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AmpFℓSTR® COfiler® PCR Amplification Kit

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SUBJECT: Kit Overview, Developmental Validation, and Performance Validation

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Using this document

The AmpFℓSTR® COfiler® PCR Amplification Kit uses protocols identical to the AmpFℓSTR® Profiler Plus® and Profiler Plus® *ID* PCR Amplification Kits. Use this document in conjunction with the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* (Pub. no. 4476688) for information on amplification, electrophoresis and data analysis protocols.

This document includes the following information for the COfiler® Kit:

- Overview of the kit
- Summary of the validation work conducted according to the guidelines established by the Technical Working Group on DNA Analysis Methods (TWGDAM)
- Performance validation for use of in-house kit components

Refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* (Pub. no. 4476688) for the following information for the COfiler® Kit:

- Instrument and Software Overview: Data Collection Software/instrument and multicomponent analysis.
- Perform PCR: Recommended reaction setup and amplification conditions.
- Electrophoresis: Protocols for detection of PCR products on the Applied Biosystems 310, 3100, 3100-*Avant*, 3130, 3130*xl*, 3500 and 3500xL Genetic Analyzers
- Data Analysis: Use of GeneMapper® *ID* Software and GeneMapper® *ID-X* Software for analysis of AmpFℓSTR® kit data
- Troubleshooting
- Ordering information
- PCR Work Areas: Work area set up and lab design, PCR setup and amplified DNA work areas
- Safety: Chemical and biohazard safety information
- Documentation

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Product overview

Purpose

The AmpF*l*STR® COfiler® PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 6 tetranucleotide repeat loci (D3S1358, TPOX, CSF1PO, D7S820, D16S539, and TH01) and the Amelogenin gender-determining marker in a single PCR amplification. The COfiler® Kit was originally designed to work in conjunction with the Profiler Plus® or Profiler Plus® *ID* Kits to amplify the 13 core loci on which the US Combined DNA Index System (CODIS) is based. Two loci (D3S1358 and D7S820) overlap between the COfiler® Kit, the Profiler Plus® Kit, and the Profiler Plus® *ID* Kit to provide evidence to the laboratory that both reactions have amplified the same sample. The COfiler® Kit can also be used independently from the Profiler Plus® or Profiler Plus® *ID* Kits for applications where a smaller multiplex may be required.

Product description

The COfiler® Kit contains all the necessary reagents for the amplification of human genomic DNA. The fluorescently labeled PCR products can be subsequently detected on an Applied Biosystems capillary electrophoresis instrument. The results are analyzed with GeneMapper® *ID* Software or GeneMapper® *ID-X* Software.

The reagents are designed for use with the following Applied Biosystems instruments:

- Applied Biosystems 3100/3100-*Avant* Genetic Analyzer
- Applied Biosystems 3130/3130*xl* Genetic Analyzer
- Applied Biosystems 3500/3500xL Genetic Analyzer
- Applied Biosystems 310 Genetic Analyzer
- GeneAmp® PCR System 9700 with the Silver 96-Well Block
- GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block
- Veriti® 96-Well Thermal Cycler

About the primers

The COfiler® Kit employs the same primer sequences for all loci common to other AmpF*l*STR® kits (except the MiniFiler™ kit).

Loci amplified by the kit

The following table shows the loci amplified, their chromosomal locations, and the corresponding fluorescent marker dyes. The AmpFℓSTR® COfiler® Allelic Ladder is used to genotype the analyzed samples. The alleles contained in the allelic ladder and the genotype of the AmpFℓSTR® Control DNA 9947A are also listed in the table.

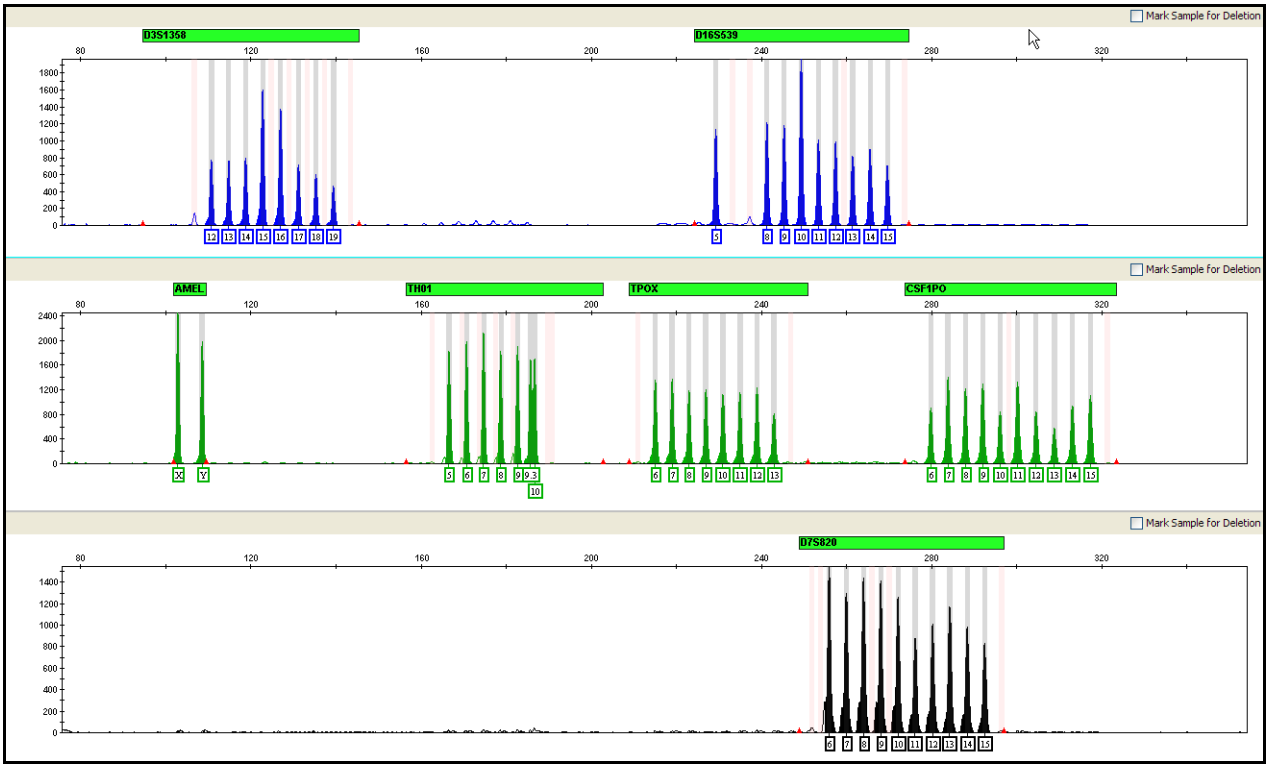
Table 1 COfiler® Kit loci and alleles

Locus designation	Chromosome location	Alleles included in AmpFℓSTR® COfiler® Allelic Ladder	Dye label	Control DNA 9947A
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	5-FAM™	14, 15
D16S539	16q24-qter	5, 8, 9, 10, 11, 12, 13, 14, 15		11, 12
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	JOE™	X, X
TH01	11p15.5	5, 6, 7, 8, 9, 9.3, 10		8, 9.3
TPOX	2p23–2per	6, 7, 8, 9, 10, 11, 12, 13		8, 8
CSF1PO	5q33.3–34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D7S820	7q11.21–22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	NED™	10, 11

Allelic ladder profile

Figure 1 shows the allelic ladder for the COfiler® Kit.

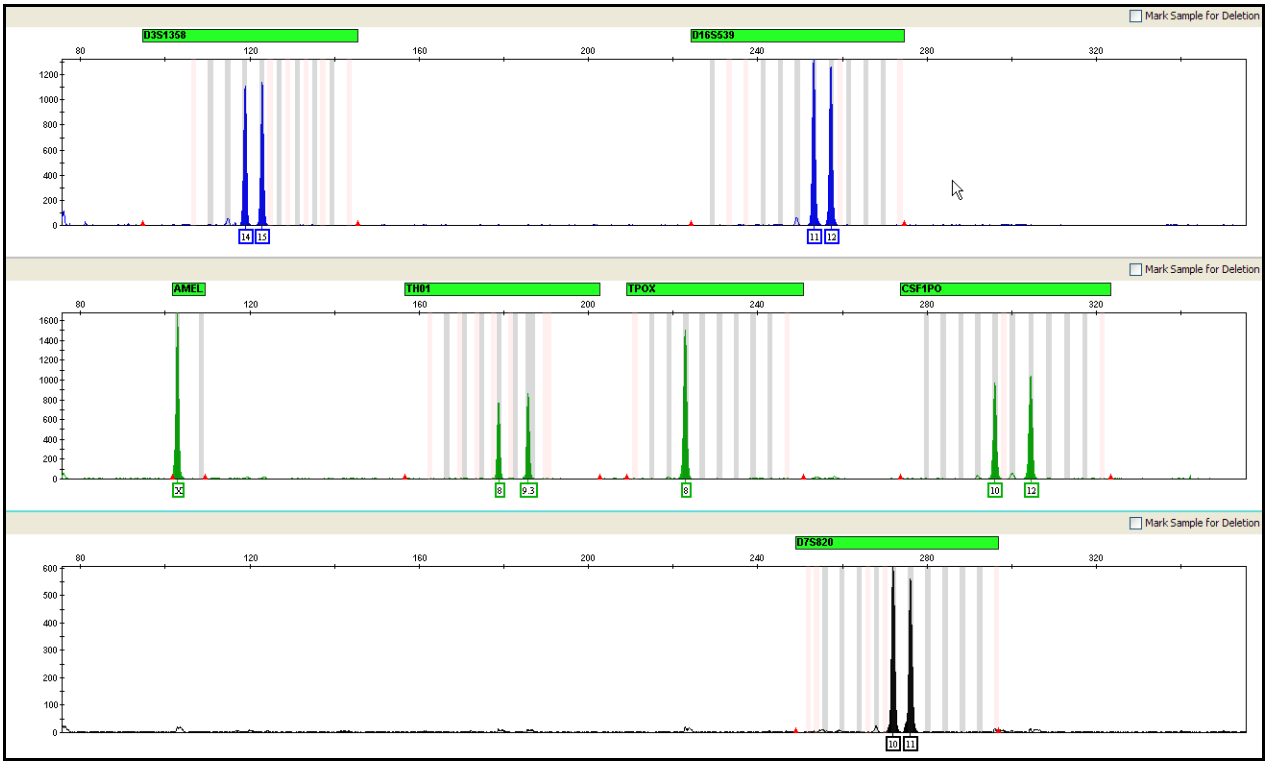
Figure 1 GeneMapper® *ID-X* Software plot of the AmpFℓSTR® COfiler® Allelic Ladder



Control DNA 9947A profile

Figure 2 shows amplification of Control DNA 9947A using the COfiler® Kit.

