QIAGEN Validation Report

Developmental validation of the Investigator® HDplex Kit

The QIAGEN® Investigator HDplex Kit is used for multiplex PCR in human identification and forensic casework. It was developed for the reliable generation of DNA profiles from blood and buccal swabs.

The Investigator HDplex Kit was validated for various reaction conditions and situations that can arise during its storage and transport, as well as during the processing of forensic samples. Optimal reaction conditions were established, and the effects of variations in those conditions were assessed. The kit was tested in house and in independent external forensic laboratories.

A range of thermal cyclers and genetic analyzers were used to demonstrate the robustness of the assay (page 2 and page 3). Its sensitivity to different cycle numbers (page 4), and to variations in PCR annealing temperature (page 5) were also assessed, as was its sensitivity to serial dilutions (page 6). The stability of the components was validated with regard to repeated freezing and thawing (page 8), long-term storage (page 9), and transport (page 10). Specific issues that can arise during forensic casework were investigated, including cross-reactivity with non-human DNA (page 11) and inhibition due to contaminants introduced during extraction (page 12). The reproducibility of the results was also verified (page 14).

The validation of the Investigator HDplex Kit showed that it yields robust and reproducible results within the normal range of conditions expected in forensic casework, as well as in long-distance transport and long-term storage.

Note: All of the electropherograms shown were generated on an Applied Biosystems® 3130 Genetic Analyzer. The standard conditions specified in the Investigator HDplex Handbook were used for the electrophoresis.

Results of developmental validation

Effect of different cycler types

Multiple PCR thermal cyclers were tested with the Investigator HDplex Kit to demonstrate its robustness. 500 pg Control DNA XY5 was used as a PCR template. The reaction took place under standard conditions 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)
- DNA Engine® PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany)
- Techne® TC-512 Thermal Cycler (biostep GmbH, Jahnsdorf, Germany)
- T1 Thermal cycler (Biometra biomedizinische Analytik GmbH, Göttingen, Germany)
- Eppendorf® Mastercycler® ep (Eppendorf AG, Hamburg, Germany)

The electropherograms in Figure 1 show comparable mean peak heights for all of the tested PCR cyclers. No imbalance, dropouts, or preferential amplification for the STR systems was observed on any of the thermal cyclers. If using the GeneAmp PCR System 9700 with an Aluminum Block use "Std Mode". If using a Silver 96-Well Block or Gold-plated Silver 96-Well Block, use "9600 Emulation Mode". Do not use "Max Mode".

