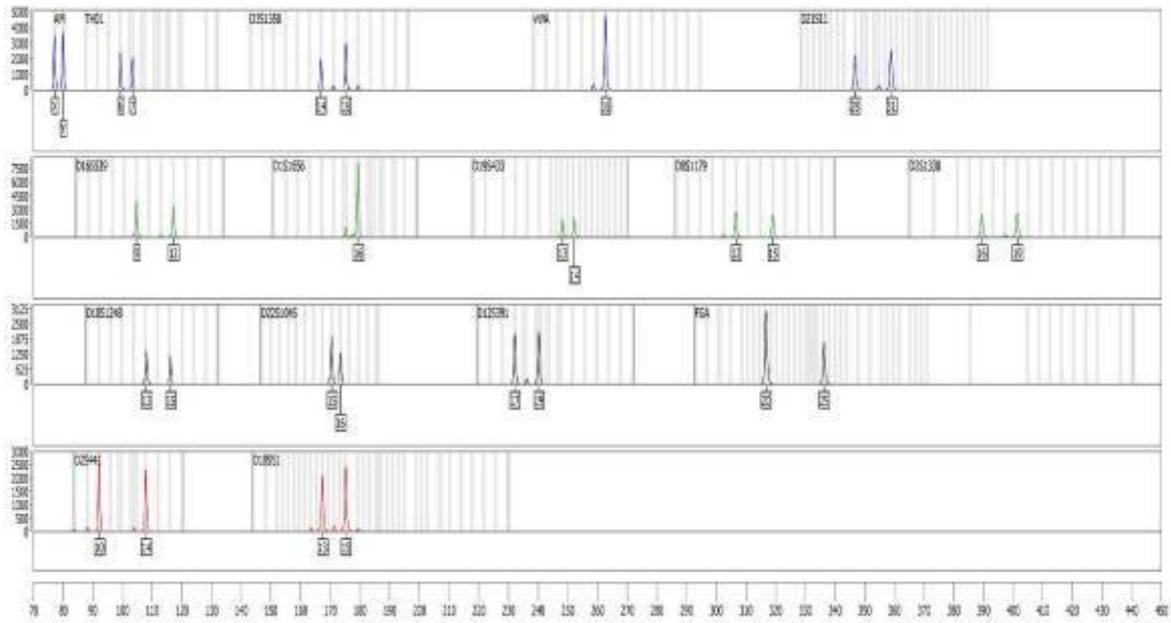
To test the robustness of the kit in the presence of inhibitors, assays were run in the presence of the following inhibitors, chosen to mimic challenging forensic sample types:

- Humic acid is a principal component of humic substances and has an inhibitory effect on PCR. It may be co-extracted from forensic samples collected from soil
- Hematin is 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 is typically present in leather. During extraction of casework evidence, tannic acid may get co-extracted with DNA
- Indigo carmine is the color of blue denim (e.g., jeans)
- Collagen is the main protein compound of many tissues
- Calcium is released during the lysis of bones

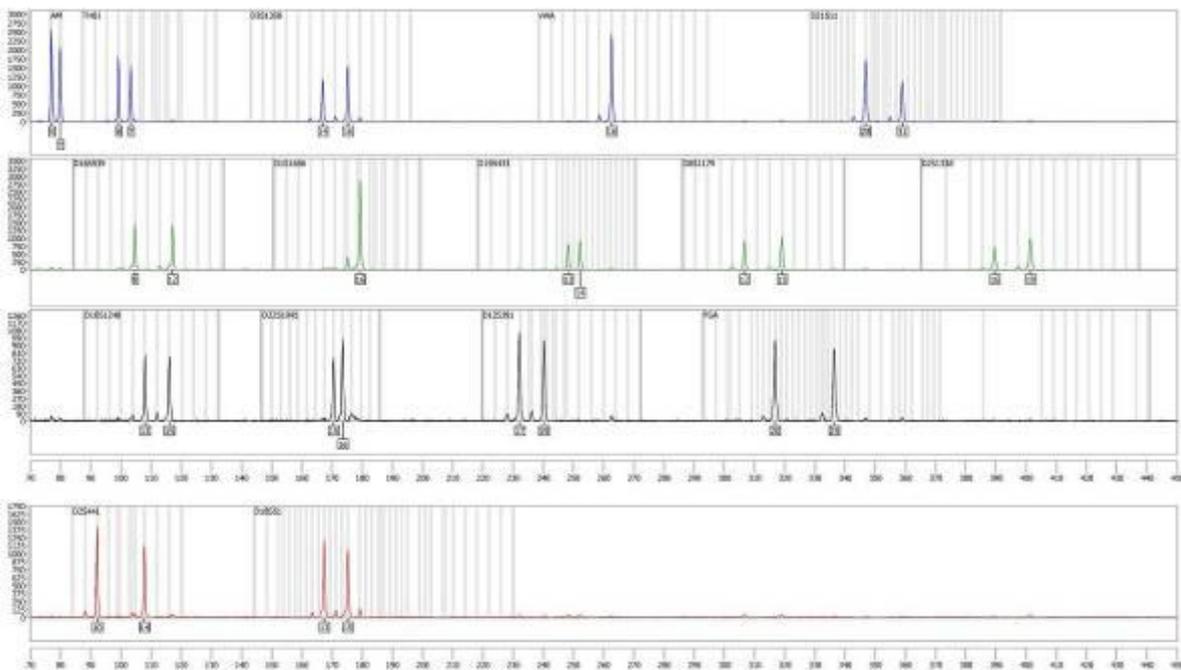
Results are shown in Figures 10–13. Figure 14 shows an overview of ESSplex Plus inhibitor resistance.