Figure 2 1 ng of Control DNA 9947A amplified with the COfiler® Kit and analyzed on the Applied Biosystems 3130*xl* Genetic Analyzer



Amplification using bloodstained FTA® cards

FTA®-treated DNA collection cards may be useful for the collection, storage, and processing of biological samples. For example, a small punch of the bloodstained card can be placed directly into an amplification tube, purified, and amplified without transferring the evidence.

Punches of 1.2-mm from a bloodstained FTA card were purified according to the "DNA Purification Using FTA™ Cards" procedure (provided with the FTA Purification Reagent). These punches were then amplified directly in the MicroAmp® tubes for only 25 cycles. Our studies have indicated that a 1.2-mm bloodstained punch contains approximately 15–20 ng DNA. Accordingly, an appropriate cycle number for this high quantity of DNA is 25 cycles.

Materials and equipment

Kit contents and storage

The COfiler® Kit (Part no. 4305246) contains materials sufficient to perform 100 amplifications at 50 µL/amplification.

IMPORTANT! The fluorescent dyes attached to the primers are light sensitive. Protect the primer set, amplified DNA, allelic ladder, and size standard from light when not in use. Keep freeze-thaw cycles to a minimum.

Component	Description	Volume	Storage
AmpFℓSTR® PCR Reaction Mix	Contains $MgCl_2$, deoxynucleotide triphosphates, and bovine serum albumin in buffer with 0.05% sodium azide.	2 tubes, 1.1 mL/tube	–15 to –25°C on receipt, 2 to 8°C after initial use
AmpFℓSTR® COfiler ® Primer Set	Contains fluorescently labeled primers and non-labeled primers.	1 tube, 1.1 mL	
AmpFℓSTR® COfiler® Allelic Ladder	Contains amplified alleles. See Table 1 on page 3 for a list of alleles included in the allelic ladder.	1 tube, 0.05 mL	
AmpFℓSTR® Control DNA 9947A	Contains 0.1 ng/µL human cell line 9947A DNA in 0.05% sodium azide and buffer†. See Table 1 on page 3 for profile.	1 tube, 0.3 mL	
AmpliTaq Gold® DNA Polymerase	Contains enzyme, with an activity of 5 U/µL.	2 tubes, 0.05 mL/tube	–15 to –25°C

† The AmpFℓSTR® Control DNA 9947A is included at a concentration appropriate to its intended use as an amplification control (i.e., to provide confirmation of the capability of the kit reagents to generate a profile of expected genotype). The AmpFℓSTR® Control DNA 9947A is not designed to be used as a DNA quantitation control, and you may see variation from the labelled concentration when quantitating aliquots of the AmpFℓSTR® Control DNA 9947A.

Standards for samples

For the COfiler® Kit, the panel of standards needed for PCR amplification, PCR product sizing, and genotyping are:

- **AmpFℓSTR® Control DNA 9947A** – A positive control for evaluating the efficiency of the amplification step and STR genotyping using the AmpFℓSTR® COfiler® Allelic Ladder.
- **GeneScan™ 500 ROX™ Size Standard** – Used for obtaining sizing results. This standard, which has been evaluated as an internal size standard, yields precise sizing results for COfiler® Kit PCR products. Order the GeneScan™ 500 ROX™ Size Standard (Part no. 4322682) separately.
- **AmpFℓSTR® COfiler® Allelic Ladder** – Allelic ladder developed by Life Technologies for accurate characterization of the alleles amplified by the COfiler® Kit. The AmpFℓSTR® COfiler® Allelic Ladder contains most of the alleles reported for the 6 autosomal loci. Refer to Table 1 on page 3 for a list of the alleles included in the AmpFℓSTR® COfiler® Allelic Ladder.

Workflow overview†

Perform PCR

Extract DNA





AutoMate *Express*™ System + PrepFiler® Express Kit

Quantify DNA



Quantifiler® Duo DNA Quantification Kit

Prepare reactions

AmpFℓSTR® COfiler® PCR Amplification Kit

Perform PCR



GeneAmp® PCR System 9700 Cycler



Veriti® 96-Well Thermal Cycler

Perform electro-phoresis



3100/3100-*Avant* Genetic Analyzer



3130/3130*xl* Genetic Analyzer



3500/3500xL Genetic Analyzer



310 Genetic Analyzer

Analyze data





GeneMapper® *ID-X* or GeneMapper® *ID* Software

† For more information, refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* (Pub. no. 4476688).

Developmental validation

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Overview

This section provides results of the developmental validation experiments we performed using the COfiler® Kit.

We performed experiments to evaluate the performance of the COfiler® Kit. The experiments were performed in part according to a subset of the guidelines recommended by the Technical Working Group on DNA Analysis Methods (TWGDAM) 1995 and American Society of Crime Laboratory Directors (ASCLD) Guidelines 1986 (indicated where applicable throughout this chapter).

Importance of validation

Validation of a DNA typing procedure for human identification applications is an evaluation of the procedure's efficiency, reliability, and performance characteristics. By challenging the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process uncovers attributes and limitations which are critical for sound data interpretation in casework (Sparkes, Kimpton, Watson *et al.,* 1996; Sparkes, Kimpton, Gilbard *et al.,* 1996; Wallin *et al.,* 1998).

Experiment conditions

This chapter discusses many of the experiments we performed and provides examples of results obtained. We chose conditions that produced optimum PCR product yield and that met reproducible performance standards. It is our opinion that while these experiments are not exhaustive, they are appropriate for a manufacturer of STR kits intended for forensic and/or parentage testing use. Each laboratory using the COfiler® Kit should perform their own internal validation studies.

Developmental validation

TWGDAM 4.4.1.3 Amplification

"*The reaction conditions such as thermocycling parameters and critical reagent concentrations (primers, polymerase and salts) needed to provide the required degree of specificity must be determined*".

TWGDAM 4.4.1.4

"*The number(s) of cycles necessary to produce reliable results must be determined*".

PCR components

The concentration of each component of the COfiler® Kit was examined. The PCR components are Tris-HCl (pH 8.3), KCl, dNTPs, primers, AmpliTaq Gold® DNA Polymerase, $MgCl_2$, bovine serum albumin, and sodium azide. The concentration for a particular component was established to be in the window that meets the reproducible performance characteristics of specificity and sensitivity.

After the optimal concentration was determined for a single component, the others were tested sequentially until it was determined that each component was at the optimal concentration relative to the concentrations of the other components in the reaction mix. The optimized COfiler® Kit provides the required degree of specificity such that it is specific to primates for the species tested (with the exception of the amelogenin locus, see "Nonhuman studies" on page 16) and does not produce nonspecific mispriming artifacts.

Thermal cycler parameters

The PCR cycle number was set to obtain reliable, specific amplification of 1 ng of AmpFℓSTR® Control DNA 9947A. Additionally, the cycle number was set to avoid detection of low quantities of DNA (≤35 pg).

Thermal cycling parameters were established for amplification of the COfiler® Kit in the DNA Thermal Cycler 480 and GeneAmp® PCR Systems 9600 and 2400. Thermal cycling times and temperatures met GeneAmp® PCR Instrument specifications. Annealing and denaturation temperature windows were tested around each setpoint to verify that a ±2°C window (DNA Thermal Cycler 480) or ±1.5°C window (GeneAmp PCR System 9600 and 2400) yielded specific PCR product with the desired sensitivity of at least 1 ng of AmpFℓSTR® Control DNA 9947A.

For example, the annealing temperatures of 57, 59, and 61°C were tested (GeneAmp® PCR Systems 9600 and 2400, and DNA Thermal Cycler 480), also for one-minute hold times. The PCR products were analyzed using either the Applied Biosystems 377 DNA Sequencer or the Applied Biosystems 310 Genetic Analyzer and GeneScan® Analysis 2.1 Software. Neither preferential nor differential amplification was observed in any of these denaturation or annealing temperature experiments.

Accuracy, reproducibility, and precision

ASCLD 4.5.3

"Precision (e.g., measurement of fragment lengths) must be determined by repetitive analyses to establish criteria for matching".

TWGDAM 4.4.2.1 Detection of PCR product

"When a PCR product is characterized directly, appropriate standards for assessing the alleles shall be established (e.g., size markers)".

Accuracy

Alleles contained in the AmpFℓSTR® COfiler® Allelic Ladder and from population samples have been sequenced to verify both length and repeat structure. The size ranges shown in Table 1 on page 3, indicate the actual number of nucleotides contained in the smallest and largest allelic ladder alleles for each locus. The size range is the actual base pair size of the sequenced alleles including the 3′ A nucleotide addition. The COfiler® Kit is designed so that a majority of the PCR products contain the non-templated 3′ A nucleotide.