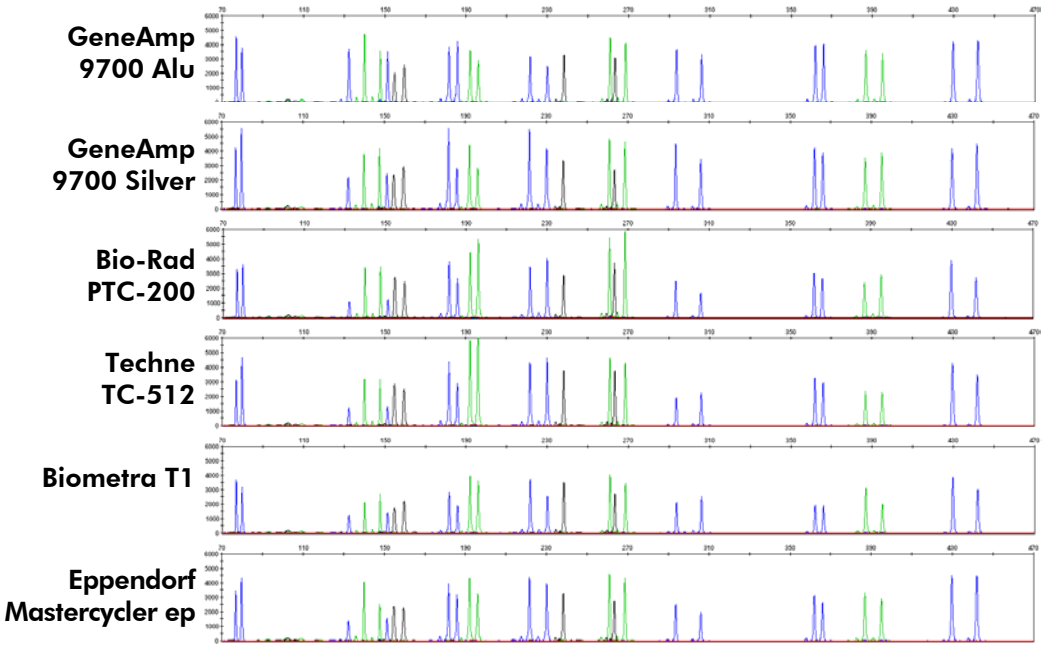

Figure 1. Effect of different cycler types on mean peak height.

Effect of different genetic analyzers

The Applied Biosystems 3130 Genetic Analyzer and ABI PRISM® 310 Genetic Analyzer were tested with the Investigator HDplex Kit in order to demonstrate its robustness. 500 pg Control DNA XY5 was used as a PCR template. The reaction took place under standard conditions and the PCR products were tested on both genetic analyzers in parallel.

The electropherograms in Figure 2 show comparable mean peak heights for both of the tested genetic analyzers. No imbalance, dropouts, or preferential amplification for the STR systems was observed on either genetic analyzer.

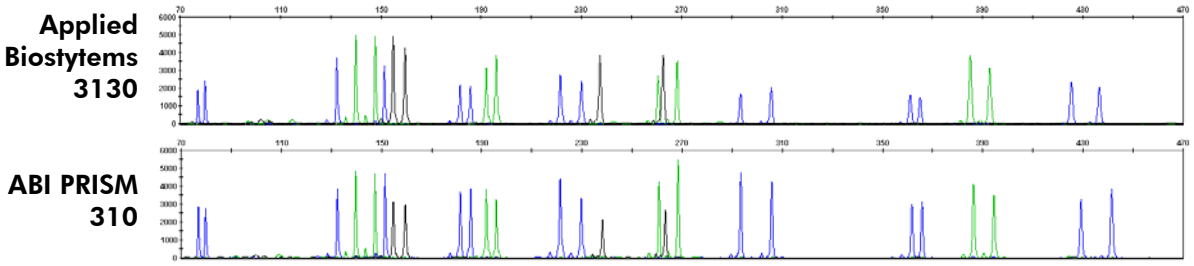

Figure 2. Effect of different genetic analyzers on mean peak height.

Effect of different cycle numbers

Altering the number of cycles can improve amplification when working with low-copy-number DNA or a large amount of template DNA (e.g., >1 ng).

To test the effect of changing the number of cycles, 1 ng Control DNA XY5 was used for 28 cycles, while 500 pg was used for 30 cycles, 100 pg was used for 32 cycles, and 50 pg was used for 34 cycles, respectively (Figure 3).

Complete profiles were generated under all of the tested conditions. It should be noted that an increase in cycle number to more than 30 may also increase the chance of unspecific products. Furthermore, because of stochastic effects, peak imbalance or dropouts may be observed for low-copy-number samples (with 100 pg or less of template DNA), regardless of any increase in cycle numbers.

