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TECHNICAL NOTE

Patrick J. Collins,[1] *B.A.; Lori K. Hennessy,*[1] *Ph.D.; Craig S. Leibelt,*[1] *A.B.; Rhonda K. Roby,*[1,†] *M.P.H.; Dennis J. Reeder,*[1] *Ph.D.; and Paul A. Foxall,*[1] *Ph.D.*

Developmental Validation of a Single-Tube Amplification of the 13 CODIS STR Loci, D2S1338, D19S433, and Amelogenin: The AmpFℓSTR® Identifiler® PCR Amplification Kit

ABSTRACT: Analysis of length polymorphism at short tandem repeat (STR) loci utilizing the polymerase chain reaction (PCR) process has proven to be an ideal assay for human identification purposes. The short length of STR loci coupled with the amplification of target sequence through PCR allows for a robust, sensitive, and specific assay for highly polymorphic markers. A multiplex containing fifteen STR loci plus the gender-determining locus Amelogenin was developed to provide a single amplification/detection of all CODIS (Combined DNA Index System) STR loci (CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA) as well as two internationally-accepted STRs (D2S1338 and D19S433). By incorporating five-dye fragment analysis technology and non-nucleotide linkers, previously optimized AmpFℓSTR® kit primer sequences have been maintained. This kit has been developed in accordance with the standards of the forensic community as defined by the DNA Advisory Board. Validation studies were performed to include developmental validation, and the results support the use of the AmpFℓSTR® Identifiler® PCR Amplification Kit for human identity and parentage testing.

KEYWORDS: forensic science, PCR, STR, multiplex, development, optimization, AmpFℓSTR

Automated, fluorescent detection of length polymorphism at short tandem repeat (STR) loci is a rapid, reliable and accurate method for gathering genetic information for a variety of purposes. When coupled with the polymerase chain reaction (PCR) utilizing fluorescently labeled primers, STR analysis has proven to be a highly sensitive and discriminating assay for human identification (1). Fields of study that employ this technology include human evolution, disease linkage analysis, gene mapping, identification of human stains and remains, and parentage testing. In the United States, the Combined DNA Index System (CODIS) was created to provide federal, state, and local laboratories with the ability to submit, search, and store STR profiles generated from human samples (2). Submission of data to CODIS requires genotypic information from 13 tetranucleotide STR loci: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA (3). STR databasing standards outside of the U.S. have also been created (e.g., ENFSI, Interpol, GITAD), and most contain a subset of the loci required for CODIS. However, the National DNA Database (NDNAD), maintained by the Forensic Science Service (FSS) in England, contains eight CODIS loci plus two additional tetranucleotide markers, D2S1338 and D19S433 (4,5).

The AmpFℓSTR® PCR Amplification kits (Applied Biosystems, Foster City, CA) were designed to amplify all of the above STR loci optimally (6). The AmpFℓSTR® Profiler Plus™ and COfiler® PCR Amplification kits must be used in combination to obtain profiles for all 13 CODIS loci for a given sample. To cover all 15 internationally accepted STR loci, the combination of the AmpFℓSTR® SGM Plus® and Profiler® PCR Amplification kits can be employed. A new multiplex PCR assay, the AmpFℓSTR® Identifiler® PCR Amplification kit, has been developed to co-amplify and simultaneously detect all 15 internationally adopted STRs.

The Identifiler kit alleviates a significant bottleneck in genotype throughput by eliminating the need for two amplifications and two lanes or injections of electrophoretic analysis to obtain complete CODIS or international profiles for a particular sample. Throughput is further enhanced by increased allelic representation in the allelic ladder, decreasing the number of "off-ladder" alleles that need to be rerun. In addition to improving throughput, this kit was designed to preserve existing AmpFℓSTR kit primer sequences and reaction conditions (reaction mix concentrations and thermal cycling parameters), as these are optimized and widely accepted (7).

[1] Human Identification Group, Applied Biosystems, Foster City, CA.
[†] Current address: Founder/Forensic Scientist, Identity Quest LLC, 13203 Astoria Hill Court, Suite J, Germantown, MD.

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The single design departure from previous AmpFℓSTR kits was the adoption and subsequent validation of a 25 μL reaction volume for the Identifiler kit, rather than the 50 μL reaction volume recommended for other AmpFℓSTR kits.

To verify that the Identifiler kit would perform as reliably and robustly as other STR multiplexes, a developmental validation was conducted according to sections 8.1.1–8.1.2.3.1 of the "Quality Assurance Standards for Forensic DNA Testing Laboratories" (8). Due in large part to the design goals mentioned above, the Identifiler kit was expected to perform as robustly as previous AmpFℓSTR kits with little or no modification to existing protocols and reagent concentrations.

Materials and Methods

Locus Selection and Characterization

Each of the 15 STR loci amplified by the Identifiler kit has been characterized extensively. The CODIS STR loci have been studied thoroughly for physical linkage, Mendelian inheritance, approximation of Hardy-Weinberg equilibrium, and independent assortment (2,9). Likewise, the D2S1338 and D19S433 loci have been validated with similar studies in both the U.S. and Europe (10,11).

The primers used to amplify the Identifiler kit loci were employed in the AmpFℓSTR Blue; AmpFℓSTR Green I; and AmpFℓSTR SGM Plus, COfiler, Profiler Plus, and Profiler kits (6). The AmpFℓSTR kit primers were designed for small amplicon sizes, specific and reliable amplification, and similar performance in a multiplex reaction. In order to accommodate allelic size ranges specified by existing AmpFℓSTR kit primer sequences, a 5-dye fragment analysis system was developed at Applied Biosystems. Where previous AmpFℓSTR kits employed Dye Set F (5FAM™, JOE, NED™, and ROX™ dyes), PCR products from the Identifiler kit are labeled with Dye Set G5, comprised of 6FAM™ (blue), VIC® (green), NED™ (yellow), and PET™ (red) dyes with the LIZ® (orange) dye used to label the GeneScan™ -500 Size Standard.

To prevent overlapping allelic size ranges for loci labeled with the same dye, monomeric non-nucleotide linkers were added to select labeled primers. When incorporated into a PCR product, these linkers alter the electrophoretic mobility of the PCR product to reproducibly retard migration in ABI PRISM® separation matrices (e.g., POP-4™ polymer, acrylamide) (12,13). Primers for five loci, CSF1PO, D2S1338, D13S317, D16S539, and TPOX, incorporate mobility modifiers to increase detected allele size by 7–26 bp depending on locus (as interpolated from the GeneScan-500 LIZ Size Standard). Sizing shifts for each locus and sizing precision data are listed in Table 1(14–17). Figure 1 depicts the yellow channel of one genomic sample amplified using TPOX primers both with and without mobility modifiers. The linkers allow for spacing between adjacent loci labeled in the same dye, permitting the use of existing primer sequences. The mobility modifiers do not greatly increase the allelic size range; the largest allele product size without mobility modifiers is 353 bp (FGA), while the largest allele product incorporating mobility modifiers is 359 bp (D2S1338).

TABLE 1—*Sizing shift and sizing precision data for loci incorporating non-nucleotide linkers on the ABI* PRISM *310 Genetic Analyzer.*

Locus	Increase in Detected Size (bp)	Range of Standard Deviation of Alleles for Identifiler Kit (bp)	Range of Standard Deviation of Alleles Previous Kit (bp)
CSF1PO	26	0.08–0.13*	0.03–0.10†
D2S1338	16	0.05–0.12*	0.02–0.15‡
D13S317	12	0.05–0.09*	0.02–0.09§
D16S539	23	0.06–0.09*	0.01–0.08‡
TPOX	7	0.03–0.08*	0.02–0.07†

* Data taken from the AmpFℓSTR Identifiler PCR Amplification Kit User's Manual (14).

† Data taken from the AmpFℓSTR COfiler PCR Amplification Kit User's Manual (15).

‡ Data taken from the AmpFℓSTR SGM Plus PCR Amplification Kit User's Manual (16).

§ Data taken from the AmpFℓSTR Profiler Plus PCR Amplification Kit User's Manual (17).