Alleles were named in accordance with the recommendations of the DNA Commission of the ISFH (DNA Recommendations, 1994; Bär *et al.*, 1997). The number of complete 4-bp repeat units observed is designated by an integer. Variant alleles that contain a partial repeat are designated by a decimal followed by the number of bases in the partial repeat. For example, a TH01 9.3 allele contains 9 complete repeat units and a partial repeat unit of 3 bp. Additional variation has been observed at some loci where alleles exist that differ from integer allele lengths by one or two base pairs.

Reproducibility

The reproducible nature of purified DNA samples from various individuals (>10), used routinely at Life Technologies, as well as validation samples processed during characterization of AmpFℓSTR® PCR Amplification Kits (for example, from body fluid mixture studies, environmental studies, matrix studies, nonprobative studies, CEPH family DNA sets) was without exception. All samples yielded the correct genotype.

Precision and size windows

The recommended method for genotyping is to employ a ±0.5-bp "window" around the size obtained for each allele in the AmpFℓSTR® COfiler® Allelic Ladder. A ±0.5-bp window allows for the detection and correct assignment of potential off-ladder sample alleles whose true size is only one base different from an allelic ladder allele. Alleles should be appropriately sized using the Local Southern sizing method. Any sample allele that sizes outside a window could be either of the following:

- An "off-ladder" allele, i.e., an allele of a size that is not represented in the AmpFℓSTR® COfiler® Allelic Ladder (see for examples of known off-ladder alleles)
- An allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement error

The measurement error inherent in any sizing method can be defined by the degree of precision in sizing an allele multiple times. Precision is measured by calculating the standard deviation in the size values obtained for an allele that is run in several injections in one capillary run. Table 2 on page 11 indicates typical precision results obtained from 30 database samples and three AmpFℓSTR® COfiler® Allelic Ladder samples analyzed on the Applied Biosystems 310 Genetic Analyzer (47-cm capillary, POP-4® polymer, GeneScan™ 500 ROX™ Size Standard).

As indicated above, sample alleles may occasionally size outside of the ±0.5-bp window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems with the smallest standard deviations in sizing. Figure 7 in the Accuracy, reproducibility, and precision section of the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* illustrates the tight clustering of allele sizes obtained on the Applied Biosystems 310 Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 bp. The instance of a sample allele sizing outside of the ±0.5-bp window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 bp or less (Smith, 1995).

For sample alleles that do not size within a ±0.5-bp window, the PCR product must be rerun to distinguish between a true off-ladder allele versus measurement error of a sample allele that corresponds with an allele in the allelic ladder.

It is important to note that while the precision within a set of capillary injections is very good, the determined allele sizes vary between platforms. Cross-platform sizing differences arise from a number of conditions, including run temperature and electrophoresis conditions. Variations in sizing can also be found between runs on the same instrument and between runs on different instruments because of these conditions. We strongly recommend that the allele sizes obtained be compared to the sizes obtained for known alleles in the AmpFℓSTR® COfiler® Allelic Ladder from the same run and then converted to genotypes. For more information on precision and genotyping, see Lazaruk *et al.*, 1998.

Table 2 Example of precision results on the Applied Biosystems 310 Genetic Analyzer

Allele	n	Mean	S.D.
D3S1358			
12	3	111.02	0.03
13	3	115.19	0.04
14	12	119.11	0.05
15	18	123.14	0.07
15	17	127.27	0.08
17	14	131.39	0.09
18	10	135.55	0.08
19	3	139.62	0.01
D16S539			
5	3	228.92	0.01
8	3	240.88	0.08
9	9	245.01	0.09
10	6	249.00	0.12
11	21	253.10	0.09
12	19	257.11	0.08
13	9	261.13	0.10
14	3	265.12	0.11
15	3	269.20	0.12

Allele	n	Mean	S.D.
Amelogenin			
X	34	103.30	0.05
Y	21	108.96	0.05
TH01			
5	3	166.91	0.04
6	19	170.97	0.05
7	7	174.99	0.05
8	12	178.99	0.05
9	14	182.95	0.05
9.3	18	185.99	0.06
10	3	186.90	0.01
TPOX			
6	3	214.96	0.05
7	3	218.90	0.02
8	32	222.82	0.06
9	8	226.80	0.05
10	4	230.81	0.05
11	15	234.75	0.07
12	5	238.72	0.03
13	3	242.71	0.03
CSF1PO			
6	3	279.65	0.03
7	3	283.77	0.08
8	3	287.86	0.06
9	5	291.93	0.08
10	19	295.97	0.07
11	14	300.05	0.07
12	22	304.58	0.08
13	10	309.00	0.10
14	4	313.36	0.09
15	3	317.67	0.04
D7S820			
6	3	256.01	0.03
7	3	260.05	0.04
8	15	264.10	0.06
9	11	268.15	0.07
10	15	272.20	0.07
11	15	276.28	0.06

Allele	n	Mean	S.D.
12	11	280.37	0.07
13	5	284.45	0.06
14	4	288.54	0.05
15	3	292.62	0.04

Extra peaks in the electropherogram

Causes of extra peaks

Peaks other than the target alleles may be detected on the electropherogram displays. Described below are several causes for the appearance of extra peaks, including the stutter product (found at the n–4 position), and incomplete 3′ A nucleotide addition (found at the n–1 position).

Stutter products

The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter (n–4) than the corresponding main allele peak. This is referred to as the stutter peak or product. Sequence analysis of stutter products at tetranucleotide STR loci has revealed that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (Walsh *et al.*, 1996).

The proportion of the stutter product relative to the main allele (percent stutter) is measured by dividing the height of the stutter peak by the height of the main allele peak. Such measurements have been made for hundreds of samples at the loci used in the COfiler® Kit.

Some of the general conclusions from these measurements and observations are as follows:

- For each COfiler® Kit locus, the percent stutter generally increases with allele length, as shown in Figure 3 through Figure 5 on page 14 through page 15. Smaller alleles display a lower level of stutter relative to the longer alleles within each locus. This is reflected in Figure 3 through Figure 5, where minimal data points are plotted for some smaller alleles, because stutter was not detected for many of these samples.

  Note that the overall trend of increasing percent stutter with increasing allele length is disrupted by the TH01 9.3 allele, where the percent stutter is similar to that for the TH01 6 allele. Interestingly, the TH01 9.3 allele contains a partial (3-bp) repeat unit after the first six consecutive core repeat units. For the COfiler® Kit loci, a positive correlation exists between the percent stutter and the number of consecutive core repeat units.
- For the alleles within a particular locus, the percent stutter is generally greater for the longer allele in a heterozygous sample (this is related to the first point above).
- The highest percent stutter observed for any TH01 or TPOX allele was <4%, for any CSF1PO allele was <7%, for any D7S820 allele <8%, and for any D3S1358 or D16S539 allele was <10%.
- Each allele within a locus displays a percent stutter that is quite reproducible. The average standard deviation is 0.4% for TH01, TPOX, and CSF1PO loci: 0.6% for D3S1358 and D7S820 loci; and 0.9% for D16S539 loci. This means that most of the time the percent stutter will be less than ±2.5% from the mean. For example, if the percent stutter for a particular allele averages 5% for multiple replicates, then it is rare that the percent stutter for this allele will ever be <2.5% or >7.5%.

- An upper-limit stutter percent interpretational threshold can be estimated for each locus as 3 standard deviations above the highest percent stutter observed at the locus (see above two observations). Peaks at the stutter position that are above this threshold are not expected to be observed in single-source samples and therefore can be noted for closer examination. The upper-limit threshold values for each locus are as follows: 7% (TH01, TPOX), 10% (CSF1PO), 12% (D7S820), or 15% (D3S1358, D16S539). For evaluation of mixed samples, see "Mixed samples" on page 21.
- The percent stutter does not change significantly with the quantity of input DNA, for on-scale data with a minimum analyzed peak height of 150 relative fluorescence units (RFU). The measurement of percent stutter may be unnaturally high for main peaks that are off-scale. Loading or injecting less of the PCR product will yield accurate quantitation. See "Effect of DNA quantity on results" on page 17 for information on off-scale data.