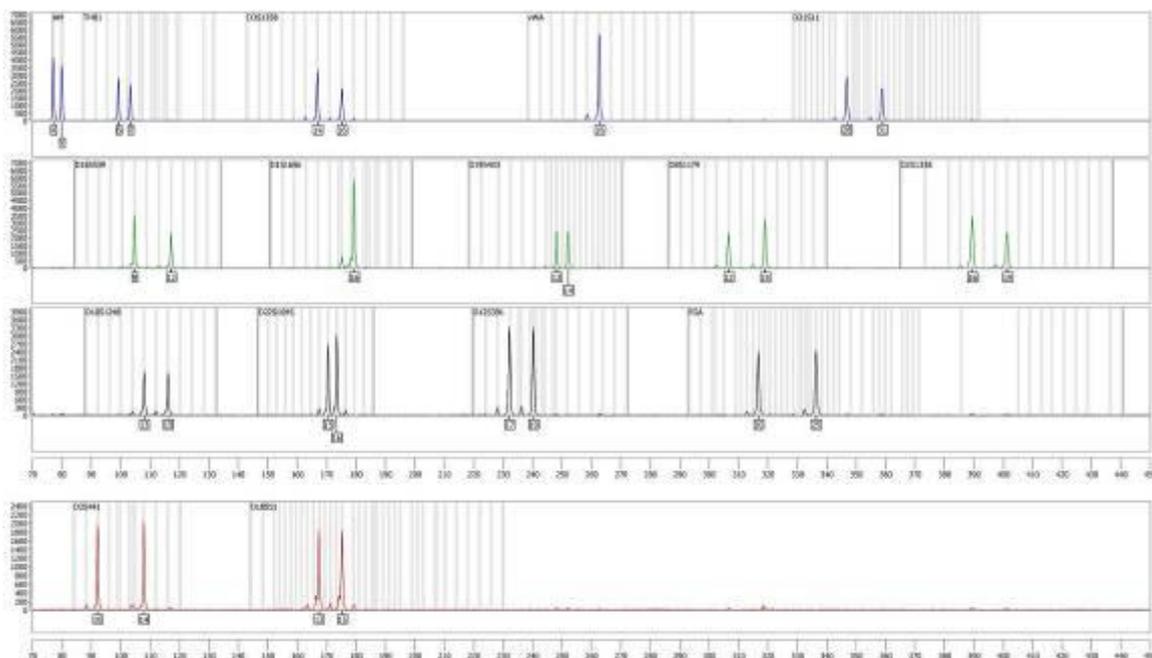
**Figure 10. Electropherogram of 500 pg Control DNA XY5 amplified in the presence of 150 ng/µl humic acid.** Data were analyzed using QIAGEN Investigator IDproof Software.



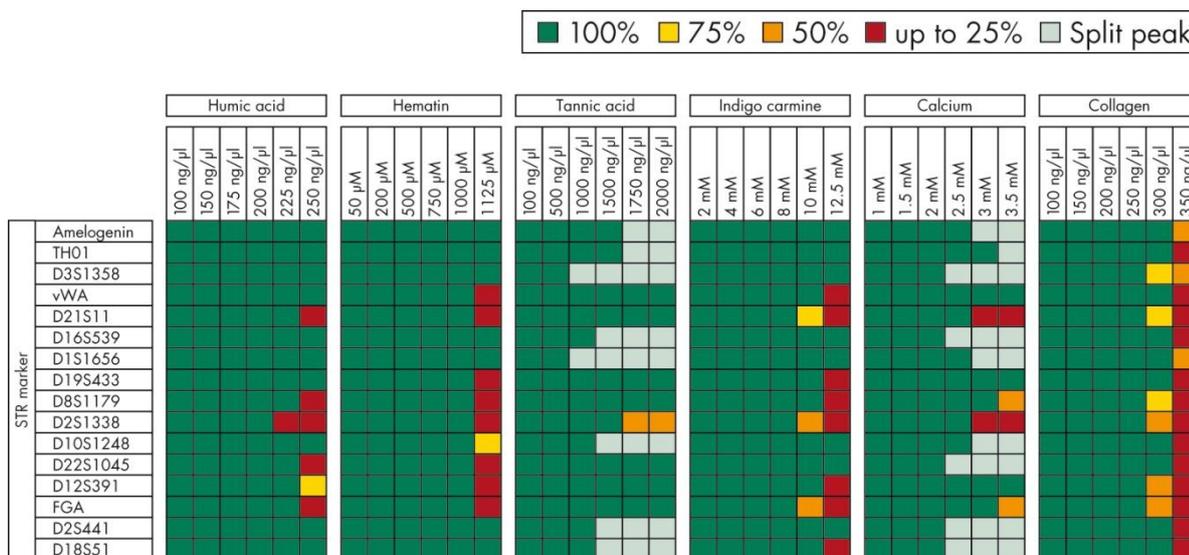
**Figure 11. Electropherogram of 500 pg Control DNA XY5 amplified in the presence of 500  $\mu$ M hematin. Data were analyzed using QIAGEN Investigator IDproof Software.**



**Figure 12. Electropherogram of 500 pg Control DNA XY5 amplified in the presence of 8 mM indigo carmine. Data were analyzed using QIAGEN Investigator IDproof Software.**



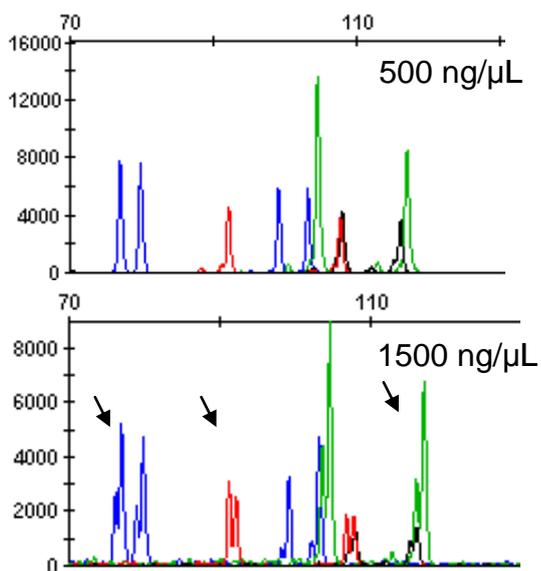
**Figure 13. Electropherogram of 500 pg Control DNA XY5 amplified in the presence of 150 ng/μl collagen.** Data were analyzed using QIAGEN Investigator IDproof Software.



**Figure 14. Overview of Investigator ESSplex Plus inhibitor resistance.** The assay was tested for its robustness toward inhibitors (humic acid, hematin, tannic acid, indigo carmine, calcium, and collagen). 500 pg of Control DNA XY5 was used as template and PCR was performed under standard conditions. 100 RFU was used as threshold for allele calling.

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. Tannic acid and calcium show 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 15). 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.