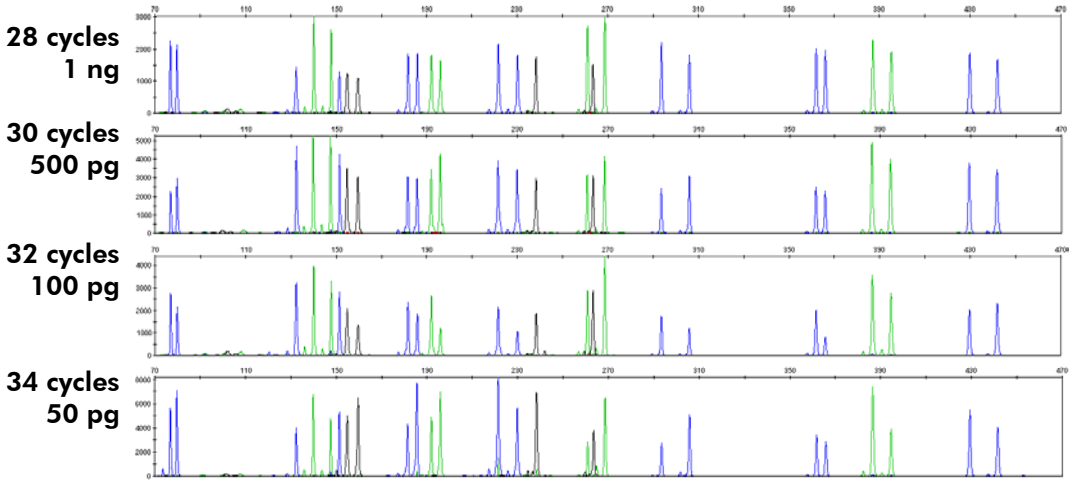

Figure 3. Effect of different cycle numbers on mean peak height. To better visualize smaller peak heights, the y-axis scales were adjusted for best fit.

Effect of PCR annealing temperature variations

Specificity and sensitivity are critical in forensic casework, and both 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 for the Investigator HDplex reaction (60°C).

Annealing temperatures of 58°C, 59°C, 60°C, 61°C, and 62°C were applied to the amplification of 500 pg Control DNA XY5. The PCR was performed on an Eppendorf Mastercycler ep and analyzed on an Applied Biosystems 3130 Genetic Analyzer.

As shown in Figure 4, no allelic dropout was detected within ±2°C of the optimal T_m of 60°C. This shows there was no loss in sensitivity under these conditions. Furthermore, no unspecific peaks occurred, indicating good specificity in this T_m range.

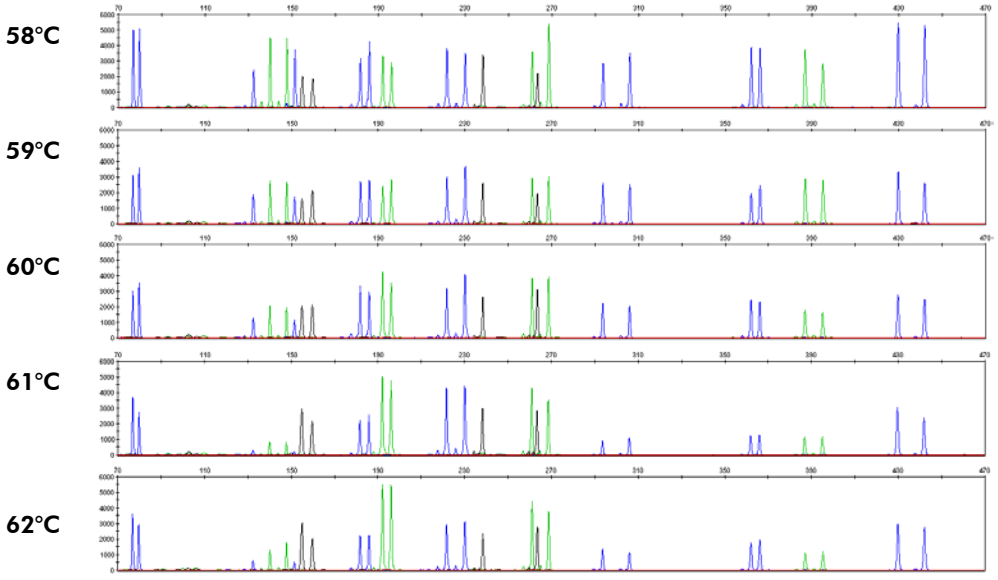

Figure 4. Effect of variations in the PCR annealing temperature.

Sensitivity to serial dilutions

The Investigator HDplex Kit is designed to detect a range of DNA quantities. The optimal amount of input DNA to yield good quality STR profiles is 500 pg. Figure 5 (page 7) shows a serial dilution of Control DNA XY5 from 500 pg to 32 pg. The optimal linear dynamic range of the assay is in the range of 500 to 125 pg. Full profiles (26 alleles) were consistently obtained at 125 pg using the standard conditions specified in the *Investigator HDplex Handbook*.