Figure 3 Stutter percentages for the D3S1358 and D16S539 loci

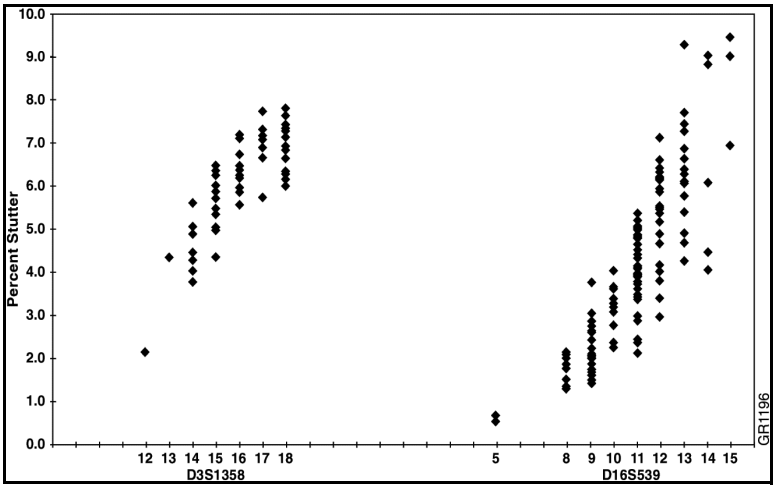


Figure 4 Stutter percentages for the TH01, TPOX, and CSF1PO loci

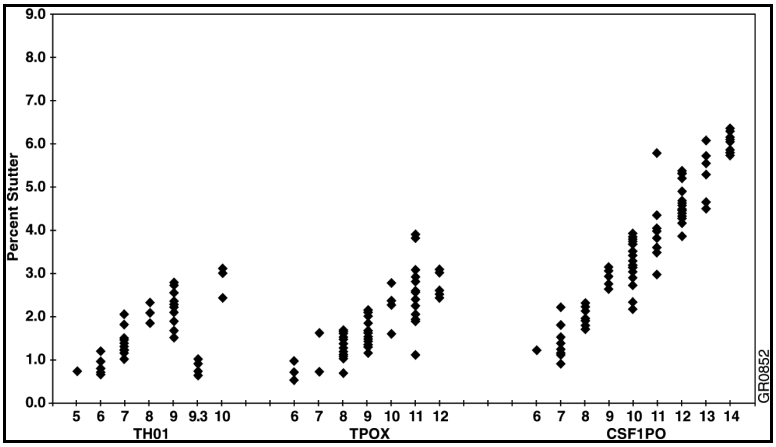
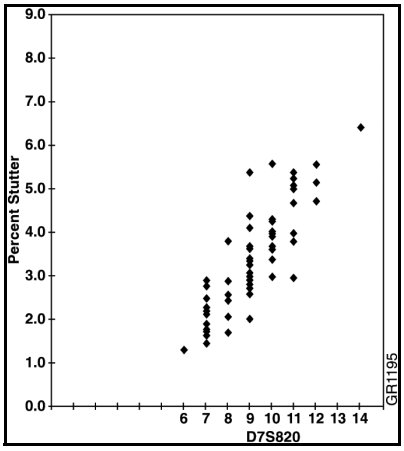


Figure 5 Stutter percentages for the D19S433, TH01, and FGA loci.



Addition of 3´ A nucleotide

Refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* for information.

Characterization of loci

TWGDAM 4.2

"*During the development of a DNA analysis system, basic characteristics of the loci must be determined and documented* (Baird, 1989; AABB Standards Committee, 1990)".

TWGDAM 4.2.4 Polymorphisms

"*DNA loci used in forensic testing shall have been validated by family studies to demonstrate the mode of inheritance. Those DNA loci used in parentage testing should have a low frequency of mutation and / or recombination*".

The primers for the amelogenin locus flank a six-base pair deletion within intron 1 of the X homologue. Amplification results in 107-bp and 113-bp products from the X and Y chromosomes, respectively. (Sizes are the actual base pair size according to sequencing results, including 3' A nucleotide addition.) The remaining COfiler® Kit loci are all tetranucleotide short tandem repeat (STR) loci. The length differences among alleles of a particular locus result from differences in the number of 4–bp repeat units (see Table 1 on page 3).

Alleles in the AmpFℓSTR® COfiler® Allelic Ladder, alleles containing partial repeat units, population database, and nonhuman primate DNA samples have been subjected to DNA sequencing at Life Technologies. In addition, other groups in the forensic community have sequenced alleles at some of these loci (Nakahori *et al.*, 1991; Puers *et al.*, 1993). Among the various sources of sequence data on the COfiler® Kit loci, there is consensus on the repeat patterns and structure of the STRs (see Table 1 on page 3).

Inheritance

The COfiler® Kit loci have been validated by family studies in order to demonstrate their mode(s) of inheritance.

The Centre d'Etude du Polymorphisme Humain (CEPH) has collected DNA from 39 families of Utah Mormon, French Venezuelan, and Amish descent. These DNA sets have been extensively studied all over the world and are routinely used to characterize the mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring. Consequently, the CEPH family DNA sets are ideal for studying inheritance patterns (Begovich *et al.*,1992).

Four CEPH family DNA sets were examined. DNA input of 1.5 ng from each sample was amplified using the COfiler® Kit, followed by analysis using an Applied Biosystems 310. The families examined included #1331 (11 offspring), #13291 (9 offspring), #13292 (9 offspring), and #13294 (8 offspring), representing 37 meiotic divisions. The results confirmed that the loci are inherited according to Mendelian rules, as has been reported in the literature (Nakahori *et al.*, 1991; Edwards *et al.*,1992; Li *et al.*,1993; Anker *et al.*, 1992; Sullivan *et al.*, 1993; Huang *et al.*, 1995).

TWGDAM 4.2.2 Gene mapping

"The chromosomal location of the polymorphic loci used for forensic testing shall be submitted to or recorded in the Yale Gene Library or the International Human Gene Mapping Workshop."

The COfiler® Kit loci D3S1358, D16S539, amelogenin, TH01, TPOX, CSF1PO, and D7S820 have been mapped and the chromosomal locations have been published (Nakahori *et al.*,1991; Edwards *et al.*,1992; Li *et al.*,1993; Anker *et al.*, 1992; Sullivan *et al.*, 1993; D7S820, Cooperative Human Linkage Center (CHLC) accession number 511, GenBank accession number G08616; Green *et al.*, 1991; Hammond *et al.*, 1994; D16S539, Cooperative Human Linkage Center (CHLC) accession number 715, GenBank accession number G07925). They are listed in Table 1 on page 3.

Species specificity

TWGDAM 4.1.5.9

"*Determine if DNA typing methods designed for use with human specimens detect DNA profiles in nonhuman source stains*".

Nonhuman studies

Nonhuman DNA may be present in forensic casework samples. The COfiler® Kit provides the required degree of specificity such that it is specific to primates for the species tested (with the exception of the amelogenin locus).

The following experiments were conducted to investigate interpretation of COfiler® Kit results from nonhuman DNA sources.

The extracted DNA samples were amplified in COfiler® Kit reactions and analyzed using the Applied Biosystems 377 DNA Sequencer.

- **Primates** – Gorilla, chimpanzee, orangutan, and macaque (2.5 ng each).
- **Non-primates** – Rat, hamster, mouse, cat, dog, rabbit, pig, chicken, fish, cow, and horse (50 ng each).
- **Bacteria and yeast** – *Escherichia coli* (2 strains), *Legionella pneumophila, Salmonella typhimurium, Listeria monocytogenes, Neisseria* lactamica, *Vibrio mimicus*, Saccharomyces cerevisiae, *Candida albicans*, (equivalent to ~50 ng human DNA), and *Rhodotorula rubra*.

The primate DNA samples all amplified, producing fragments within the 75–350 base pair region (Wallin *et al.*,1998). The primate samples were subsequently sequenced by Life Technologies scientists. The data revealed significant sequence homology between the primate and human DNA for the COfiler® Kit loci.

The bacteria, yeast, cat, chicken, and mouse samples did not yield detectable product. The dog, pig, cow, and samples produced a 103-bp fragment. This 103-bp fragment was also amplified using the amelogenin primers alone. This confirms amplification of the product obtained by Buel *et al.*(1995). The 103-bp fragment is 4 bp shorter than the primate 107-bp X-specific product (including +A addition).

Sensitivity

TWGDAM 4.1.5.10 Minimum sample

"*Establish quantity of DNA needed to obtain a reliable result*".

Importance of quantification

The amount of input DNA added to the PCR reaction should be 1.0–2.5 ng. The DNA sample should be quantitated prior to amplification using a system such as the Quantifiler® Human DNA Quantitation Kit (Part no. 4343895) or Quantifiler® Duo DNA Quantitation Kit (Part no. 4387746). In Figure 6 on page 18, a range of AmpFℓSTR® Control DNA 9947A concentrations from 16 pg–2 ng was amplified. Partial or no profiles resulted at lower concentrations.

Effect of DNA quantity on results

The final DNA concentration should be in the range of 0.05–0.125 ng/µL so that 1.0–2.5 ng of DNA will be added to the PCR reaction in a volume of 20 µL. If the sample contains degraded DNA, amplification of additional DNA may be beneficial.

If too much DNA is added to the PCR reaction, then the increased amount of PCR product that is generated can result in the following:

- Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument ("off-scale" data)

  Off-scale data is a problem for two reasons:

  – Quantification (peak height and area) for off-scale peaks is not accurate. For example, an allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.

  – Multicomponent analysis of off-scale data is not accurate, which results in poor spectral separation ("pull-up").

  Identification of off-scale peaks and multicomponent analysis are discussed in the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide*.