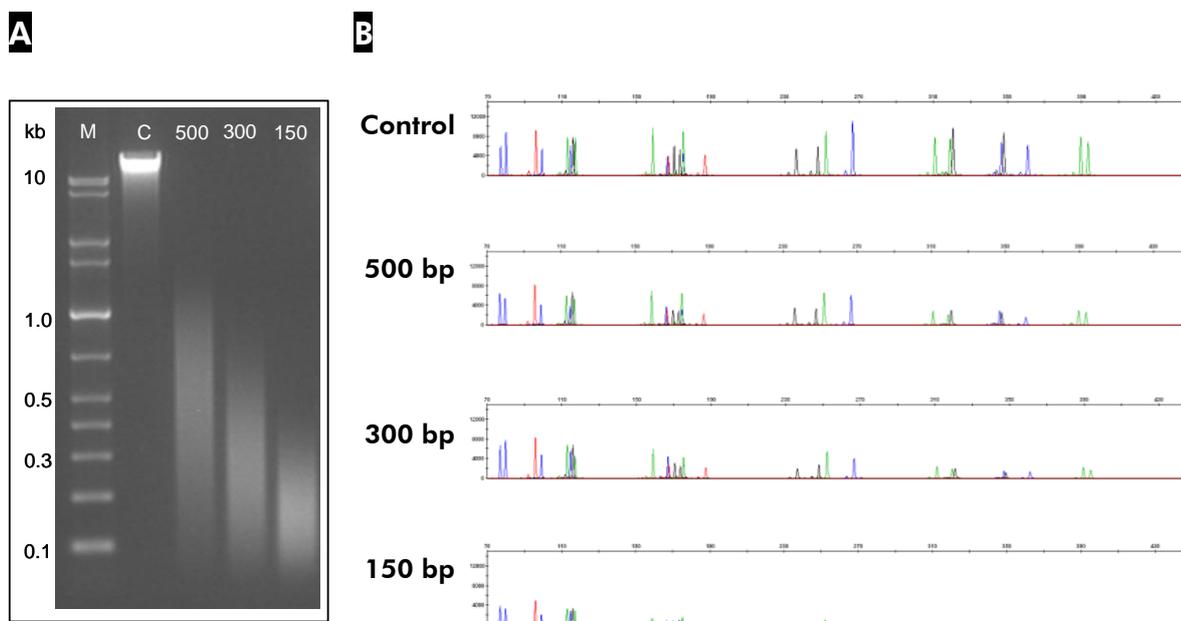


**Figure 15. Effect of high levels of tannic acid.** 500 pg Control DNA XY5 was amplified in the presence of 500 ng or 1500 ng/μl tannic acid. Arrows indicate PCR products showing split peaks caused by partial adenylation.

## Stability with degraded DNA

Casework evidence has often been exposed to adverse environmental conditions, e.g., UV light, humidity, or microbial growth. Human DNA extracted from those samples may 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. Fragment lengths were verified on an agarose gel and 500 pg DNA was used as template for amplification (all samples in duplicate; Figure 16A). 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, 45 out of 56 expected PCR products were detected using a threshold of 50 RFU (Figure 16B). Increased amounts of template (up to 2 ng), can be used to improve results for heavily degraded DNA.



**Figure 16. 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 an average fragment length of 300 bp.

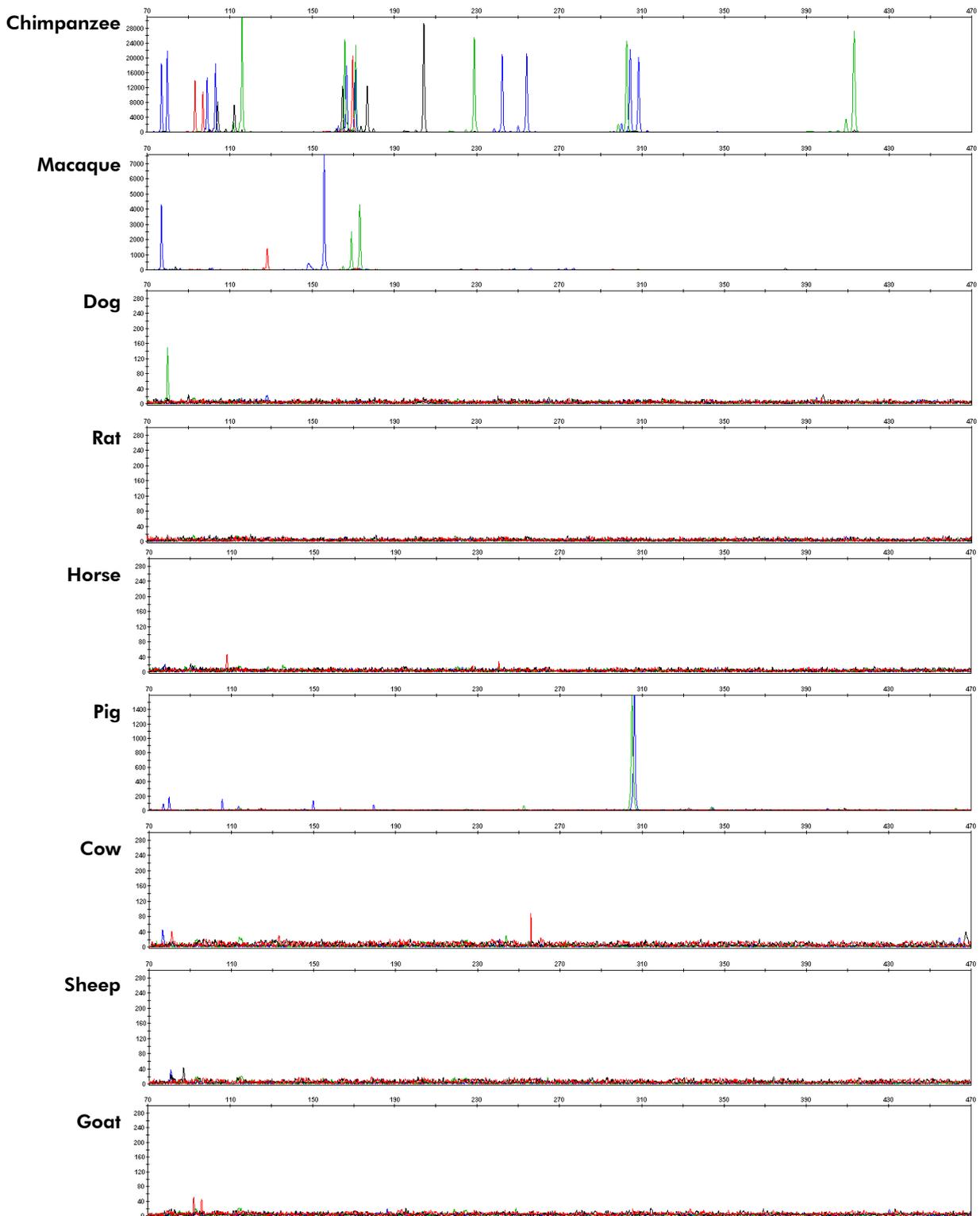
## Species specificity

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 Plus species specificity for human DNA, DNA from other species was each tested following the standard assay protocol (Figure 17).

Besides common pets and farm animals, some primates were also tested. As expected for primates, amplification of some products is possible. Chimpanzee, bonobo, orangutan, and to a lesser degree, gorilla give rise to several peaks within marker ranges in all channels, some of which match the size of human STR products. Macaque DNA produced an Amelogenin X-peak and further off-ladder peaks in the FAM™ and BTG panel.

Cat, rabbit, hamster, bird, mouse, rat, cow, horse, goat, and sheep did not give rise to any reproducible peaks in the analysis range.