Issues with very high amounts of DNA

An amount of DNA above the optimal range (>1 ng) may give inaccurate or unusable data. The fluorescence intensity may cause peaks to go off the scale. Such data cannot be quantified accurately based on peak height and area. As a secondary effect, irregular stutter-peak heights may occur.

"Off scale" peaks are often accompanied by "pull up" peaks. This effect hinders the multi-component analysis, and is caused by an inaccurate spectral separation. 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 DNA.

Issues with very low amounts of DNA

An amount of DNA in the PCR below the optimal range (<125 pg) may lead to incomplete profiles, where partial profiles lacking alleles occasionally occur. 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 analyzer runs of the same sample to complete the partial profiles, or by adding the maximum volume of the DNA template to the PCR.

The Investigator HDplex Kit was developed for use with reference samples, especially for applications in paternity testing, rather than casework samples that may involve low amounts of DNA.

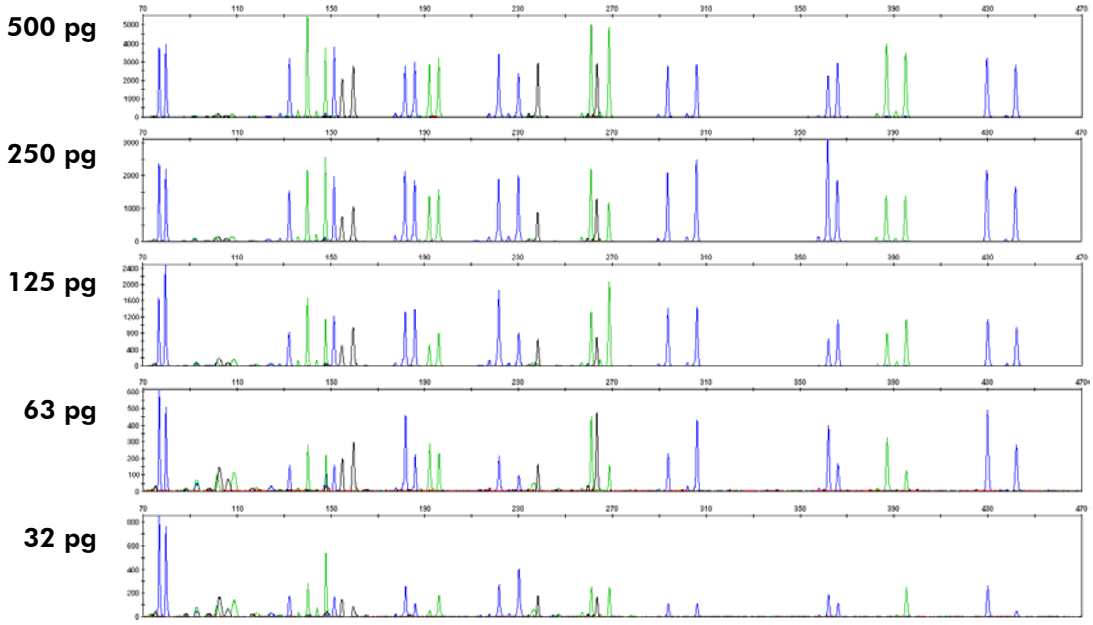

Figure 5. Effect of serial dilution of control DNA XY5 during amplification. The y-axis scales were adjusted for best fit.

Stability

20 freeze/thaw cycles

In a forensic lab, it is possible that the maximum number of reactions of a kit will not be performed in a single day. Therefore, the Investigator HDplex components were tested to prove 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 6 shows the electropherograms obtained by amplifying 500 pg Control DNA XY5 with fresh kit components (No Freeze/Thaw) and with kit components stressed by 20 rounds of freezing and thawing (20x 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.

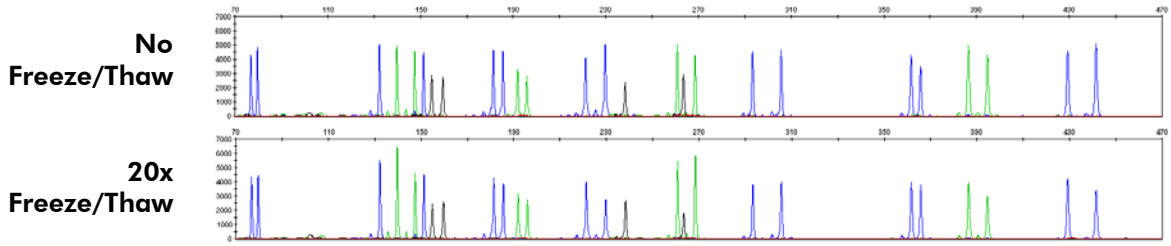

Figure 6. Results of a simulated freeze/thaw stability test of kit components.