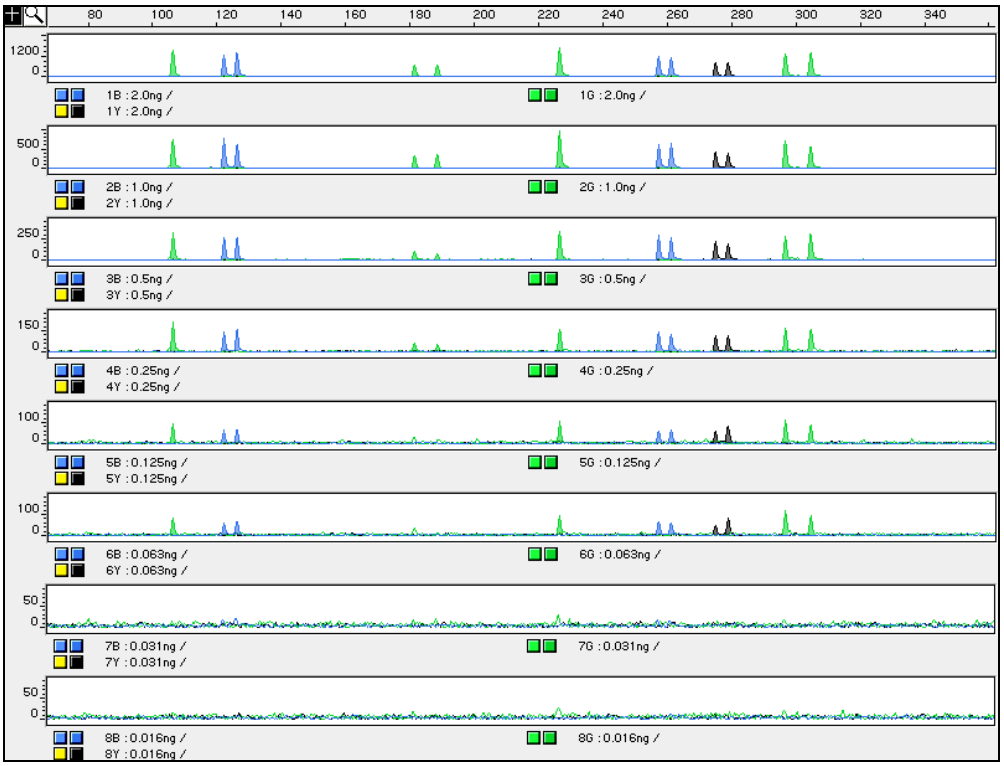
- Incomplete A nucleotide addition

To avoid these issues, the sample can be re-amplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the two alleles of a heterozygous individual may occur (Wallin *et al.*, 1998; Walsh *et al.*, 1992). This is due to stochastic fluctuation in the ratio of the two different alleles (Sensabaugh *et al.*, 1991).

Individual laboratories may find it useful to determine an appropriate minimum peak height interpretational threshold based on their own results using low amounts of input DNA. The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing 50 pg human genomic DNA. Low peak heights should be interpreted with caution.

Figure 6 Effect of amplifying various amounts of AmpFℓSTR® Control DNA 9947A ranging from 16 pg to 2 ng. Note that the y-axis scale differs in many of these panels.



Stability

Lack of amplification of some loci

As with any multi-locus system, the possibility exists that not every locus will amplify. This is most often observed when the DNA substrate has been severely degraded or when the DNA sample contains PCR inhibitors. Because each locus is an independent marker, results generally can still be obtained from the loci that do amplify.

Degraded DNA

Refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* for information.

Effect of inhibitors

Heme compounds have been identified as PCR inhibitors in DNA samples extracted from bloodstains (Akane *et al.*, 1994., DeFranchis *et al.*, 1988). It is believed that the inhibitor is co-extracted and co-purified with the DNA and subsequently interferes with PCR by inhibiting polymerase activity.

Bovine serum albumin (BSA) can prevent or minimize the inhibition of PCR, most likely by binding to the inhibitor (Comey *et al.*, 1994). Because the presence of BSA can improve the amplification of DNA from blood-containing samples, BSA has been included in the AmpFℓSTR® PCR Reaction Mix at a concentration of 8 µg per 50-µL amplification. BSA has also been identified as an aid in overcoming inhibition from samples containing dyes, such as in denim (Comey *et al.*, 1994).

To examine the effects of hematin on the COfiler® Kit amplification results, DNA samples were amplified using the COfiler® Kit reagents (including the BSA-containing PCR reaction mix) in the presence of varying concentrations of purified hematin. The concentrations of hematin used were 0 µM, 16 µM, 18 µM, 20 µM, 22 µM, 24 µM, 26 µM, 28 µM, and 30 µM. As the concentration of hematin increased, the overall yield of product at each locus was reduced. D7S820 was the first locus to become undetectable; followed by CSF1PO; D16S539; D3S1358, TH01, and TPOX; and finally amelogenin (Figure 7). No advantage in amplifying each locus alone was observed (Figure 8 on page 20).

Figure 7 DNA amplified with the COfiler® Kit in the presence of varying concentrations of hematin: 0, 16 µM, 18 µM, 20 µM, 22 µM, 24 µM, 26 µM, 28 µM, and 30 µM

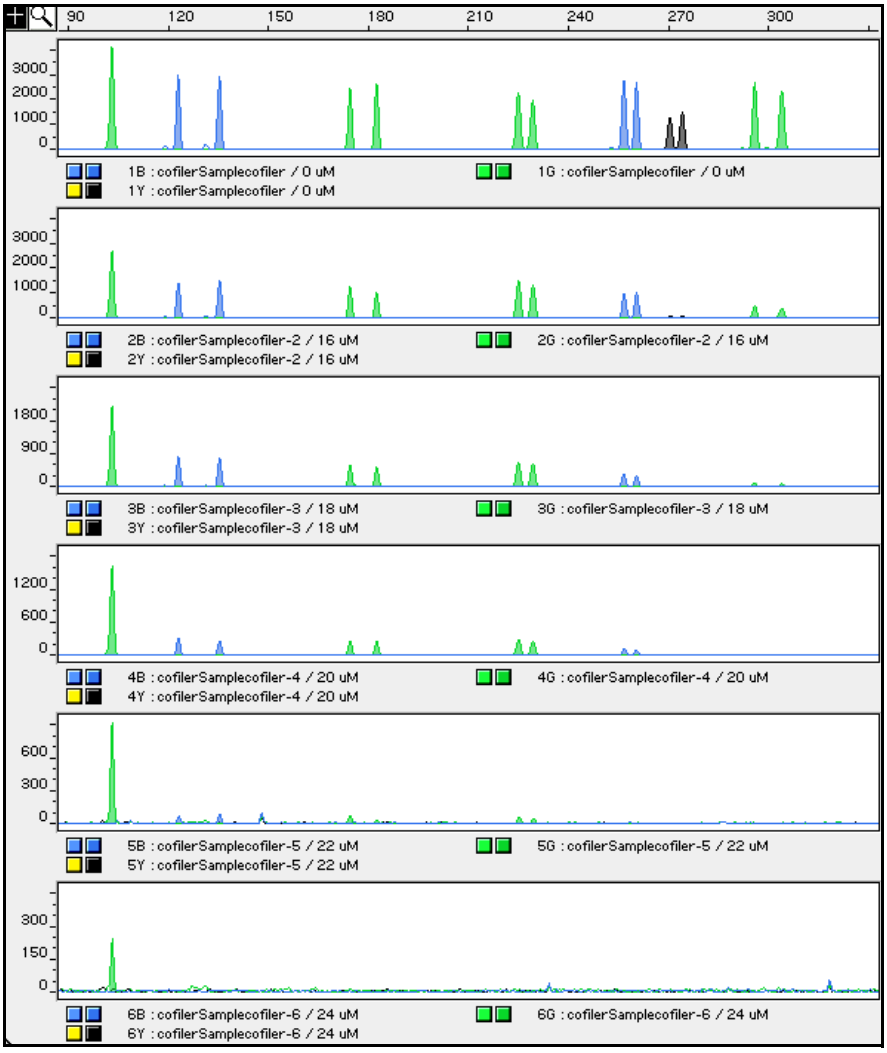
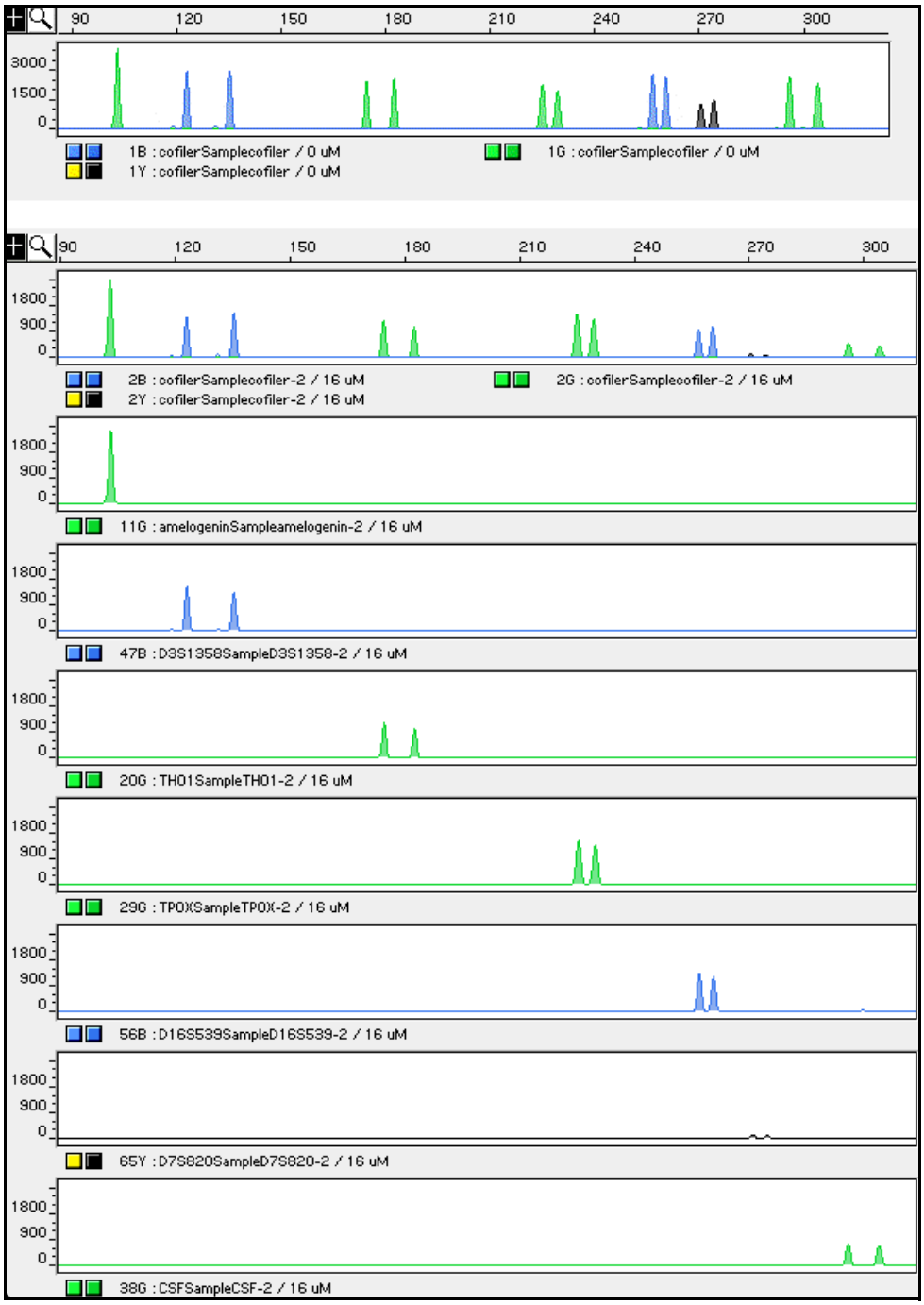


Figure 8 DNA amplified in multiplex and single-locus amplifications in the presence of 0 µM hematin (top panel) and 16 µM hematin (all panels except top panel) using COfiler® Kit reagents and primer sets.