Pig DNA produced a single peak of about 300 bp that is detected in the FAM and BTG panel. Dog produced a single peak of about 80 bp visible in the BTG panel, but it does not overlap with known human alleles.



**Figure 17. Representative results of the species specificity assessment.** 500 pg primate DNA and 2.5 ng DNA from all other species was used as a 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.

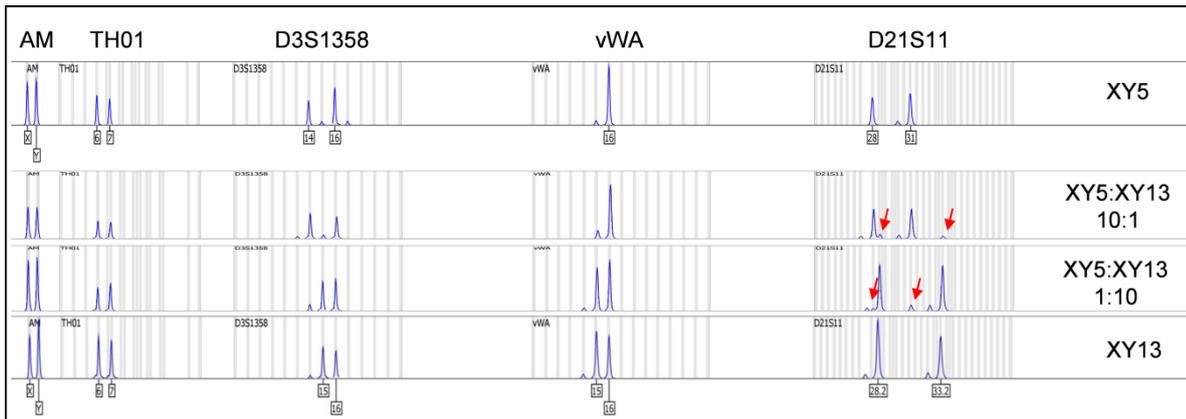
Samples were created by mixing control DNA XY5 and XY13 in ratios of 1:1, 3:1, 7:1, 10:1, 15:1, and vice versa (see Table 1 for the genotypes of the mixed samples). The total amount of mixed DNA used in this study was 500 pg; a 15:1 mixture thus contains 31 pg of the minor component DNA and 469 pg of the major component (Table 2). The limit of detection of the minor component was determined by analyzing non-overlapping alleles of both types of DNA. All of the expected alleles were found for the minor components of the 3:1 and 7:1 mixtures. 10:1 and 15:1 typically resulted in partial profiles of the minor component. An example for 10:1 mixtures is shown in Figure 18. Since these contain  $\leq 50$  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 (Figure 19) if the amount of available DNA is not limited. See "Sensitivity", page 11, for general considerations.

**Table 1. Genotypes of the DNA used for mixed samples**

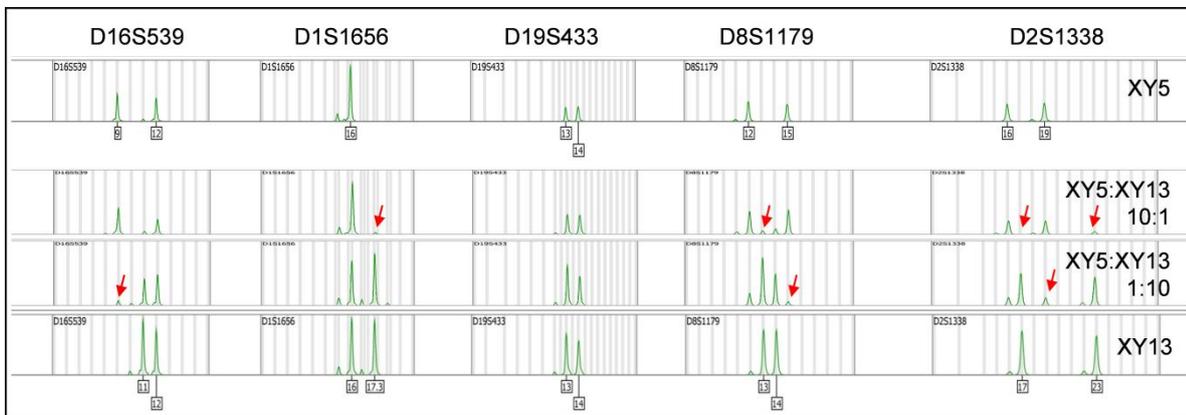
<b>Locus</b>	<b>XY5 genotype</b>	<b>XY13 genotype</b>
Amelogenin	X/Y	X/Y
D1S1656	16/16	16/17.3
D2S441	10/14	10/11
D2S1338	16/19	17/23
D3S1358	14/16	15/16
D8S1179	12/15	13/14
D10S1248	13/15	14/15
D12S391	17/19	16/18
D16S539	9/12	11/12
D18S51	13/15	14/15
D19S433	13/14	13/14
D21S11	28/31	28.2/33.2
D22S1045	15/16	17/18
FGA	20/25	22/23
TH01	6/7	6/7
vWA	16/16	15/16

**Table 2. Amounts of DNA template in the mixtures**

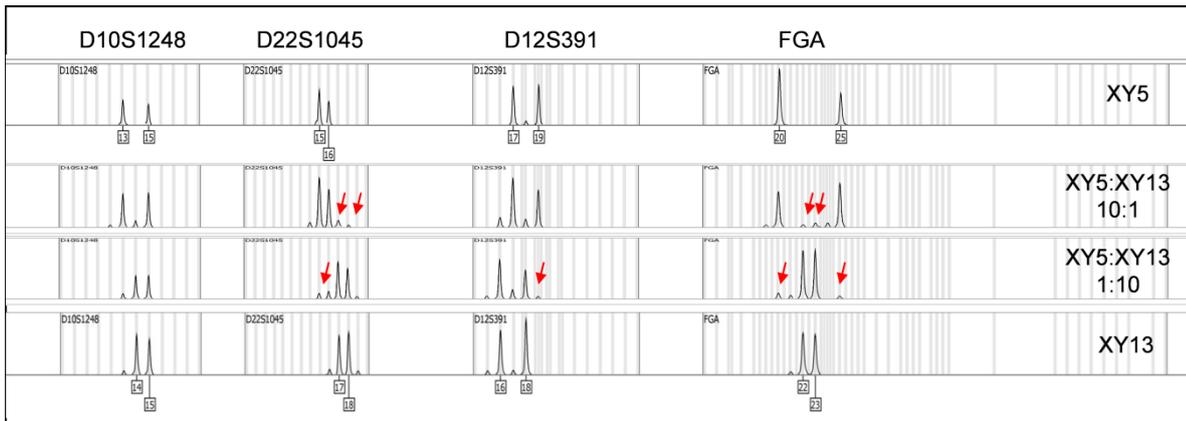
<b>Mixture ratio</b>	<b>Major component</b>	<b>Minor component</b>
1:1	250 pg	250 pg
3:1	375 pg	125 pg
7:1	437 pg	63 pg
10:1	450 pg	50 pg
15:1	469 pg	31 pg