Long-term stability

Forensic kits must be viable after long-term storage. Investigator HDplex Kit components were stored for 1 year at –20°C. After 1 month, 2 months, 3 months, 6 months, and 12 months, the kit performance was tested by amplifying 500 pg control DNA XY5 under standard PCR conditions. The electrophoresis was performed on an Applied Biosystems 3130 Genetic Analyzer. Over the course of the experiment, the overall kit performance was found to be stable (Figure 7).

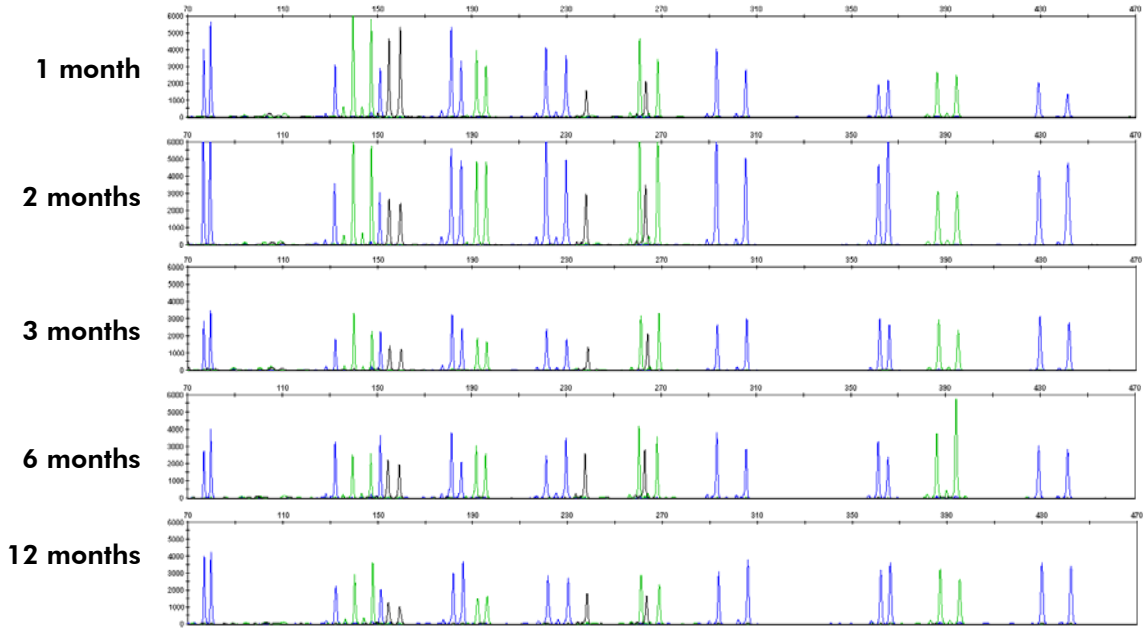

Figure 7. Effects of long-term storage. The kit components were tested at the indicated times.

Simulated shipment condition on dry ice

Investigator HDplex Kits are shipped on dry ice. To assess the performance of the kit after such transportation, a 5-day storage on dry ice and at –20°C was simulated.

Kits were stored for 16 h on dry ice, then transferred to –20°C for 8 h. This cycle was repeated for 5 days. Each day, components from these kits were used to amplify 500 pg Control DNA XY5. The electrophoresis was performed on an Applied Biosystems 3130 Genetic Analyzer.

The results indicate that the performance before and after storage on dry ice was comparable (Figure 8).