Sizing information for loci amplified with non-nucleotide linker-containing primers. Approximate sizing shift relative to previous kits imparted by the linkers is listed in the first column. Standard deviation ranges for the alleles present in the allelic ladder for these loci are listed for both the Identifiler kit and for previous kits. Where possible, the most recent data was taken for each locus. In all cases, standard deviations for mobility modified loci are similar to previous ranges and meet the 0.15 bp standard deviation specification necessary for detection of single base pair variants.

Sample Source and Extraction Protocols

Extracted human DNA samples were either obtained from the Serological Research Institute (Richmond, CA) and quantified

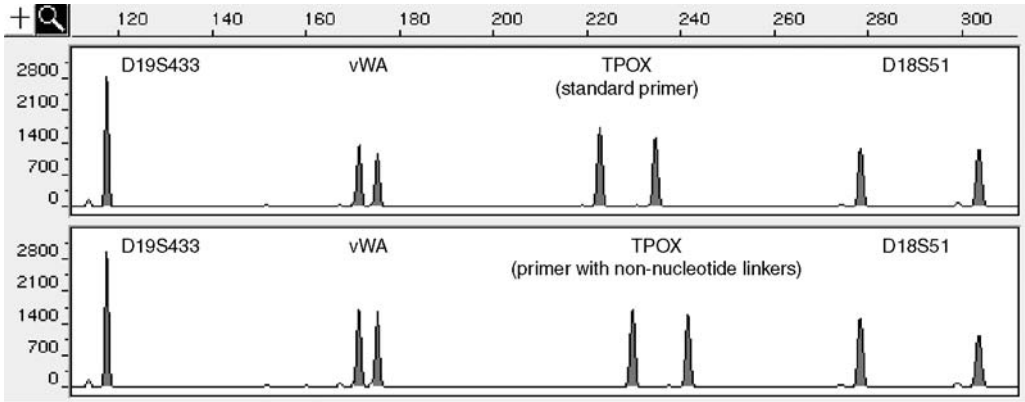

FIG. 1—*NED dye labeled loci from two amplifications of a single sample using TPOX primers both with and without non-nucleotide linkers. The X-axis indicates base pair size and the Y-axes RFU. The top panel depicts the amplification without non-nucleotide linkers. Sizes for the TPOX alleles for this panel were 222.93 and 234.81 bp. Sizes for the TPOX alleles in the amplification using the modified primer, depicted in the bottom panel, were 229.85 and 241.71 bp, indicating an average shift of 6.91 bp. Peaks heights, intralocus balance, and intracolor balance were similar in both amplifications.*

using the QuantiBlot® Human DNA Quantitation Kit (Applied Biosystems) or were AmpFℓSTR Control DNA from the Profiler Plus, Cofiler, and SGM Plus kits (Applied Biosystems). Primate DNA samples were obtained from BIOS Laboratories, Inc. (New Haven, CT). Non-primate DNA samples were obtained from Celera AgGen (Davis, CA) or Applied Biosystems. Non-primate samples were extracted using either phenol/chloroform (18) or IsoQuick™ Nucleic Acid Extraction kit (Orca Research, Inc., Bothell, WA). Samples used for genotyping concordance verification were those included in the National Institute of Standards and Technology Standard Reference Material® 2391a (NIST, Gaithersburg, MD).

PCR Amplification

Unless stated otherwise, a master mix was prepared as follows for all amplifications: 10.5 μL AmpFℓSTR® PCR Reaction Mix, 0.5 μL AmpliTaq Gold® DNA Polymerase, and 5.5 μL Identifiler® Primer Set multiplied by the number of samples. Fifteen μL of this mix were then aliquotted into either MicroAmp® 8 Strip Reaction Tubes with MicroAmp® Caps (8 Caps/strip) or MicroAmp® Reaction Tubes with Cap (Applied Biosystems). Ten μL of dilute DNA were then added to each tube so that the final template input was in the range of 0.5–1.25 ng for a total reaction volume of 25 μL. Eight strip reaction tubes were placed in a 96-well tray/retainer and capped or, alternatively, individual reaction tubes with caps were placed in a 96-well tray (Applied Biosystems). All reactions were performed in either a GeneAmp® PCR System 9600 or 9700 (Applied Biosystems) operating in 9600 emulation mode. Unless stated otherwise, the thermal profile consisted of an 11 min incubation at 95°C, followed by 28 cycles of three-step PCR at 94°C, 59°C, and 72°C each for 1 min, and concluded with a final hold at 60°C for 60 min. A final hold was added if the PCR product was to remain in the thermal cycler at 25°C for up to 18 h or 4°C for longer

Electrophoresis, Detection, and Analysis

All PCR products were separated on the ABI PRISM® 310 Genetic Analyzer using POP-4™ polymer (Applied Biosystems). 1.5 μL PCR product was combined with 0.5 μL GeneScan-500 LIZ Size Standard and 24.5 μL Hi-Di™ Formamide in either a Genetic Analyzer Sample Tube (for use with the 48-well autosampler tray) or the MicroAmp 8 Strip Reaction Tubes (for use with the 96-well autosampler tray) (Applied Biosystems). One tube was prepared additionally as above, but 1.5 μL of AmpFℓSTR® Identifiler® Allelic Ladder was substituted for sample product. Tubes were capped with either Genetic Analyzer Septa for 0.5 mL Sample Tubes or Genetic Analyzer Septa Strips (Applied Biosystems). Samples were then denatured at 95°C for 3 min and snap-cooled for 3 min. Injections were performed using the GS STR POP4 (1 mL) G5 module and, in most cases, each sample was injected twice. After collection, data were analyzed using GeneScan® Software v3.1 (Applied Biosystems) with peak amplitude threshold set at 50 relative fluorescence units (RFU) for all colors. Genotypes were generated using Genotyper® Software v2.5.2 (Applied Biosystems). Samples where only one allele of a heterozygous locus was called by analysis software were reanalyzed with a threshold of 25 RFU to determine if the other allele was detectable. While peaks could occasionally be detected below 25 RFU, random baseline noise can also be detected in this range. The low analytical threshold has been employed to capture as much data as possible and should not be considered an interpretational threshold.

Balance Calculations

Three types of balance were monitored in evaluating the performance of the Identifiler kit: intralocus, intracolor, and intercolor. Peak heights were exported from GeneScan software into Microsoft® Excel and, unless otherwise stated, multiple injections were averaged prior to subsequent calculations. Intralocus balance, or heterozygote peak height ratio, was calculated by dividing the lower allele of a heterozygous individual by the higher and then expressed as a percentage. Intracolor balance was assessed by first averaging heterozygous peaks and dividing homozygous peaks in half. Once normalized for diploidy, the lowest score for a locus labeled with a given dye was divided by the highest, and the result was reported as a percentage. Intercolor balance was calculated in a similar manner to intracolor, although the comparison was made across all loci regardless of dye label.

Primer Set Concentration

Minor changes were made to the AmpFℓSTR primer concentrations for the Identifiler kit to account for the use of different reporter dyes. Final primer reaction concentrations ranged from 0.24–0.44 μM depending on locus for Dye Set F primers, whereas for the Identifiler kit (Dye Set G5) concentrations ranged from 0.20–0.44 μM. Two samples were amplified in triplicate at the final concentrations and at 10% intervals to ±4g0% to test primer set performance. The primer set was diluted 1:2 in modified TE buffer (10 mM Tris, 0.1 mM EDTA) and between 6–14 μL dilute primer set was then added to individual reactions to achieve desired primer set concentrations at 10% intervals.