Mixture studies

Mixed samples

Evidence samples may contain DNA from more than one individual. The possibility of multiple contributors should be considered when interpreting the results. In the discussion below, a peak is defined as any peak that is greater than 150 RFU. We suggest you determine an appropriate minimum peak height threshold based on your own results using low amounts of input DNA (see "Effect of DNA quantity on results" on page 17).

Detection of mixed samples

Each of the following can aid in determining whether a sample is a mixture:

- The presence of more than two alleles at a locus
- The presence of a peak at a stutter position that is significantly greater in percentage than what is typically observed in a single-source sample (see "Stutter products" on page 13 and Figure 3 through Figure 5).
- Significantly imbalanced alleles for a heterozygous genotype

  The peak height ratio is defined as the height of the lower peak (in RFU) divided by the height of the higher peak (in RFU), expressed as a percentage. Mean peak height ratios and standard deviations observed for alleles in the COfiler® Kit loci in unmixed population database samples are as follows:

Allele	Mean Peak Height Ratio	Number of Observations (n)
D3S1358	93 ± 4%	68
D16S539	92 ± 6%	70
Amelogenin	90 ± 6%	46
TH01	92 ± 6%	70
TPOX	92 ± 5.5%	78
CSF1PO	92 ± 6%	84
D7S820	93 ± 6%	79

- For all seven loci, the mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced. Ratios <70% are rare in normal, unmixed samples.

  Other possible causes of imbalance at a locus are degraded DNA, PCR inhibition, low template copy number, or the presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele. Amplification and analysis of additional loci may assist in the interpretation of the difficult samples.

Resolution of genotypes in mixed samples

Refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* for information.

Limit of detection of the minor component

Refer to the *AmpFℓSTR® Profiler Plus® and Profiler Plus® ID Kits User Guide* for information.

Population data

TWGDAM 4.1.5.3 Population studies

"Establish population distribution data in different racial and/or ethnic groups".

Overview

The COfiler® Kit contains loci for which extensive population data are available.

Population samples used in these studies

The COfiler® Kit was used to generate the population data provided in this section. Samples were collected from individuals throughout the United States with no geographical preference.

Population	Number of samples	Samples provided by
African-American	195	Laboratory Corporation of America
U.S. Caucasian	200	

In addition to the alleles that were observed and recorded in the Life Technologies databases, other known alleles have either been published or reported to us by other laboratories (go to **http://www.cstl.nist.gov/strbase/**).

Allele frequencies

Table 3 shows the COfiler® Kit allele frequencies in two populations, listed as percentages.

Table 3 COfiler® Kit allele frequencies

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
D3S1358		
9	0.26†	†
10	†	†
11	0.26†	0.25†
12	0.51†	0.25†
13	†	0.50†
14	11.80	11.25
15	27.95	28.25
15.2	0.26†	†
16	32.31	22.25
17	21.80	22.25
18	4.62	14.50
19	0.26†	0.50†
D16S539		
5	0.26†	†
8	3.85	1.5

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
9	18.21	10.75
10	11.28	4.25
11	30.00	29.75
12	18.46	34.25
13	15.64	17.5
14	2.05	1.75
15	0.26†	0.25†
TH01		
5	0.26†	†
6	13.08	25.25
7	40.00	16.00
8	22.82	9.25
9	13.08	13.75
9.3	10.00	35.00
10	0.77†	0.75†
CSF1PO		
6	0.26†	†
7	7.18	†
8	7.44	0.25†
9	3.85	2.75
10	30.51	26.75
10.3	†	0.25†
11	21.28	25.75
12	22.05	35.75
13	6.92	7.00
14	0.51†	1.50
15	†	†
D7S820		
6	0.51†	†
6.3	†	0.25†
7	†	2.50
8	17.95	17.50
9	11.80	13.00
10	33.59	24.00
11	22.82	23.00
12	9.49	16.00
13	3.59	2.75

Allele	African-American (n = 195)	U.S. Caucasian (n = 200)
14	0.26†	0.75†
15	†	0.25†

† A minimum allele frequency of 1.3% is suggested by the National Research Council in forensic calculations using either of these African American or U.S. Caucasian databases analyzed using the COfiler® Kit.

Analyzing the two databases

Analysis across both databases of 790 total chromosomes (per locus) revealed a total of 11 different D3S1358 alleles, 9 different D16S539 alleles, 7 different TH01 alleles, 8 different TPOX alleles, 10 different CSF1PO alleles, and 11 different D7S820 alleles.

In addition to the alleles that were observed and recorded in the Life Technologies databases, other known alleles (listed in Table 1, "COfiler® Kit loci and alleles" on page 3) have either been published or reported to us by other laboratories.

Evaluation of Hardy Weinberg Equilibrium

Independence of allelic frequencies within a locus can be expressed by the Hardy-Weinberg (HW) relationship. Approximation of HW expectations in a sample population allows estimation of genotypic frequencies (HW proportions) from observed allelic frequencies using the HW equation (expanded binomial square law) (Hartl and Clark, 1989; Weir, 1996).

Several biostatistical tests were used to survey HW relationships at the COfiler® Kit STR loci in each sample population. Independence was found between alleles within each locus, as p values >0.05 were obtained from the homozygosity test (Chakraborty *et al.*, 1988; Nei and Roychoudhury, 1974; Nei, 1978), likelihood-ratio test (Edwards *et al.*, 1992; Weir, 1992), and Guo-Thompson exact test (Guo and Thompson, 1992).

Additionally, allele frequency data were analyzed for independence based on the total number of observed distinct homozygous and heterozygous genotype classes (Nei, 1978; Chakraborty *et al.*, 199). Observed values were within two standard errors of expected values for each locus. These sets of data demonstrate that appropriate estimations of COfiler® Kit genotype frequencies are generated from allele frequencies observed in the Life Technologies African-American and U.S. Caucasian databases.

Random association

Existence of random association (linkage equilibrium) between all six STR loci was established through two separate statistical tests. Results of the first test, which considers the observed variance of the number of heterozygous loci (Brown *et al.*, 1980; Budowle *et al.*, 1995) indicate that in both population samples, all COfiler® Kit loci are inherited independently.

Pairwise interclass correlation tests were performed between every possible two-locus combination across the African-American and U.S. Caucasian databases (Karlin *et al.*, 1981). Mendelian behavior between the ten STR loci was observed. COfiler® Kit multilocus genotype frequency estimates may be derived through direct multiplication of each single-locus genotype frequency (the "product rule") estimated from the Life Technologies African-American and U.S. Caucasian databases.

Low frequency alleles

Some alleles of the COfiler® Kit loci occur at a low frequency (less than five times in either database). For these alleles, a minimum frequency of 0.013 (five divided by 2n, where n equals the number of individuals in the database) was assigned for the COfiler® Kit African-American and U.S. Caucasian databases, as suggested in the 1996 report of the Committee on DNA Forensic Science (National Research Council, 1996). These databases are summarized in Table 3, "COfiler® Kit allele frequencies" on page 22. The minimum reportable genotype frequency at each locus is then 1.69×10^{-4}, giving a minimum combined multilocus genotype frequency of 2.33×10^{-23} for both the African-American and U.S. Caucasian databases.

Probability of identity

Table 4 shows the Probability of Identity (P_I) values of the COfiler® Kit loci individually and combined.

Table 4 Probability of Identity values for the COfiler® Kit STR loci

Locus	African-American	U.S. Caucasian
D3S1358	0.102	0.078
D16S539	0.066	0.103
TH01	0.102	0.094
TH01	0.102	0.094
TPOX	0.081	0.211
CSF1PO	0.070	0.122
D7S820	0.081	0.061
Combined	3.15×10^{-7}	1.19×10^{-6}

The P_I value is the probability that two individuals selected at random will have an identical COfiler® Kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/3.2 \times 10^6$ (African-American) and $1/8.4 \times 10^{12}$ (U.S. Caucasian).

Of 18,915 and 19,900 pairs of COfiler® Kit profiles represented by the African-American and U.S. Caucasian databases, respectively, no 6-locus matches were observed.

Linkage disequilibrium between the COfiler® Kit loci and the unique STR loci included in the Profiler Plus® Kit (vWA, FGA, D8S1179, D21S11, D18S51, D5S818, and D13S317) was not detected. The combination of these 13 COfiler® Kit and Profiler Plus® Kit loci offers an average probability of identity of approximately $1/1.8 \times 10^{15}$ (African-American) and $1/3.8 \times 10^{14}$ (U.S. Caucasian).