**A**

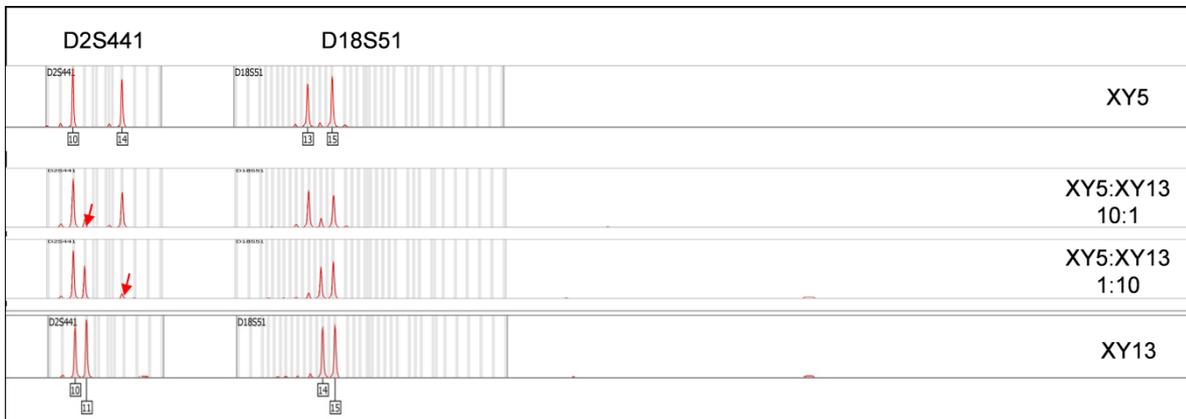
Amelogenin, TH01: Heterozygote + heterozygote, 4 overlapping alleles. Major component completely covers minor component. D3S1358: Heterozygote + heterozygote, 2 overlapping alleles, 2 alleles overlapping with stutter positions. Minor component is covered by major component, or visible as elevated stutter. vWA: Homozygote + heterozygote, 2 overlapping alleles, 1 allele overlapping with stutter position. Minor component is covered by major component, or visible as elevated stutter. D21S11: Heterozygote + heterozygote, no overlapping alleles. All minor component peaks can be assigned (arrows).

**B**

D16S539: heterozygote + heterozygote, 2 overlapping alleles, one allele overlapping with stutter position. The non-overlapping minor component peak can be assigned (arrow). D1S1656: homozygote + heterozygote, 2 overlapping alleles. The non-overlapping minor component peaks can be assigned (arrows). D19S433: heterozygote + heterozygote, 4 overlapping alleles. Major component completely covers minor component. D8S1179: heterozygote + heterozygote, no overlapping alleles, 2 alleles overlapping with stutter positions. The 2 non-overlapping minor component peaks can be assigned (arrows). D2S1338: heterozygote + heterozygote, no overlapping alleles, 1 allele overlapping with stutter position. 2 out of 3 non-overlapping minor component peaks can be assigned; allele 17 of XY13 dropped out (arrow).

**C**

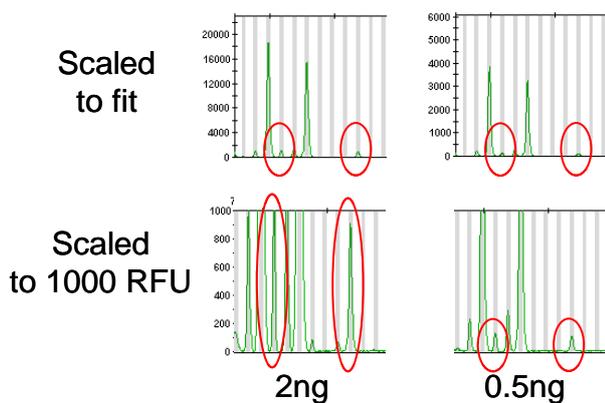
D10S1248: Heterozygote + heterozygote, 2 overlapping allele, 2 alleles overlapping with stutter position. Minor component is covered by major component, or visible as elevated stutter.  
D22S1045: Heterozygote + heterozygote, no overlapping alleles, 1 allele overlapping with stutter position. The 3 non-overlapping minor component peaks can be assigned (arrows).  
D12S391: Heterozygote + heterozygote, no overlapping alleles, 3 alleles overlapping with stutter positions. The non-overlapping minor component peaks can be assigned (arrows).  
FGA: Heterozygote + heterozygote, no overlapping alleles. All 4 non overlapping minor component peaks can be assigned (arrows).

**D**

D2S441: Heterozygote + heterozygote, two overlapping alleles. The 2 non-overlapping minor component peak can be assigned (arrow).  
D18S51: Heterozygote + heterozygote, 2 overlapping alleles, 2 alleles overlapping with stutter positions. Minor component is covered by major component, or visible as elevated stutter.

**Figure 18. Results of the mixture analysis.** Figures show 1:10 mixtures of DNA XY5 and XY13 (middle panels of each figure) and the corresponding single source samples as a reference (upper and lower panel of each figure). Red arrows indicate the expected positions of a peak of the minor component DNA that does not overlap with a main peak or of a forward stutter peak of the major component. A brief description of the mixture situation given for each individual marker is given below the figures. All but one of the expected peaks were found with a threshold of detection of 50 RFU. In this example, only allele 17 of D2S1338 of DNA XY13 dropped out (Figure 18B, second panel).

Figure 19 shows an example of how such dropouts can be recovered by using more template DNA.