PCR Reaction Mix

Reaction mix titrations were performed in accordance with the goal of keeping reaction conditions constant, relative to existing AmpFℓSTR kits. For each component, a range of concentrations was tested around the standard condition, as defined by the AmpFℓSTR PCR Reaction Mix. Typically, concentrations were tested at ±10%, as well as at larger deviations from the standard to test for both reliability within a normal use range and robustness under extreme conditions. Each concentration was tested with three DNA samples and amplified in duplicate with a targeted input of 1 ng. The following components were tested at the indicated concentrations, with all other reagent concentrations held constant (AmpFℓSTR kit standard indicated in bold):

- AmpliTaq Gold DNA Polymerase—1.5, 2, **2.25**, 2.5, 3, and 4.5 U per reaction
- $MgCl_2$—0.8, 1.0, 1.15, **1.25**, 1.35, 1.50, 1.75, and 2.0 mM
- dNTP mixture (equimolar dATP, dCTP, dGTP and dTTP)—600, 720, **800**, 880, and 1000 μM
- KCl—25, 40, **50**, 60, and 75 mM
- BSA—0, 2, 3.2, **4**, 4.8, 8, and 16 μg per reaction
- Sodium azide—0%, **0.05%**, 0.10%, 0.15%, and 0.20%

Thermal Cycling Parameters

As with reaction mix component optimizations, thermal cycling parameters were tested with the goal of preserving the standard AmpFℓSTR kit reaction conditions. To verify optimal performance for the Identifiler kit, two or more cycling parameters were tested around the AmpFℓSTR kit standard. For each study, 1 ng of three DNA samples was prepared in duplicate,

and then stored at 4°C for no more than 24 h while awaiting amplification. Variable conditions were then tested by consecutively amplifying the previously prepared samples in the same thermal cycler for each parameter. The following thermal cycle parameters were examined, with the previous standard indicated in bold:

- AmpliTaq Gold DNA Polymerase Activation Temperature—94°, **95°C**, and 96°C
- Denaturation temperature—92.5°, **94°**, and 95.5°C
- Annealing temperature—55°C, 57°C, **59°C**, 61°Cs and 63°C
- Final extension time—15, 30, **45**, 60, 75, and 90 min
- Cycle number—27, **28**, 29, 30, and 31 cycles

Species Specificity, Sensitivity, Stability, and Mixture Studies

The AmpFℓSTR kit primers were designed to be primate specific with no cross-reaction with other animal or microbial species. Amplifications were performed on 1 ng of genomic DNA from chimpanzee, orangutan, macaque, and gorilla to determine the completeness of profiles for these primate species. Reactions for non-primate samples were performed with more DNA, 2.5–10 ng, than recommended to detect potential low-level amplification. Representatives from the following groups were tested: vertebrates (dog, mouse, horse, cow, cat, pig, and chicken), yeast (*Saccharomyces cerevisiae*), and bacteria (*Bacillus subtilis, Brochothrix campestris, Pseudomonas aeruginosa, Escherichia coli, Neisseria gonorrheae,* and *Staphylococcus aureus*).

Assessing amplification performance with a range of DNA input amounts is helpful to understand potential interpretational limitations of a PCR-based typing system. Due to system variation, sensitivity studies aimed at producing interpretation guidelines are best performed by the individual testing laboratory. However, to demonstrate Identifiler kit performance, triplicate amplifications were performed on a dilution series of a genomic sample (0.03125, 0.0625, 0.125, 0.20, 0.25, 0.5, 1.0, and 1.25 ng).

Stability studies were conducted to characterize amplification performance in the presence of either degraded or inhibited DNA, both of which have been known to alter the efficiency of both single-plex and multiplex reactions (19–23). Bovine serum albumin has been added to the AmpFℓSTR PCR Reaction Mix to improve PCR efficiency in the presence of some inhibitors (24). To assess the effects of inhibition on amplification, porcine hematin (Sigma-Aldrich, St. Louis, MO), a heme-containing known inhibitor was added to 1 ng amplifications prior to PCR in concentrations varying from 10–36 μM (25). To determine the efficiency of amplification in the presence of degraded DNA, deoxyribonuclease (DNase I; Gibco BRL, Gaithersburg, MD) was used to digest DNA for 30 s; for 1, 4, 8 12, 20, and 30 min; and for 1, 2, 4, 8, and 24 h (25). One ng of undigested DNA or 4 ng of digested DNA from each timepoint was then added to individual reactions.

Analysis of mixture data (profiles generated from a sample containing DNA from more than one individual) is ultimately guided by examiner interpretation and discretion. However, as part of our developmental validation, a mixture study was conducted. Two genomic samples were mixed in the following proportions, along with controls, so that the total DNA input for the reaction was 1.0 ng: 20:1, 10:1, 3:1, 1:1, 1:3, 1:10, and 1:20. Amplifications were performed in triplicate.

Population and Concordance Studies

Allele frequency distributions in major population groups and relevant statistics for the loci amplified by the Identifiler kit were documented in the Identifiler kit User's Manual (N = 1187) (14). A subset of these samples (N = 461) was tested further for expectations of independence and approximation of Hardy-Weinberg equilibrium with no significant deviations noted (11).

In the course of a population/concordance study, a significant difference in genotype between commercial kits was noted at the D8S1179 locus in a population from Guam (approximately 5% of alleles, 10% of individuals) (26). A mutation was found in the AmpFℓSTR primer binding region for the reverse primer at this locus. Although this mutation affects only a small percentage of the world population, a degenerate primer was added to the Identifiler primer set to rescue amplification of mutant alleles. The experiments detailed in this study were conducted without the use of the degenerate primer. To ensure the addition of the primer did not alter performance of the Identifiler kit multiplex, a number of the experiments described here have been repeated, and no ill effects were observed as a result of the primer addition (Leibelt et al., submitted). Two other degenerate primers, for D16S539 and vWA, have been included in previous AmpFℓSTR primer sets with no deleterious effects (6,27).

Genotypes obtained with the Identifiler kit were tested for concordance using NIST Standard Reference Material 2391a. Genotypes for all fifteen STR loci, as well as sex information obtained from the Amelogenin sex marker, amplified by the Identifiler kit are provided in the Certificate of Analysis. One ng of eight genomic and two cell line-derived DNA samples were amplified, genotyped, and compared to the manufacturer's data.

Additionally, the samples used to generate allele frequency distributions were typed with the Profiler Plus, Profiler, and/or COfiler kits (3) (M. Adkins and A. Gross, personal communication). Identifiler genotypes were tested for concordance with previous results.

Results

Primer Set Concentration

Peak heights were generally found to increase linearly with increasing primer set concentration. However, little or no improvement in signal strength was observed above the standard concentration at CSF1PO, D7S820, D18S51, and FGA (data not shown). Full profiles were generated at all primer concentrations tested without any spurious amplification or effect on intralocus balance (data not shown). Between both the minimum and standard and the standard and the maximum concentration tested, intracolor balance decreased approximately 9% on average. Guided by the previous AmpFℓSTR kit standard, final reaction concentrations for primers were chosen as a compromise between robust amplification of individual loci and intracolor balance.

PCR Reaction Mix

AmpliTaq Gold DNA Polymerase—Similar results were observed when testing AmpliTaq Gold DNA Polymerase concentrations in the ±10% range around the recommended 2.25 U in a 25 μL reaction. At the lowest and highest levels tested, peak heights were respectively lower and higher than at the standard input level, but with no effect on the completeness of profiles (data not shown). As the amount of enzyme increased, loci larger than 200 bp benefited from increased peak height more than smaller loci. At the standard condition, 2.25 U, the lowest observed heterozygote peak height ratio was 61% while the average was 88% (n = 72). Average intracolor balance improved between 1.5 U and 2.25 U by 21% and a further 9% between that value and 4.5 U.

$MgCl_2$—As a DNA polymerase cofactor, $MgCl_2$ functions as a critical reagent for the PCR amplification. A sigmoidal relationship of signal strength and concentration was observed, with little amplicon detected at low $MgCl_2$ and rapid increase to an upper bound for most loci around the standard condition of 1.25 mM. At 0.8 mM $MgCl_2$, amplification above the standard peak amplitude threshold was only observed at D8S1179 and Amelogenin (data not shown). At 1.0 mM $MgCl_2$, smaller fragments were preferentially amplified, and low signal was observed at some loci (e.g., D13S317, D18S51, and FGA). Between 1.15–1.35 mM, results were very similar by all measures, and the multiplex performed optimally in this range. The loci D3S1358, D13S317, and D16S539 continued to increase in signal with added $MgCl_2$, resulting in an unbalanced profile for VIC dye-labeled loci at higher concentrations.There were no non-specific amplicons detected in the read region, even at the maximum concentration tested, 2.0 mM. At 1.25 mM $MgCl_2$,the average observed intralocus balance was 90% with a minimum of 70% (N = 66). Due to lack of amplification at most loci, intracolor balance could not be calculated at 0.8 mM but grew rapidly from an average of 23% at 1.0 mM to a plateau of 60–70% for all other concentrations tested. Representative electropherograms for 1.0–1.5 mM $MgCl_2$ are shown in Fig. 2.