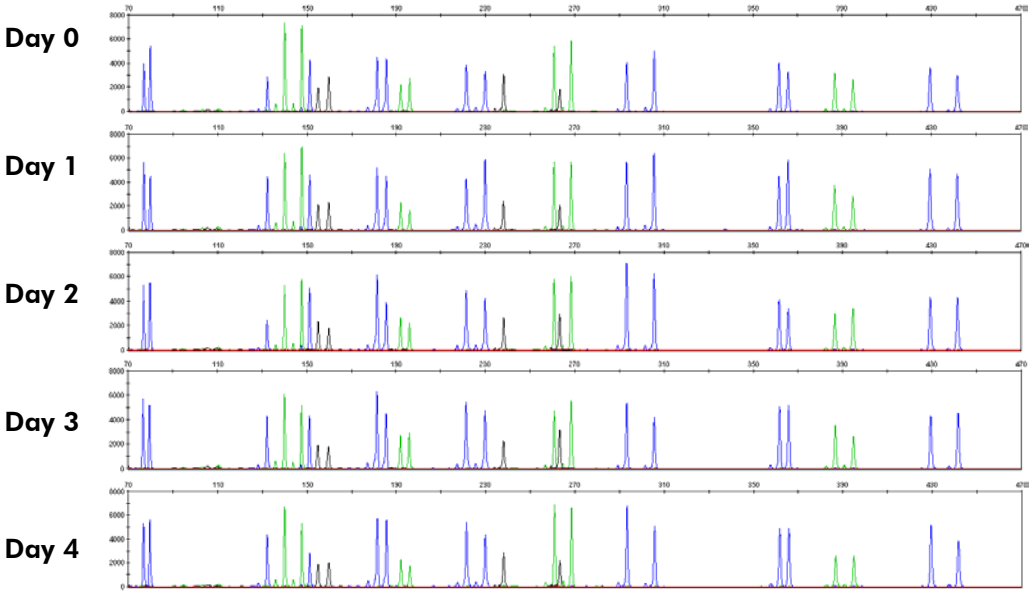

Figure 8. Prolonged storage of Investigator HDplex components on dry ice.

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 HDplex species specificity, 2.5 ng of DNA from dog, cat, rabbit, cow, and pig were each tested following the standard assay protocol, with 500 pg Control DNA XY5 as a positive control.

The presence of any amplified peaks in the electropherograms would have suggested cross reactivity with DNA from the non-human species. None of the tested DNA yielded any detectable product peaks under the chosen conditions, as shown in Figure 9.

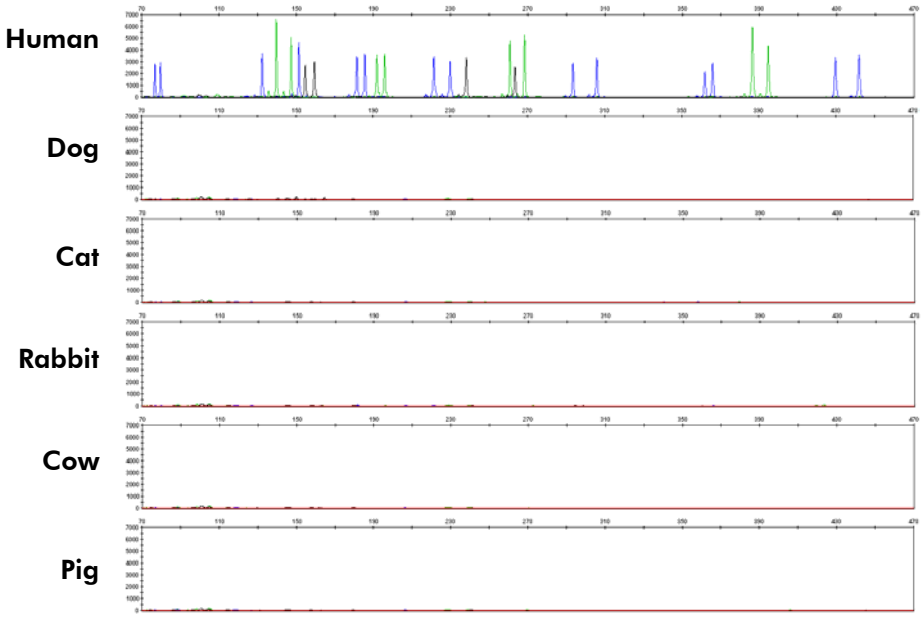

Figure 9. Results of the species specificity assessment.