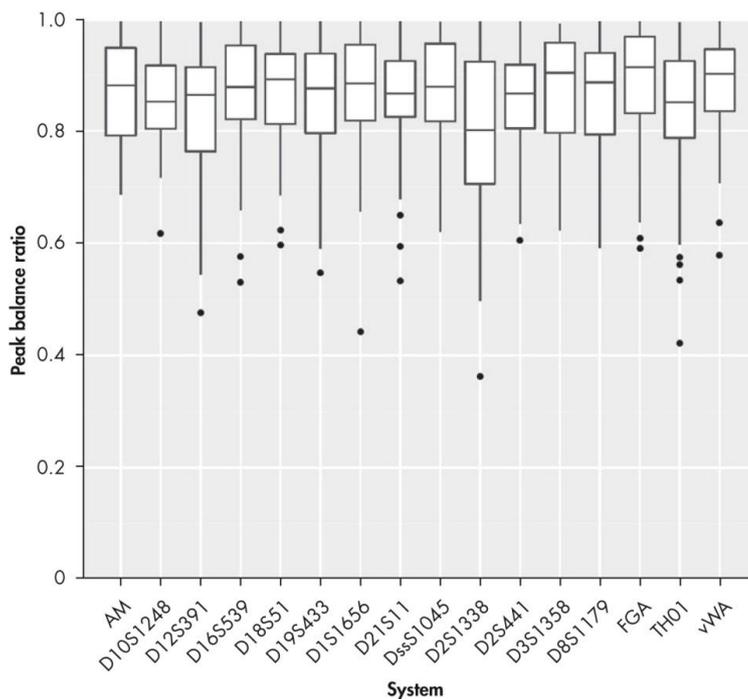
**Figure 19. Analysis of DNA mixtures — effect of increased template amount.** Control DNA XY13 and XY5 were mixed in a 1:10 ratio. 0.5 ng or 2 ng of the mixture was amplified. Marker D2S1338 is shown; the main peaks correspond to allele 16 and 19 of DNA XY5. Allele 17 and 23 of the minor component DNA XY13 are indicated with red circles. Signal ratios of minor and major components are unchanged (upper panel), while the signal of the minor component is pushed up against background when the increased template is used (lower panel).

## Reproducibility — concordance test

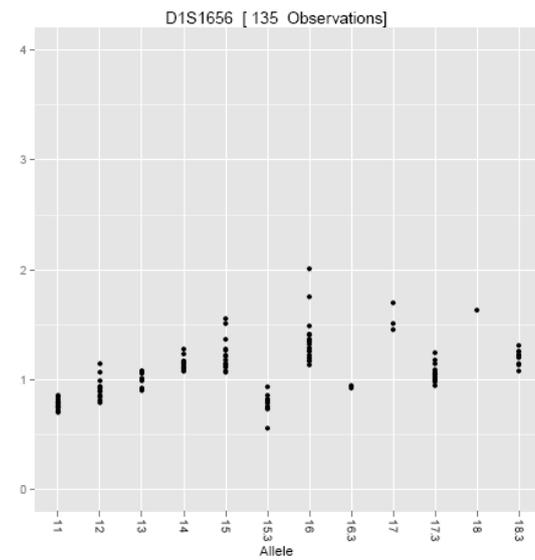
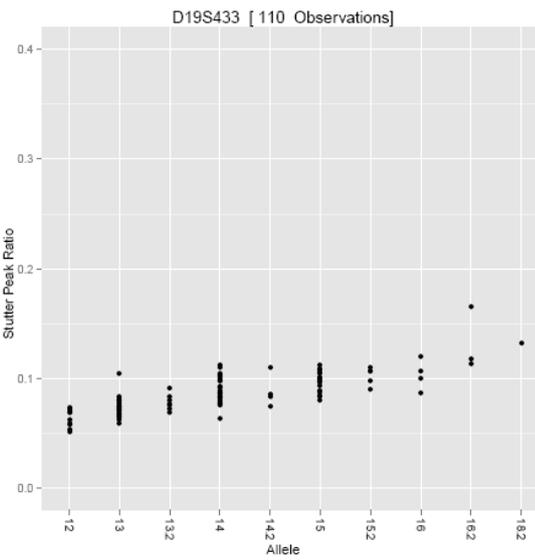
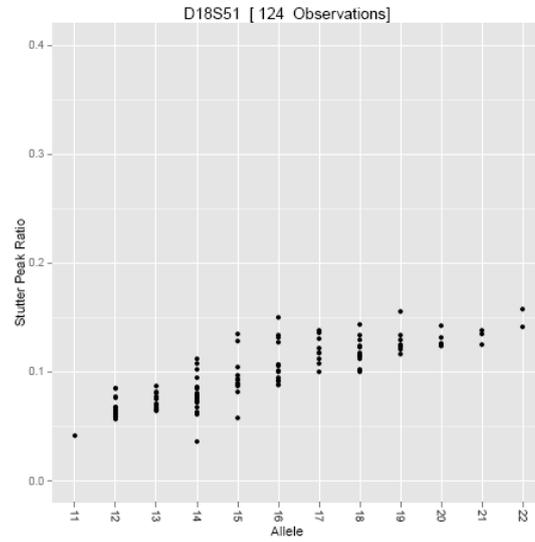
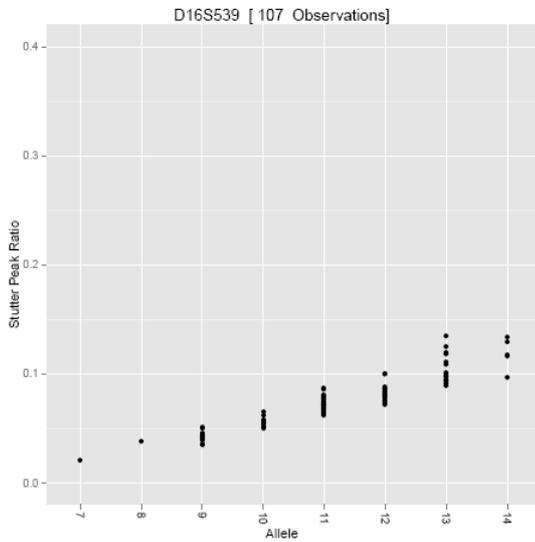
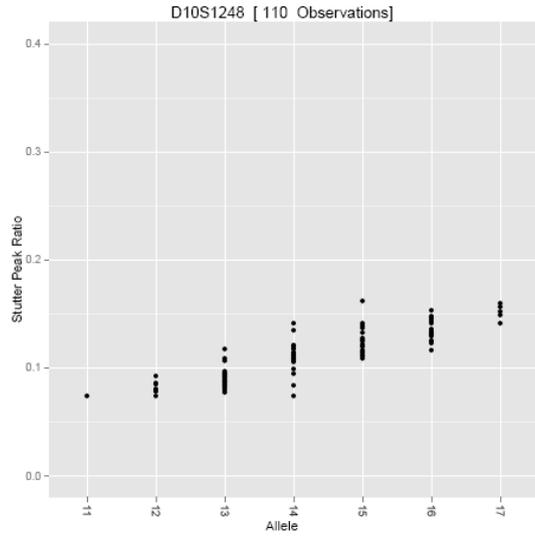
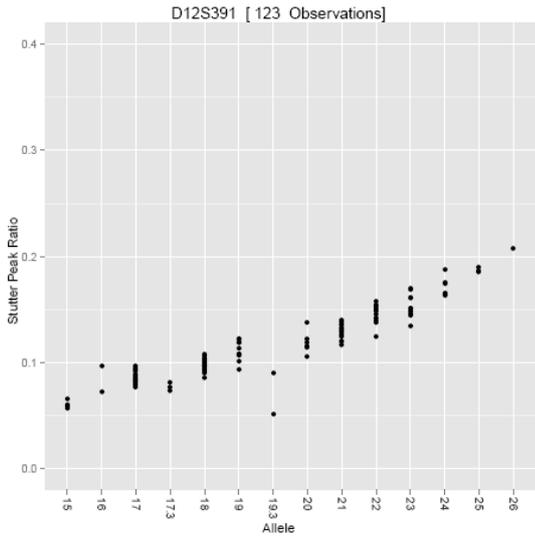
All of the primer binding sites of the Investigator ESSplex Plus Kit are the same as those of the Investigator ESSplex assay. Minor changes (5' tailing of unlabeled primers) have been introduced to some sequences in order to optimize adenylation of PCR products and allow faster PCR cycling.