DNTPs—Deoxyribonucleoside triphosphate concentration was noted to have an inverse relationship with amplification performance. At 600 μM dNTP, D3S1358, D13S317, and D16S539 peak heights were higher than other VIC dye-labeled loci by an average of 70%, resulting in poor intracolor balance. Between 720–880 μM, results were very similar. One millimolar dNTP amplification exhibited slightly decreased peak heights, but no dropout was observed. Intralocus balance did not vary with dNTP concentration; heterozygote peak height ratio averaged 87% at 800 μM with a minimum of 63% (N = 66). Although intracolor balance in the green dye layer improved approximately 10% through increased dNTP concentration to a plateau around 800 μM, on average intracolor balance was constant until a 20% reduction at 1 mM (data not shown).

KCl—Varying potassium chloride concentration resulted in a parabolic distribution of peak heights, although the degree of the effect depended largely on locus size. Diminished peak heights and very poor amplification of D13S317 were evident at 25 mM KCl. Peak heights were optimal for the multiplex in the 40–60 mM range. At 75 mM, peak heights were reduced for all loci, most dramatically for those sizing 250 bp or larger. Intralocus balance was best in the 40–60 mM range with greater heterozygote peak height imbalance noted at 75 mM than at 25 mM. The lowest observed intralocus balance at 50 mM was 72% with an average of 89% (N = 66). Intracolor balance, optimal in the 40–60 mM range, decreased 20% from the standard concentration to 25 mM and to less than 10% at 75 mM (data not shown).

Other Tested Components—No meaningful differences were seen with varying bovine serum albumin concentrations around the standard of 4 μg per 25 μL reaction. Similarly, sodium azide results were comparable to standard (0.05% v/v) at all concentrations tested (data not shown).

Thermal Cycling Parameters

AmpliTaq Gold DNA Polymerase Activation and Denaturation Temperatures—AmpliTaq Gold DNA Polymerase had previously

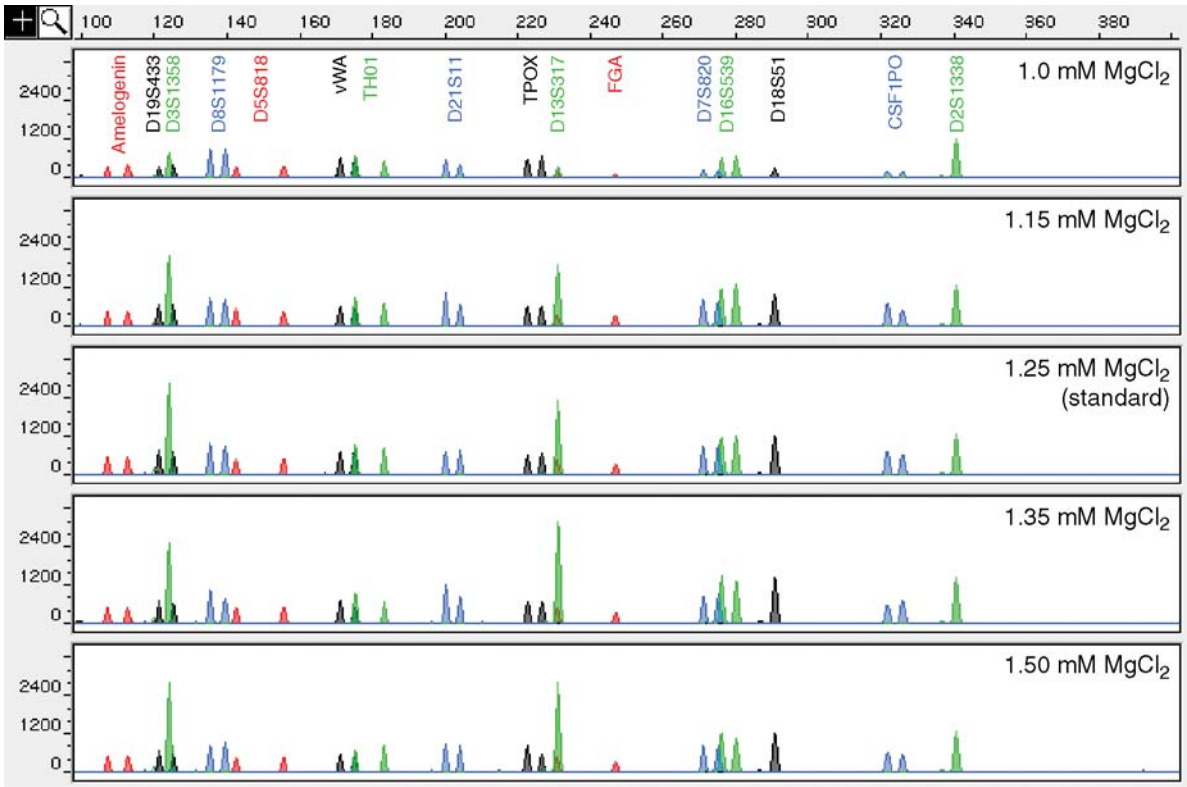

FIG. 2—*Representative electropherograms from a PCR reaction mix $MgCl_2$ titration. The panels depict 1.0, 1.15, 1.25, 1.35, and 1.50 mM $MgCl_2$ in order from top to bottom. The X-axis indicates base pair size and the Y-axes RFU. At the lowest depicted concentration, several loci were not performing optimally. However, all higher tested concentrations resulted in robust performance.*

been observed to perform optimally with AmpFℓSTR primers and reaction mix using an 11 min activation time (25). Two activation temperatures, 94 and 96°C, were tested around the standard of 95°C for 11 min to ensure robust enzyme activation. Additionally, two denaturation temperatures, 92.5 and 95.5°C, were tested against the recommended 94°C. All tested temperatures performed similarly in terms of peak height, intralocus, and intracolor balance (data not shown).

Annealing Temperature—The PCR primers employed in the Identifiler kit were designed and selected for efficient multiplexing. This strategy resulted in an optimal performance window of 59°C, around which all markers performed similarly (6). At 55°C, strong performance of some loci (e.g., D13S317, average peak heights, 177% of those of 59°C) compared to poor performance of others (e.g., D16S539, 42% of standard) resulted in a poorly balanced multiplex. At 57°C, CSF1PO, D3S1358, D7S820, D19S433, and TH01 performed optimally, although this led to widely disparate peak heights within and between colors. At 59°C, peak heights were most balanced for the multiplexug At 61°C, diminished peak heights were evident at some loci, although full profiles were generated for all samples. At the highest temperature tested (63°C), 10 STR loci nearly or completely fell below 50 RFU. At the standard annealing temperature (59°C), intralocus balance averaged 90% with an observed minimum of 65%. Intracolor balance increased approximately 18% from 55°C to an optimum at 59°C and then decreased to 0% at 63°C, such that full profiles were reliably obtained at all but the highest tested temperature (data not shown).

Final Extension—The primers used in the Identifiler kit were optimized for non-specific terminal nucleotide addition by AmpliTaq Gold DNA Polymerase (28,29). To ensure complete terminal nucleotide addition, a final 60°C hold was added. At 15 min final extension, terminal nucleotide addition was incomplete, but at all subsequent tested timepoints, results were comparable at most loci. For the majority of samples, after 15 min of final extension, incomplete nucleotide addition was detected only at the vWA locus. With more than 15 min final extension, less than 10% incomplete product was observed at the vWA locus except for a single amplification of one sample at 30 and 45 min. Sixty min provided a small but uniform benefit over 30 and 45 min in terms of nucleotide addition with a maximum measured –N of 8.2%. Final extensions longer than 60 min did not provide worthwhile benefits (7.1% versus 7.6% for the least amount of incomplete addition detected). Although the standard 60 min extension time will facilitate terminal nucleotide addition, some incomplete products will remain detectable under standard conditions, typically at the vWA locus (data not shown).

Cycle Number—As expected, most peak heights increased with each additional PCR cycle. Results were very similar with 27, 28, and 29 cycles in terms of balance, although incrementally increased peak heights were observed with each added cycle. At 29 cycles, one offscale, homozygous peak was detected in three of six amplified samples. At 30 and 31 cycles, several peaks at different loci were offscale, and an abundance of incomplete terminal addition products was observed with 1 ng of template. Minimum intralocus balance at 28 cycles was 66% with an average heterozygote peak height ratio of 88% (N = 66). Intracolor balance was similar between 27 and 29 cycles but decreased an indeterminate amount at the two highest cycle numbers due to offscale data (data not shown).