Performance with simulated inhibition

While the Investigator HDplex has been optimized for use with reference samples in applications such as paternity testing, the kit has been tested with various concentrations of inhibitors as sometimes seen in difficult samples. If the DNA extraction from forensic casework samples is done using inappropriate methods, Investigator HDplex assay performance may be compromised. QIAGEN sample preparation technology is recommended for extraction, as it yields pure DNA free of inhibitors.

Humic acid, a principle component of humic substances, has an inhibitory effect on PCR. It is often co-purified and co-extracted from forensic samples collected from soil.

To test the robustness of the kit in the presence of typical inhibitors, the assay was run in the presence of 0, 20, and 30 ng/µl humic acid under standard conditions (500 pg Control DNA XY5, 30 cycles).

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. Its interfering effect is related to the inhibition of polymerase activity.

Investigator HDplex Kit performance was assessed in the presence of increasing concentrations of hematin: 0, 100, and 150 µM under standard conditions (500 pg Control DNA XY5, 30 cycles).

Full profiles (>100 RFU) were obtained in the presence of 30 ng/µl humic acid and 100 µM hematin. The results for these maximum inhibitor concentrations from both studies are shown in Figure 10 (page 13).

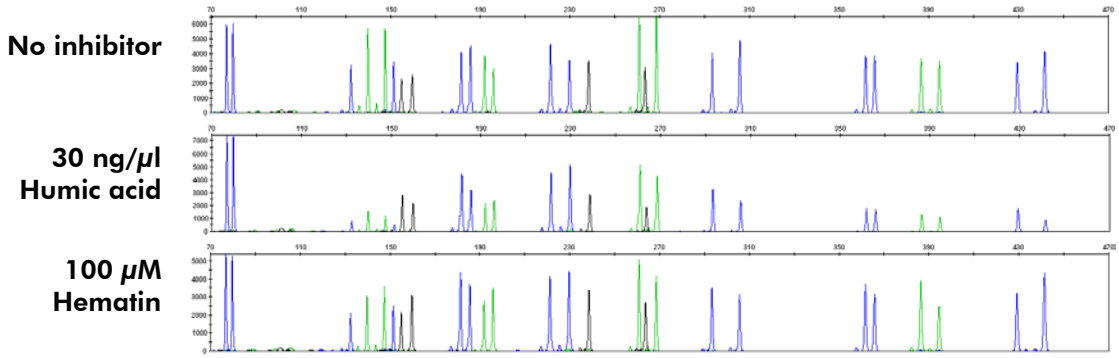

Figure 10. Impact of hematin and humic acid on performance. The y-axis scales were adjusted for best fit.

Reproducibility

Concordance test with an internal DNA pool

The reproducibility of results generated by the Investigator HDplex was validated under standard conditions. This included a test of automatic allele calling based on the allelic ladder, and a concordance analysis of the allele correlation compared to control DNA that had been pre-validated in additional forensic assays. These assays covered of the STR systems of interest partially or completely. The following kits were used:

- Investigator ESSplex SE (QIAGEN GmbH, Hilden, Germany)
- Investigator Nonaplex ESS (QIAGEN GmbH, Hilden, Germany)
- AmpFℓSTR® SEfiler Plus™ (Applied Biosystems Inc., Foster City, USA)
- PowerPlex® 16 (Promega Corporation, Madison, USA)
- Mentype® Chimera® (Biotype GmbH, Dresden, Germany)

A total of 80 DNA samples (40 male, 40 female) of different origin (blood, saliva) were quantified, and profiles were generated with each assay in quadruplicate. All of the assays generated full profiles with steady peak heights of >50 rfu. The profiles generated with Investigator HDplex kits concurred with those generated by the listed assays.

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Ordering Information

Product	Contents	Cat. no.
Investigator HDplex Kit (25)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	381213
Investigator HDplex Kit (100)	Primer mix, reaction Mix, DNA Polymerase, Control, allelic ladder, DNA Size Standard, and nuclease-free water	381215

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Service or your local distributor.

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