Probability of paternity exclusion

Table 5 shows the Probability of Paternity Exclusion (P_E) values of the COfiler® Kit STR loci individually and combined.

Table 5 Probability of paternity exclusion values for the COfiler® Kit loci

Locus	African-American	U.S. Caucasian
D3S1358	0.5260	0.5797
D16S539	0.6146	0.5252
TH01	0.5250	0.5418
TPOX	0.5764	0.3589
CSF1PO	0.6048	0.4854
D7S820	0.5742	0.6307
Combined	0.9938	0.9889

The P_E value is the probability, averaged over all possible mother-child pairs, that a random alleged father will be excluded from paternity after DNA typing of the COfiler® Kit STR loci (Chakraborty and Stivers, 1996).

The P_E value offered by the combination of the 13 COfiler® Kit and Profiler Plus® or Profiler Plus® *ID* Kits loci is approximately 99999581 for the African American database and approximately 0.99999156 for the U.S. Caucasian database.

Additional studies

TWGDAM 4.1.5.8 Nonprobative evidence

"*Examine DNA profiles in nonprobative evidentiary stain materials. Compare the DNA profiles obtained for the known liquid blood versus questioned blood deposited on typical crime scene evidence*".

DNA extracts from three adjudicated sexual assault cases were prepared by DNA analysts at the Santa Clara County Crime Laboratory. Sexual assault evidence materials were processed using the differential lysis and organic extraction procedure, while victim/suspect reference blood samples were processed using the Chelex® extraction procedure. Following amplification with the COfiler® Kit reagents, the PCR products were analyzed using the Applied Biosystems 377 DNA Sequencer.

- Case 1 and Case 2 contained a victim reference blood sample, a suspect reference blood sample, and a victim vaginal swab. The COfiler® Kit genotype of the epithelial cell fraction was the same as that of the victim reference and did not contain alleles foreign to the victim. The COfiler® Kit genotype of the sperm cell fraction did not contain detectable epithelial cell fraction DNA and included the suspect as a possible semen donor.
- Case 3 contained a victim reference blood sample and a victim vaginal swab. The COfiler® Kit genotype of the epithelial cell fraction was the same as that of the victim reference and did not contain alleles foreign to the victim. In accordance with the victim's account, COfiler® Kit genotypes of the sperm fraction revealed DNA from multiple semen donors. No suspect(s) were developed in this case.

Performance validation after buffer and enzyme component replacement

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- Experiments 27
- Reproducibility study 28
- Sensitivity study 30
- Conclusions 32

Overview

As part of an ongoing program to exercise greater control over raw materials used in the AmpFℓSTR® PCR Amplification Kits, manufacturing of the AmpliTaq Gold® enzyme and 10X PCR Buffer II (Tris-KCl buffer) components is transitioning from Roche Molecular Systems to Life Technologies. Manufacturing of both components by Life Technologies will be conducted according to the same specifications used previously by Roche. The in-house components are established raw materials in our next generation kits (for example, the NGM™, NGM SElect™ and Identifiler® Plus Kits).

Experiments

We performed studies to compare the performance of the COfiler® Kit containing the in-house components (updated kit) with the performance of the original kit, focusing on studies most relevant to forensic DNA testing (see SWGDAM Guidelines effective January 1, 2011). These studies, while not exhaustive, are in our opinion appropriate for a manufacturer.

Our studies compared the performance of two Roche-manufactured enzyme and buffer lots (Control mixes) with three new lots of buffer and two new lots of enzyme manufactured by Life Technologies (Test mixes). Studies were performed using Test mixes containing both the enzyme and buffer manufactured by Life Technologies.

Test Material	Control A mix	Control B mix	Test A mix	Test B mix	Test C mix
Buffer	Control Buffer Lot 1	Control Buffer Lot 2	Test Buffer Lot 1	Test Buffer Lot 2	Test Buffer Lot 3
Enzyme	Control Enzyme Lot 1	Control Enzyme Lot 2	Test Enzyme Lot 1	Control Enzyme Lot 2	Test Enzyme Lot 2

Each of the five mixes listed above were used to conduct reproducibility, and sensitivity studies. All amplifications were performed using a GeneAmp® PCR System 9700 with either silver or gold-plated silver block using the recommended amplification conditions and cycle number for the COfiler® Kit. All data was run on an Applied Biosystems 3130*xl* Genetic Analyzer running Data Collection Software v3.0

and analyzed using GeneMapper® *ID-X* Software. Subsequent data analysis was performed using Minitab® Statistical Software. For information on performance of the in-house components on inhibited samples see the SGM Plus® or Identifiler® Kit User Guides.

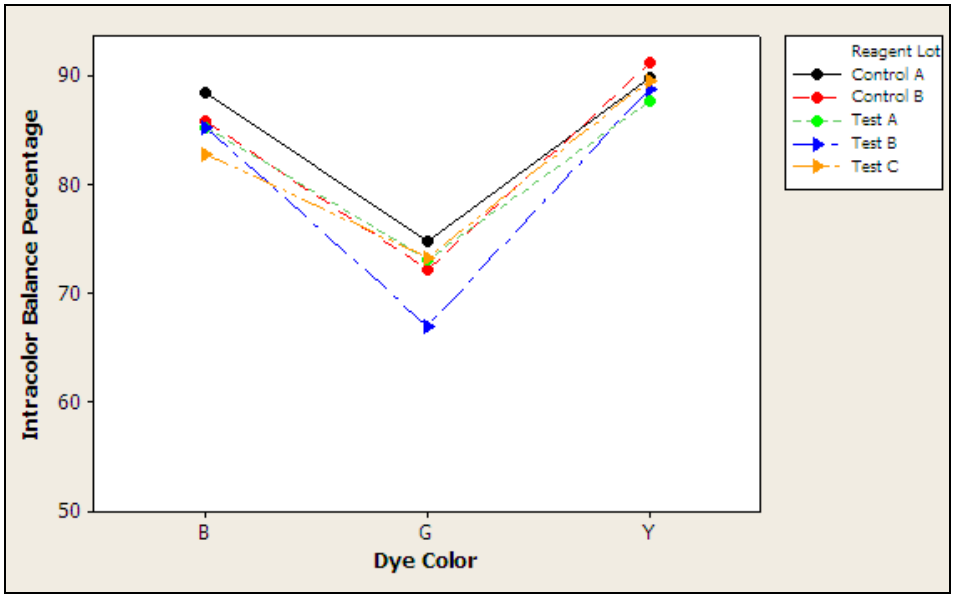
Reproducibility study

For the reproducibility study, 12 replicates of control DNA 007 at 2 ng input and three negative control replicates were amplified. The results were evaluated for intracolor balance, stutter percentage, and the presence, signal intensity, and location of artifacts.

Intracolor balance

No significant difference (<10% increase or decrease) in the level of intracolor balance was observed between the Test and Control mixes (Figure 9).

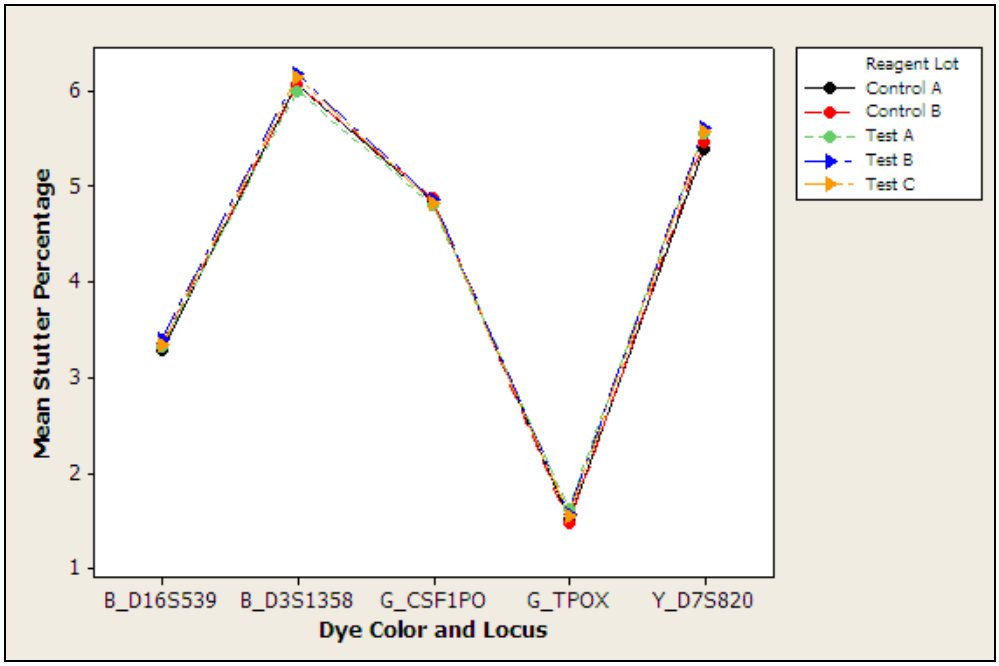
Figure 9 Reproducibility study: intracolor balance results.



Stutter percentages

Stutter percentage results for each marker were comparable across all Test and Control mixes (Figure 10). Results are shown for the 5 of the 6 STR loci contained in the COfiler® Kit. The remaining locus did not exhibit measurable stutter above the detection threshold used for this study.