Species Specificity, Sensitivity, Stability, Mixture, and Population Studies

Species Specificity—When tested with a range of animal and microbial templates, amplification was observed only among primates with one notable exception. One ng of primate DNA (chimpanzee, orangutan, macaque, and gorilla) produced partial profiles. At most, 11 loci were amplified for chimpanzee and gorilla DNA. The only non-primate amplification observed was for an approximately 103 bp-sized, PET dye-labeled fragment in equine, porcine, and canine (at reduced peak height) samples. With increased template, amplification of this fragment from bovine DNA was also observed. This fragment has been attributed to a monomorphic product amplified from certain mammals by the Amelogenin primers (30). By comparison, Amelogenin amplification of male human DNA resulted in 107 and 112 bp PET™ dye-labeled fragments. The remainder of tested species exhibited no amplification (chicken, cat, mouse, *B. subtilis*, *B. campestris, P. aeruginosa, E. coli, N. gonorrheae, S. aureus,* and *S. cerevisiae*). Representative electropherograms for several species are illustrated in Fig. 3.

Sensitivity—For triplicate amplifications performed on a genomic source, with input ranging from 0.03125–1.25 ng, all data were onscale, and the highest analyzed peak was observed at 5148 RFU for a 1.25 ng amplification of a D8S1179 13 allele. Figure 4 depicts peak heights obtained in this study grouped by dye label and template amount. Full profiles were generated for all but the two lowest template levels, assuming a 50 RFU peak amplitude threshold. At 62 pg input DNA, 144 of 174 peaks (injections not averaged) were detected above 50 RFU with a maximum of 239 RFU. The 30 remaining peaks were all visible above 25 RFU. Eighteen of these 30 peaks were observed with a sister allele that measured over 50 RFU (the highest such peak was 108 RFU). At 31 pg input DNA, the maximum peak height observed was 171 RFU, and 65 of 174 peaks were detected above 50 RFU. Sixty-six peaks were observed between 25–50 RFU, and a further 43 were not visible above 25 RFU. Twenty of the peaks not detected above 50 RFU had sister alleles that measured greater than 50 RFU. Results are summarized for all but the 31 pg input in Table 2 with respect to minimum observed peak height, average, and minimum observed intralocus balance. As several peaks were not visible at 31 pg, accurate calculations could not be made for average and minimum intralocus balance. Figure 5*a* contains a scatter plot of heterozygote peak height ratios from this study. The accompanying scatter plot, Fig. 5*b*, illustrates peak

TABLE 2—*Minimum peak height and average and minimum heterozygote peak height ratios for 0.0625–1.25 ng of template.*

Template Input (ng)	Min. Peak Height (RFU)	N	Avg. Heterozygote Peak Height Ratio (%)	N	Min. Heterozygote Peak Height Ratio (%)
1.25	640	145	89	65	68
1.0	420	174	88	78	59
0.5	231	174	86	78	61
0.25	121	174	82	78	56
0.2	102	174	78	78	44
0.125	50	174	73	78	30
0.0625	25	174	73	78	31

Descriptive statistics for sensitivity study without averaging multiple injections of the same sample. As some peaks were not detected at 0.03125 ng, the data from this template amount have not been included. Average heterozygote peak height ratios decreased directly with template input, however minimum intralocus balance was affected more heavily.

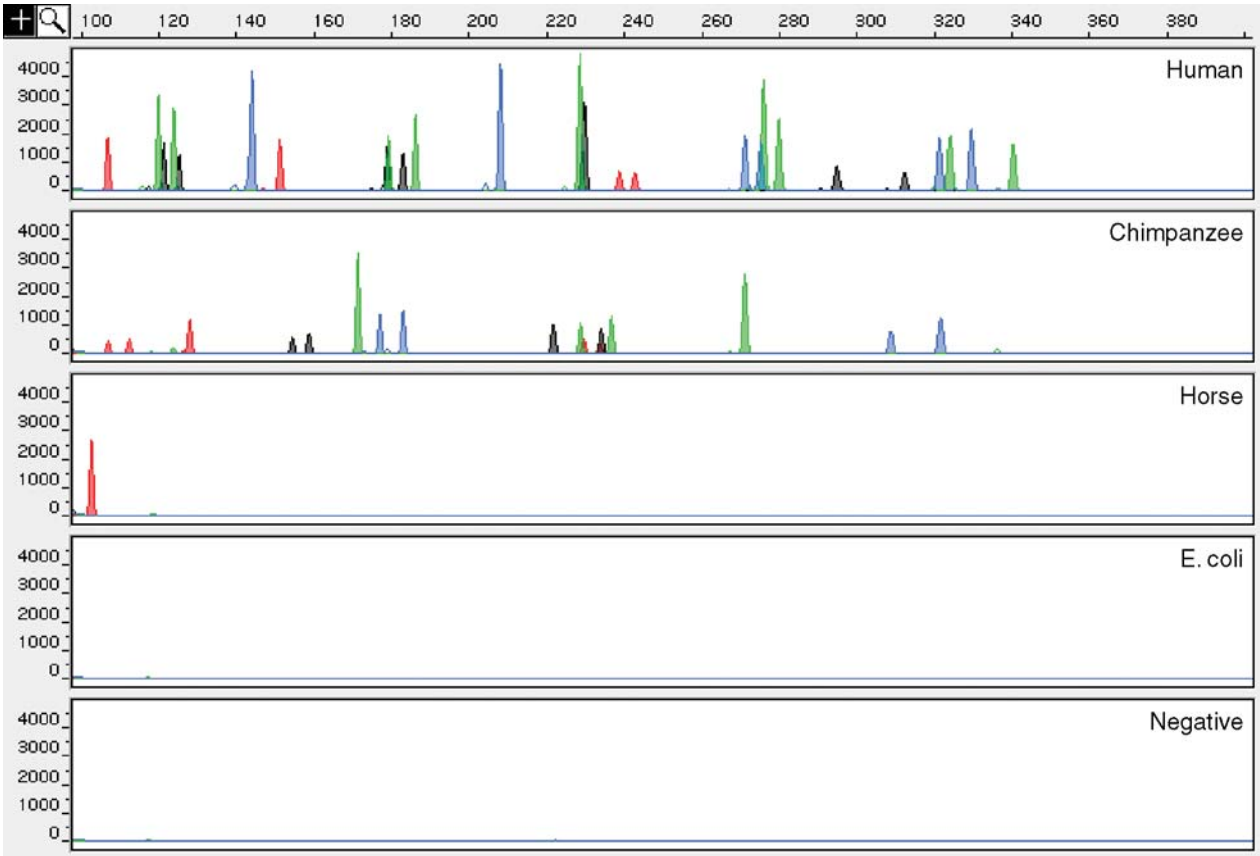

FIG. 3—*Representative results from a species specificity study including positive and negative controls. The top panel shows a 1 ng amplification of AmpFℓSTR Control DNA 9947A, panel 2 chimpanzee (1 ng), panel 3 horse (2.5 ng), panel 4 E. coli (5 ng), and the bottom panel the negative control. The X-axis indicates base pair size and the Y-axes RFU. Except for the monomorphic, Amelogenin-like product seen in panel 3, amplification was only observed for primates among all tested species.*

height ratios for all 15 STR loci obtained from concordantly-typed population samples with template inputs ranging from 0.25 to greater than 3 ng. When all peaks were detected for a given sample, intracolor balance did not vary with template amount, averaging 61–73% for 0.0625–1.25 ng.

Some minor artifacts were routinely observed with the Identifiler kit that could possibly complicate interpretation of amplifications with low levels of input template. These artifacts were uniformly present in the negative control, and this should be used to facilitate interpretation of samples (Fig. 6). Note that the morphology of these artifact peaks differs from true product peaks. The artifacts are dye-labeled primer byproducts and were either present prior to amplification or generated in part by the thermal cycling process. Within a run, artifacts did not vary greatly in migration or peak height, and this should be taken into account when analyzing and interpreting data. However, artifacts did vary in migration and peak height depending on separation medium and detection platform (e.g., POP-6™ polymer and ABI PRISM® 3700 DNA Analyzer) and should be evaluated by testing laboratories that employ more than one instrument type (data not shown).