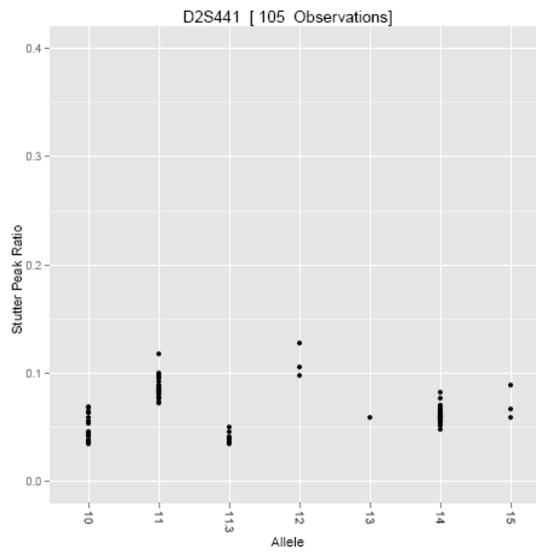
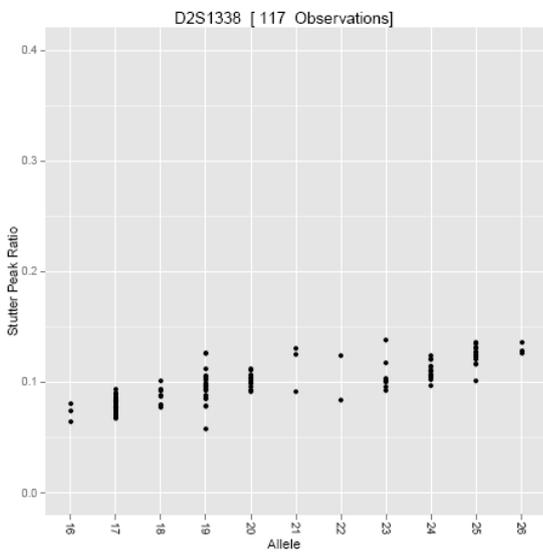
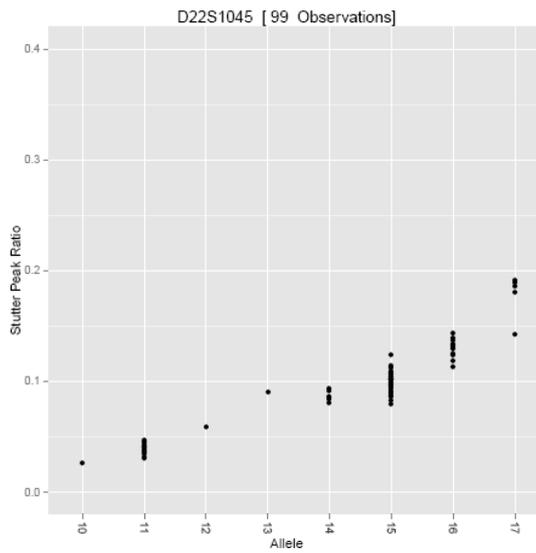
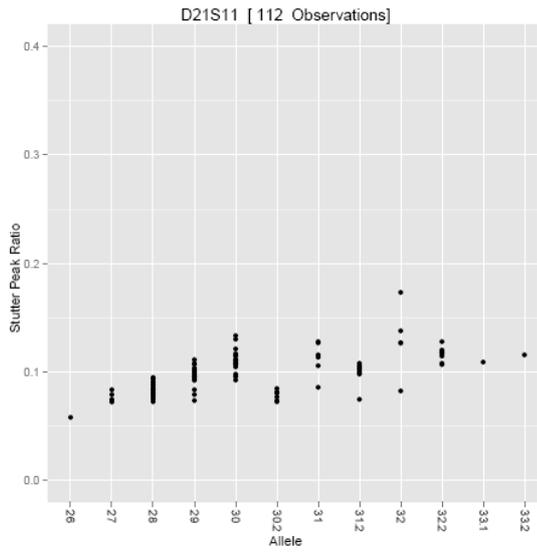
A concordance study with an internal DNA pool of 632 Caucasian samples in total was conducted. Identical DNA profiles were obtained for all samples using either the Investigator ESSplex or the ESSplex Plus Kit, indicating that the minor primer changes between the two primer sets do not affect allele calls. The internal DNA pool had previously been analyzed using the Applied Biosystems AmpF $\phi$ STR<sup>®</sup> Identifiler<sup>™</sup> PCR Kit. Genotypes obtained for all 10 overlapping loci and Amelogenin were fully concordant for all but 3 samples. These nonconcordant samples were previously typed for D19S433 as homozygous allele 13, but revealed a heterozygous 12/13 genotype when analyzed with the ESSplex Plus Kit. No nonspecific amplification was observed in any of the samples.