Figure 10 Reproducibility study: mean stutter percentage results

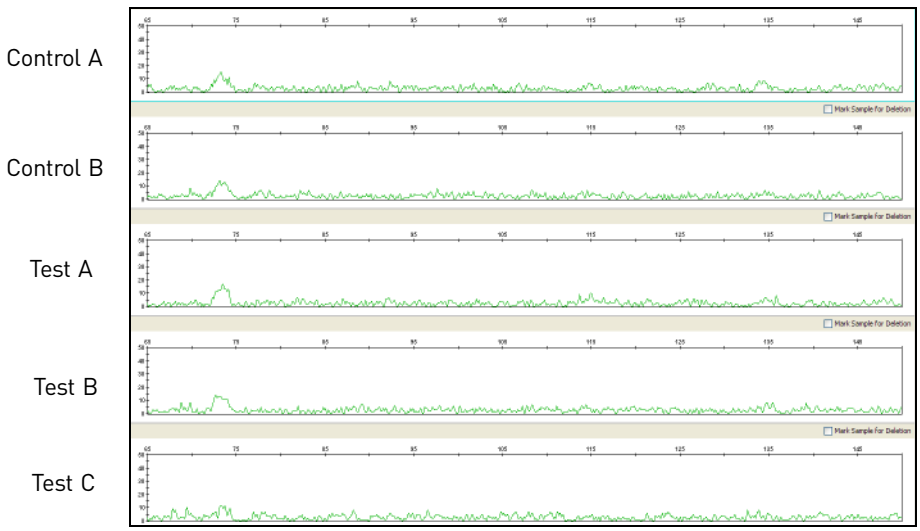


Artifacts

Known artifacts observed showed the same morphology, signal intensity, and location in all Test and Control mixes and do not exceed 50 RFU (Figure 11). No new artifacts were observed in the Test mixes.

Figure 11 Reproducibility study: known artifacts (Y-scale 100 RFU)

VIC® dye labeled artifacts at ~70 bp and ~115 bp



Sensitivity study

For the sensitivity study, dilution series of three genomic DNA samples were amplified: 2 ng (three replicates each), 1 ng, 0.5 ng, and 0.25 ng (four replicates each). The results were evaluated for mean peak height, degree of linearity between input DNA concentration and peak height, level of allelic dropout at 250 pg, and genotype concordance.

Mean peak height

Mean peak height observations were consistent between all Test and Control mixes (Figure 12) demonstrating equivalent performance (Figure 13).

Figure 12 Sensitivity study: mean peak heights for three genomic DNA samples

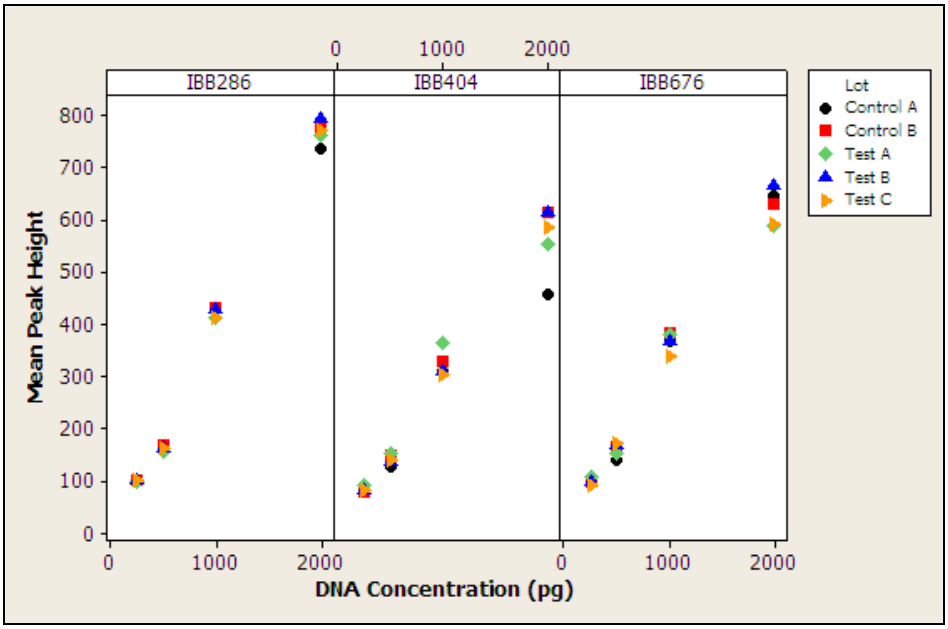
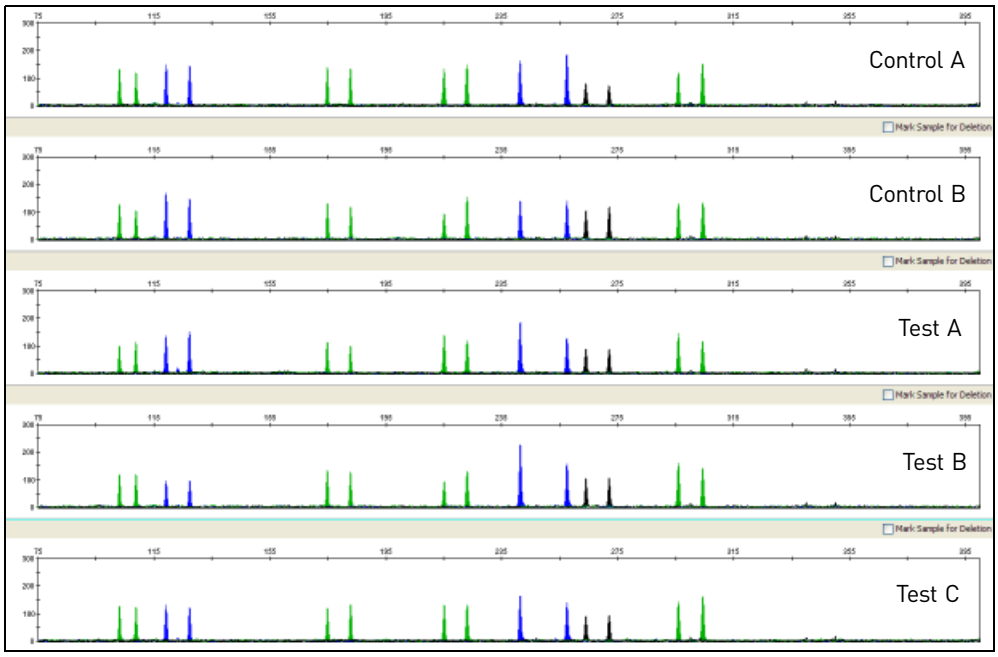


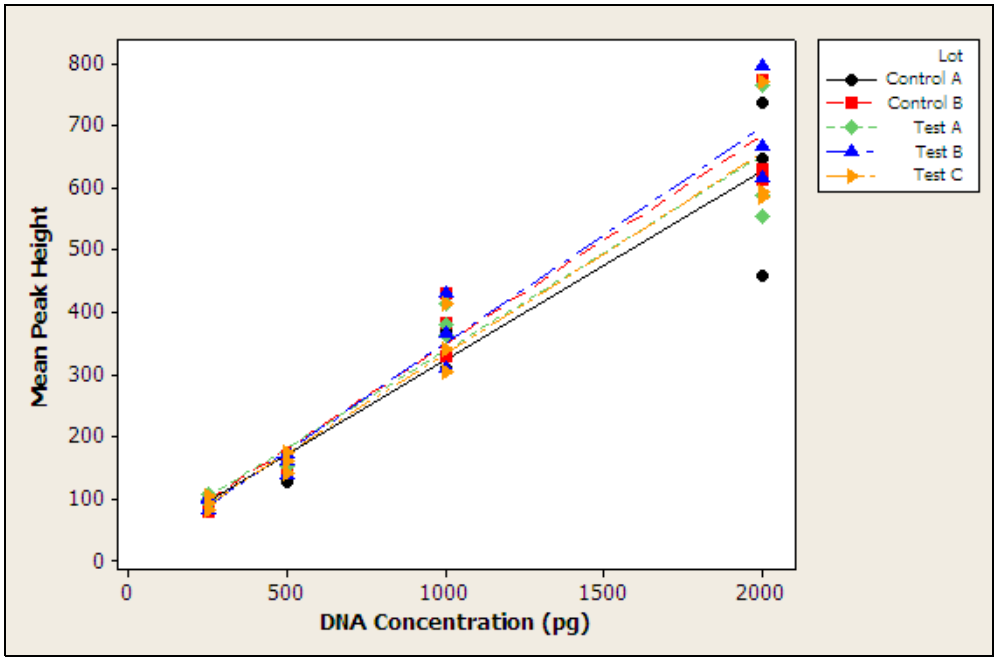
Figure 13 Sensitivity study: representative electropherograms for Sample 2 amplified using 250 pg input DNA (Y-scale 500 RFU)



DNA concentration and peak height

The calculated slope and R^2 values for each of the plotted curves are equivalent, showing comparable relationships between peak height and DNA input amount for the Test and Control mixes (Figure 14).

Figure 14 Sensitivity study: linear regression plot of combined mean peak height for three genomic DNA samples



Allelic dropout

No allelic dropout was observed for any of the Test or Control mixes.

Genotype concordance

Genotypes for Test and Control mixes were 100% concordant (Table 6).

Table 6 Sensitivity study: genotype concordance

DNA Input Amount	Reagent Mix	Genotype Concordance
250 pg	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
500 pg	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
1 ng	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%
2 ng	Test A	100%
	Test B	100%
	Test C	100%
	Control A	100%
	Control B	100%

Conclusions

Laboratories can expect to obtain equivalent quality profiles across a wide range of forensic samples when using the COfiler® Kit containing the AmpliTaq Gold® enzyme and 10X PCR Buffer II manufactured by Life Technologies as compared to the original COfiler® Kit containing AmpliTaq® Gold enzyme and 10X PCR Buffer II manufactured by Roche Molecular Systems.

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