Stability—When challenged with hematin, decreased peak heights were evident at the lowest concentration (10 μM), relative to the inhibitor-free control. For one sample heterozygous at the loci D7S820 and D18S51, one allele was detected above 50 RFU and one below at these loci with 12 μM hematin in the reaction. Locus dropout increased successively with hematin concentration until no loci were amplified at 22 μM or above. Locus dropout proceeded from larger loci to smaller loci as hematin concentration increased (data not shown).

For samples that had been incubated for 1 min or less with DNase I, full profiles were generated, except for one instance of a heterozygote observed to have one allele above 50 RFU and another below but still visible. With increased digestion, peak heights fell below 50 RFU beginning with larger loci and then progressing incrementally to smaller loci. As signal at a locus approached 50 RFU, examples of one allele being detected above that level and the sister allele visible below or not at all were noted. No amplification was observed for samples digested with DNase I for longer than 8 min (data now shown).

Mixtures—Table 3 depicts genotyping results for several mixture ratios of two individuals at seven loci. The loci contained in the table were based upon non-overlapping genotypes (no shared alleles) determined from the non-mixed controls. Deconvolution of low level mixtures where there is considerable overlap in genotypes can be more challenging to the analyst and was not undertaken in this study. Where there were no minor component alleles in the stutter position, analyses of 1:10 mixtures were robust. Genotypes from mixtures of 1:20 were detectable, but not without exception. For each sample at the 1:20 ratio, one minor contributor allele for one replicate (at the D7S820 locus for one and the D19S433 locus for the other) fell below 50 RFU (48 RFU). When considering minor component alleles that fall in the stutter position of a major component, allele labels from 1:20 mixtures were filtered by the Identifiler Kazam macro at the D16S539, D19S433, D21S11, and

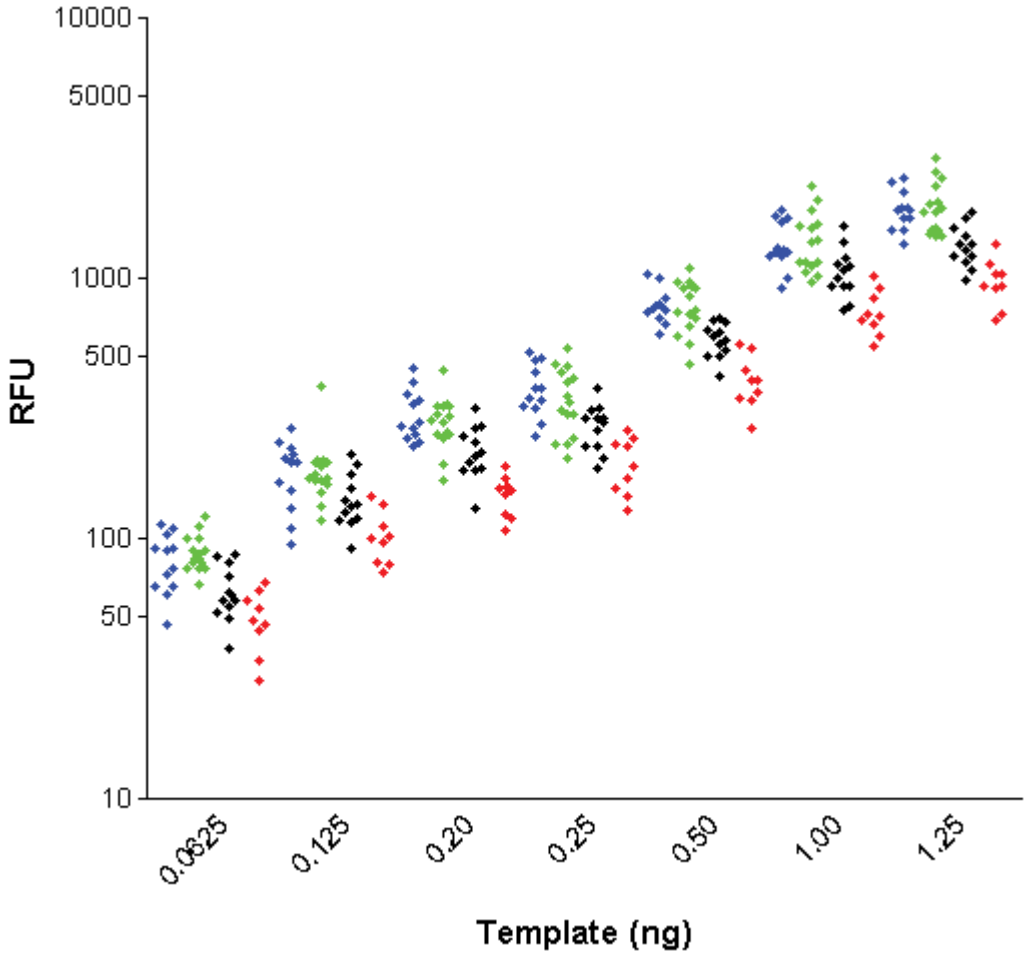

FIG. 4—*Normalized peak heights (average of heterozygote alleles or one-half of homozygote allele) separated by dye label and template input. The results depicted are from three amplifications of a single genomic DNA at 0.03125 (not shown), 0.0625, 0.125, 0.2, 0.25, 0.5, 1.0, and 1.25 ng. Each data point represents the normalized locus score from averaged injections of a replicate amplification. The X-axis indicates template amount and the Y-axis RFU on a* log_{10} *scale. Variation in signal for a given color can be attributed to both intracolor and inter-amplification performance fluctuations.*

TABLE 3—*Minor component genotypes at non-overlapping STR loci from replicate mixture amplifications.*

Mixture Ratio	Locus						
	D2S1338	D7S820	D18S51	D16S539	D19S433	D21S11	vWA
20:1	20,23	7,12	12,15	9,10	14,15	28,31	14,16
20:1	20,23	7,12	12,15	9,10	14,15	28,31	14*
20:1	20,23	7,12	12,15	9,*	14	28,31	14*
10:1	20,23	7,12	12,15	9,10	14,15	28,31	14,16
10:1	20,23	7,12	12,15	9,*	14,15	28,31	14,16
10:1	20,23	7,12	12,15	9,10	14,15	28,31	14,16
3:1	20,23	7,12	12,15	9,10	14,15	28,31	14,16
1:3	17,25	9,10	17,19	11,12	13	30,32.2	17,19
1:10	17,25	9,10	17,19	11,12	13	30,32.2	17,19
1:10	17,25	9,10	17,19	11,12	13	30,32.2	17,19
1:10	17,25	9,10	17,19	11,12	13	30,32.2	17,19
1:20	17,25	9,10	17,19	11,12	13	32.2*	17,19
1:20	17,25	9,10	17,19	11,12	13	32.2*	17,19
1:20	17,25	9	17,19	11,12	*	32.2*	17,19

* Allele present but below stutter filter threshold.

Detected genotype of the minor component using a peak amplitude threshold of 50 RFU and the IDENTIFILER_V1 Kazam macro. The markers in italics do not have overlapping genotypes between the two mixture components, and no true allele is detected in the stutter position of another true allele. The four other markers listed have one minor component allele in the stutter position of the major component. The genotype of the minor component at 3:1 or 1:3 (displayed only once as triplicate results were identical) was the correct genotype as determined by reference sample (data not shown).