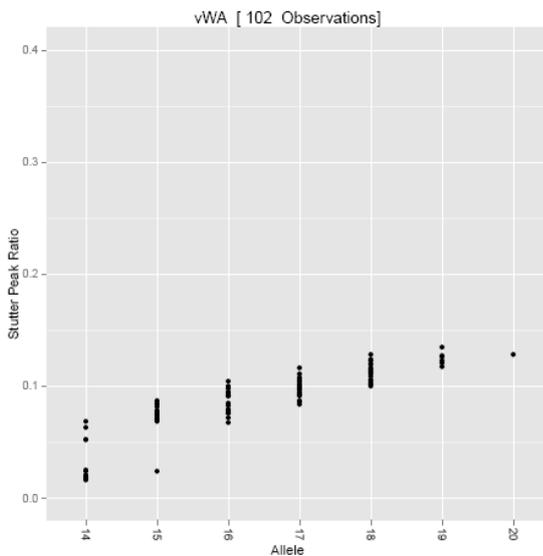
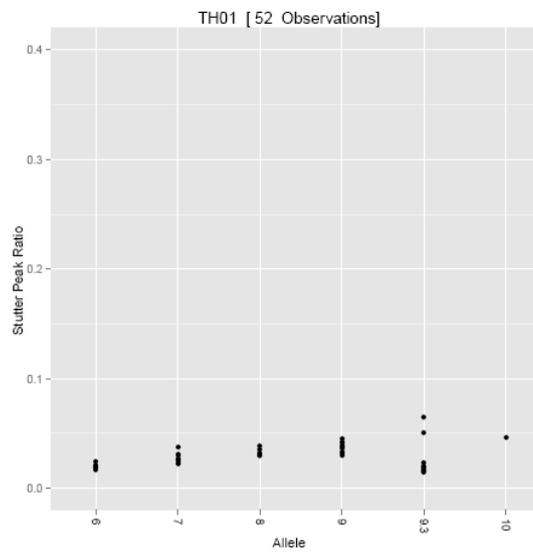
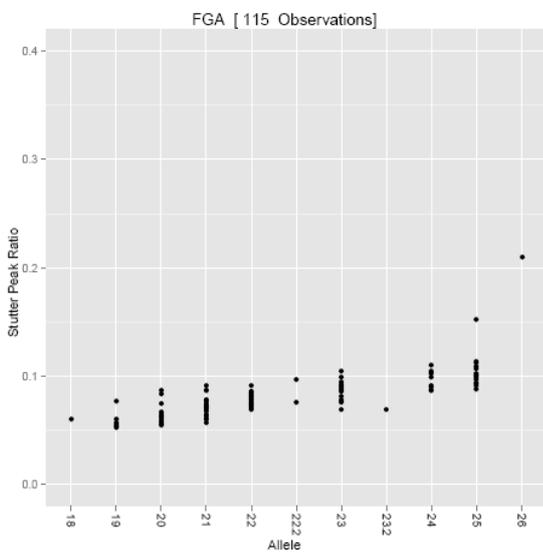
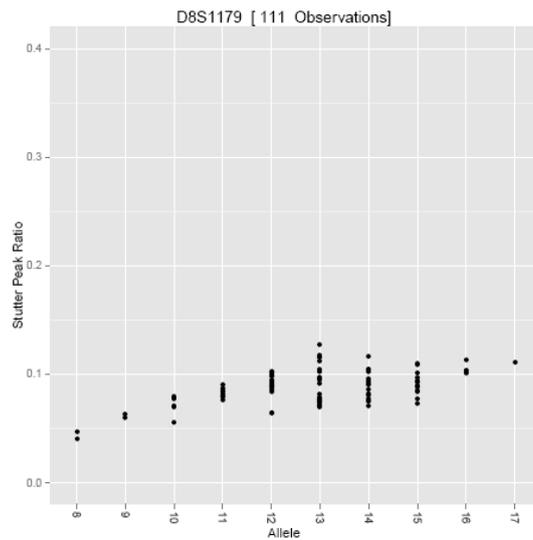
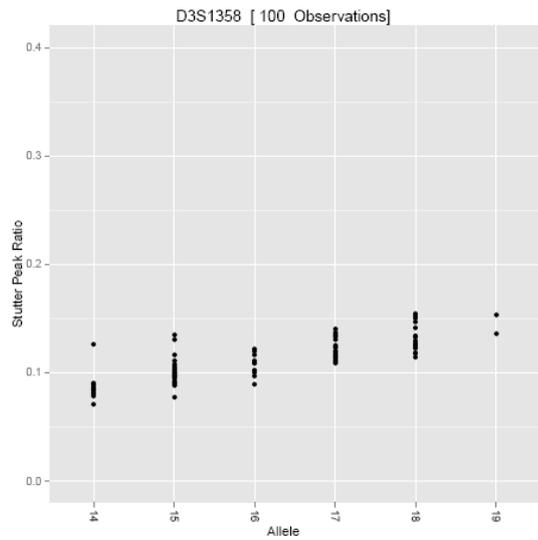
Peak height ratios of heterozygous markers (Figure 20) and stutter peak heights (Figure 21) were analyzed for a subset of 92 samples of the internal DNA pool.



**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.



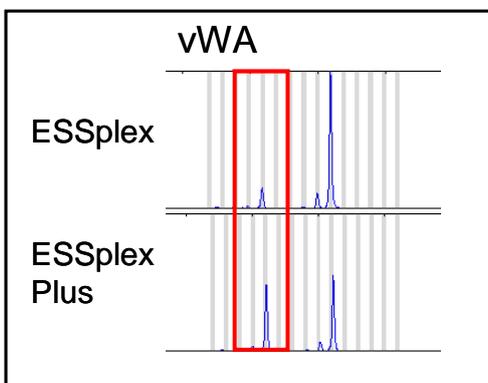




**Figure 21. Stutter peak height ratios.** Peak heights of forward stutters compared to main peaks were analyzed for 92 DNA samples from different donors.

Stutter peak heights are characteristic for each marker and the number of repeat motifs of an allele. TH01 in general shows the lowest stutter ratio of all STR markers of the Investigator ESSplex Plus Kit. Smaller alleles display lower stutter levels than longer alleles of the same marker. Only forward stutter positions were analyzed, as backward stutters usually are of very low height, although present. Please note that marker D22S1045 shows a significantly elevated backward stutter. This is intrinsic and due to the fact that the marker consists of trinucleotide instead of tetranucleotide repeats. This may also lead to unexpectedly high forward stutter peaks if alleles differ by two repeat units and the backward stutter of the smaller allele overlaps with the forward stutter of the longer allele. This is true for Control DNA 9948 (see Figure 6 for an example).

A previous concordance study on an American population set (conducted by Rebecca Hill and John Butler, NIST) using the Investigator ESSplex Kit revealed a primer binding-site mutation affecting the amplification efficiency of vWA alleles in 22 of 255 samples of African-American origin (Figure 22).



**Figure 22. vWA amplification of a sample carrying a primer binding site mutation.** 500 pg of DNA OT05600 (kindly provided by Rebecca Hill, NIST) was amplified with the Investigator ESSplex or the ESSplex Plus Kit. The allele boxed in red shows less efficient amplification in the ESSplex Kit due to a sequence variation in this African-American sample.

## References

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### Cited references

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

## Ordering Information

Product	Contents	Cat. no.
Investigator ESSplex Plus Kit (100)	Primer mix, Fast Reaction Mix including HotStarTaq <i>Plus</i> DNA Polymerase, Control DNA, allelic ladder ESSplex SE Plus, DNA size standard 550 (BTO), and nuclease-free water	381535
Investigator ESSplex Plus Kit (400)	Primer mix, Fast Reaction Mix including HotStarTaq <i>Plus</i> DNA Polymerase, Control DNA, allelic ladder ESSplex SE Plus, DNA size standard 550 (BTO), and nuclease-free water	381537

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