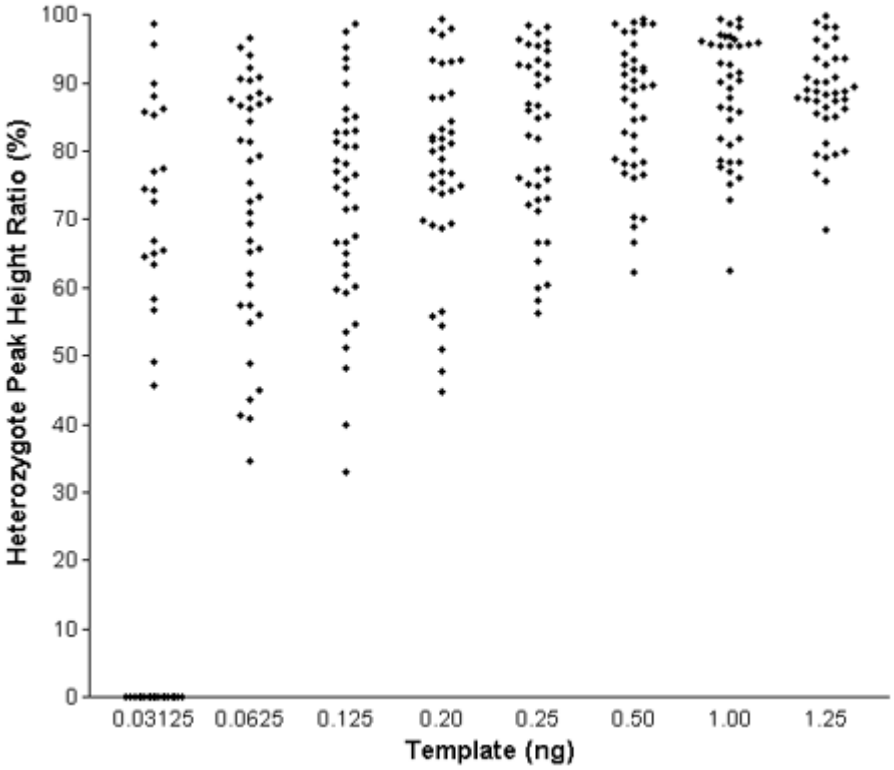

FIG. 5*a*—*Heterozygote peak height ratios with varying inputs of template DNA. The results depicted are from three amplifications of a single genomic DNA at 0.03125, 0.0625, 0.125, 0.2, 0.25, 0.5, 1.0, and 1.25 ng. Multiple injections were averaged, resulting in a total of 39 datapoints per input amount (13 heterozygous markers × 3 repetitions). Data were analyzed with a peak amplitude threshold of 25 RFU. A heterozygote peak height ratio (PHR) of 0% was obtained when one allele fell below 25 RFU, whether or not the allele was visible below that level. For two instances at 31 pg, when one injection had a PHR of 0% and the other non-zero, 0% was considered the average for that datapoint.*

vWA loci. Additionally, one allele at the D16S539 locus in a major peak's stutter position was filtered at the 1:10 level. These data demonstrate the lower limits of mixture detection observed in this study.

Population and Concordance Studies—Population distribution data was documented in the Identifiler User's Manual (14).

Amplification of the standards contained in the SRM 2391a produced correct genotypes for all 16 markers (data not shown). Additionally, no discrepancies were observed upon comparison of Identifiler kit genotypes and genotypes from the same samples generated with previous STR kits (N = 1187). In certain cases, the Identifiler kit was able to provide a more complete genotype at D18S51, D21S11, FGA, TH01, or vWA based upon expanded, physical representation in the allelic ladder at these loci (e.g., FGA 44.2 genotype with the Identifiler kit instead of FGA > 30 with the Profiler Plus or Profiler kits). Based upon these and other concordant results (data not shown, over 2000 samples total, and casework validation studies contributed by three forensic laboratories), the Identifiler kit was accepted by the National DNA Index System (NDIS) for DNA databasing of both offender and forensic samples to the COmbined DNA Index System (CODIS) (B. Brown, NDIS custodian, personal communication).

Discussion

In determining the optimal condition for the Identifiler kit components, several criteria were considered as previously used for evaluation of AmpFℓSTR PCR Amplification kits. Performance criteria included overall peak heights, intercolor, intralocus, and intracolor balance. With 1 ng of template, peak heights were evaluated to verify that homozygous peaks were not offscale and overall peak amplitude was sufficient to allow for detection of 0.5 ng of template or less. Intercolor balance was also monitored in the course of these studies in relation to the ability to produce full profiles within the recommended input range. With 1 ng single-source controls, intralocus balance of greater than 70% was desired to ensure accurate heterozygote genotyping with a range of template amounts and to facilitate mixture interpretation. Additionally, an intracolor balance goal of greater than 50% was adopted to ensure complete results from degraded or inhibited samples. Figure 7 depicts a representative, 1 ng amplification from the Identifiler kit, along with balance calculations, performed in accordance with the procedures described in Materials and Methods. These studies demonstrate that the Identifiler kit is capable of producing robust, reliable profiles under a wide range of reaction conditions, DNA input concentrations, and in the presence of confounding factors (e.g., degraded DNA, PCR inhibitors).

Peak height results from the sensitivity study confirmed reliable generation of full profiles within the recommended input range for the Identifiler kit (0.5–1.25 ng). This range corresponds to the proper template concentration for previous AmpFℓSTR kits, 0.02–0.05 ng/μL final reaction concentration, although the absolute quantity has been halved due to the 25 μL reaction volume. The sensitivity study also demonstrated the ability to produce full profiles from samples with less template than recommended. The lower limit of detection will depend on a number of factors, which should be taken into account when setting the interpretational threshold. The 50 RFU analytical threshold employed here should not be considered a recommendation for an

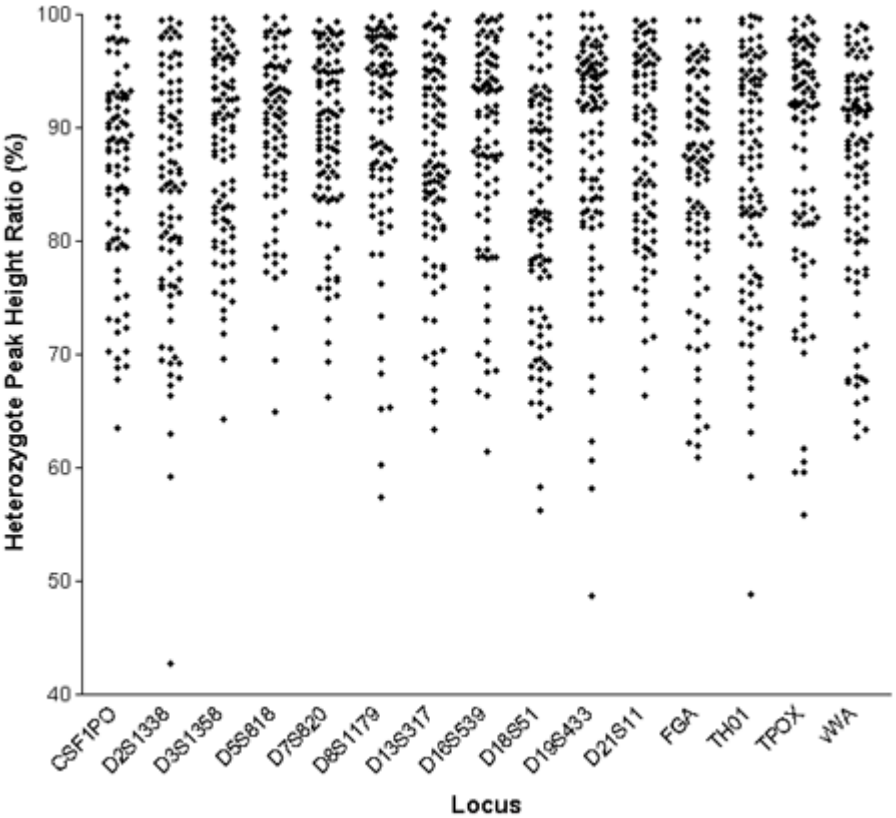

FIG. 5*b*—*Peak height ratios for 15 STR loci obtained from 116 correctly genotyped population samples (n = 69–101, depending on locus). Template inputs varied from approximately 250 pg to greater than 3 ng. The X-axis indicates the locus and the Y-axis heterozygote peak height ratios (PHR) observed at individual loci. Locus means ranged from 82.4–89.8% with medians typically slightly higher. The three outliers (<50% PHR) were attributed to two samples amplified at approximately 250 and 350 pg. Reamplification with a targeted input of 1 ng yielded PHRs of 78–92%.*

interpretational threshold. We have proceeded with a low analytical threshold, functionally less than 50 RFU, to provide as much data as possible to aid laboratories in the process of setting an interpretational threshold for the Identifiler kit. Laboratories should perform sensitivity and mixture studies to correlate peak heights and peak height ratios as well as to capture the limit of detection using their systems and then develop interpretation guidelines accordingly.

Intercolor balance is only of interest in that as the disparity in the signal of the dyes increases, the dynamic range of the assay decreases. Intercolor balance was maximized through careful selection of dye/marker combinations, taking into account the relative performance of a particular locus and the reporting efficiency of a given dye. Additionally, primer titrations were undertaken to narrow the signal range between dyes. However, in order to permit equal reporting in all colors, PCR efficiency would have been compromised. As a result, intercolor balance for the assay is generally in the 20–40% range. The specified template input range of the kit promotes intralocus and intracolor balance, maximum terminal nucleotide addition, and complete profiles. Results of the sensitivity study indicate that within this range, all loci, regardless of dye label, are detectable down to the level of stochastic amplification.

Low heterozygote peak height ratios under standard conditions can increase the possibility of detecting only one allele at a heterozygous locus when the reaction is challenged and can lead to difficulty in characterizing mixtures with overlapping genotypes. For the end user, the most important factor influencing intralocus balance is the quantity of amplifiable DNA present. As template copy number decreases, stochastic sampling can result in one allele of a heterozygote falling below an interpretational threshold, or in more extreme cases, not being visible at all (31). Several of the studies detailed here indicate that although intralocus balance can occasionally be observed below 70%, heterozygote peak height ratios average 86–90% within the recommended DNA input range.

Intracolor balance is important in assessing the condition of the sample and contributes to accurate mixture interpretation. A well-balanced amplification provides the user confidence in the completeness of the profile. Reagent concentrations play a large role in the determination of intracolor balance, although these results demonstrate a window of performance around the recommended condition, ensuring reproducibility in multiple laboratory environments. Other factors that can influence intracolor balance fall into two categories: 1) sample condition and 2) inappropriate template diluent/dilution. Samples encountered in the human identification field are often degraded or contain PCR inhibitors, either of which can lead to deterioration of intracolor balance. Due to the ubiquitous nature of these factors, achieving good intracolor balance with control samples is essential. To ensure good balance, we recommend the use of modified TE buffer (10 mM Tris, 0.1 mM EDTA) as a diluent for extracted template DNA. The use of TE with 1 mM EDTA can lead to inhibition of the reaction through chelation of ionic enzyme co-factors. Inappropriate dilution (too much template) can lead to offscale data, excessive amounts of incomplete terminal nucleotide addition products, and degeneration of intracolor balance. When amplifying non-inhibited, non-degraded samples, the Identifiler kit is able to produce intracolor balance greater than 50%.

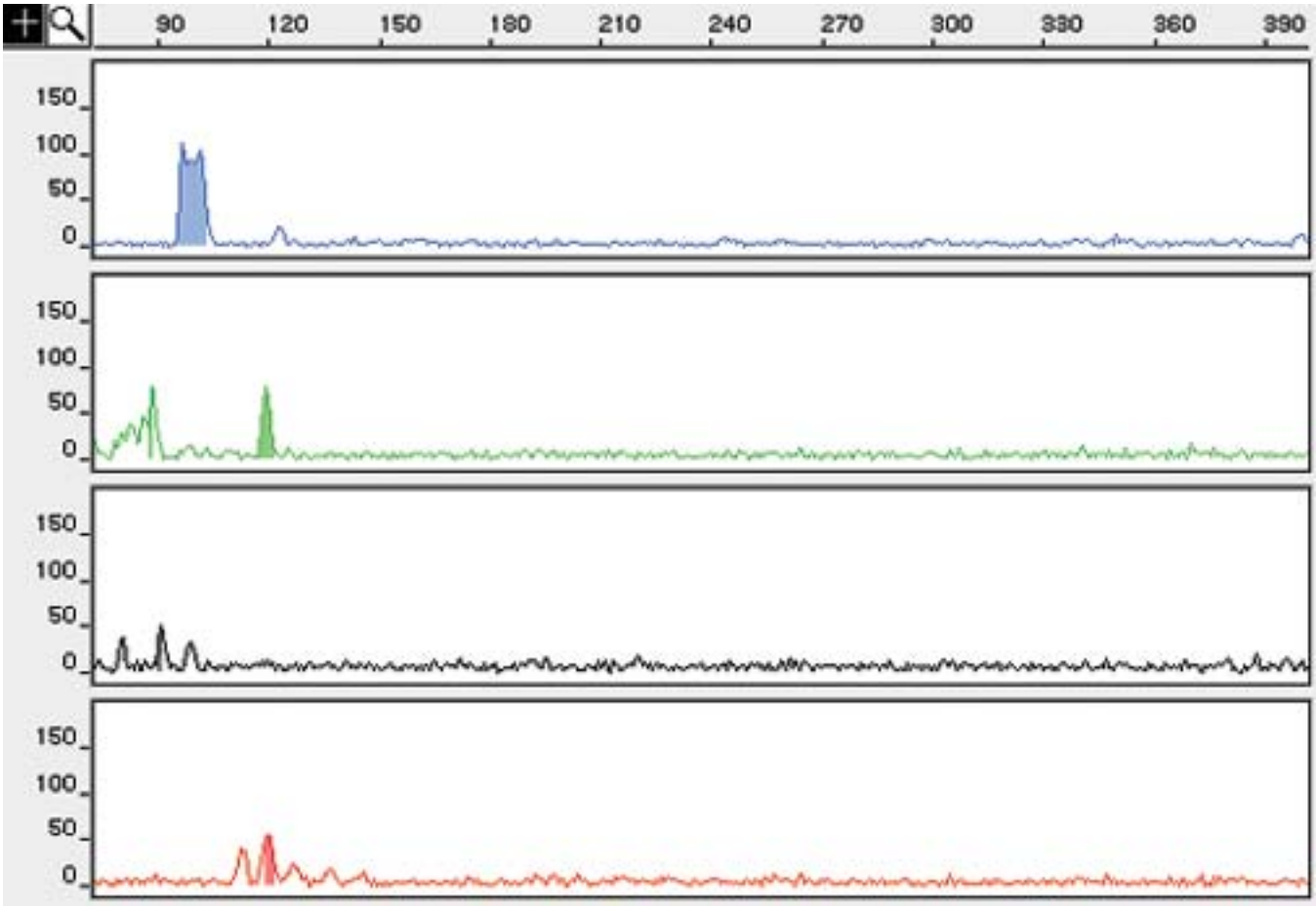

FIG. 6—*Artifacts detected in a negative amplification control. Artifacts detected above 50 RFU have been highlighted in GeneScan software. The X-axis indicates size in base pairs and the Y-axes signal strength (RFU). GeneScan software detected multiple peaks for single artifacts in blue, green, and red. The blue artifact in this instance sized from 96.74–101.63 bp, the green artifact 118.78–119.54 bp, and the red artifact 119.64–120.30 bp. Artifacts detected as single peaks were observed in green (88.68 bp) and yellow (91.18 bp). Migration of artifacts in POP-4 polymer on the ABI Prism 310 Genetic Analyzer varied slightly, approximately ±2 bp (data not shown). Depending on migration, only those artifacts detected above 50 RFU in green and red are labeled by Genotyper software; in this instance, the green artifacts were labeled as D3S1358 "OL Allele?".*

As reported here, the incorporation of novel technologies into the Identifiler kit enabled performance similar to other AmpFℓSTR kits. Previously determined concentrations for PCR reaction mix components performed optimally for the Identifiler kit. Additionally, AmpFℓSTR kit thermal cycling parameters were not altered, other than to increase the final extension hold to ensure complete, non-specific, terminal nucleotide addition. To ensure accurate sizing of D2S1338 and expanded FGA alleles larger than 350 bp using the Local Southern sizing method, the run time was increased relative to the GS STR POP4 (1 mL) F module to routinely collect the 450 bp fragment of the GeneScan-500 LIZ Size Standard. The inclusion of additional alleles at D18S51, D21S11, FGA, TH01, and vWA will provide more specific allele designation and lessen the need to re-run "off-ladder" alleles. Further differences between this kit and previous AmpFℓSTR kits worth noting are: 1) narrowed template input range (although input and final concentration remain the same), 2) larger signal variation between dyes, and 3) slightly decreased heterozygote peak ratios for single-source samples. These minor differences, albeit worthy of consideration, emphasize that the Identifiler kit is simply an expanded AmpFℓSTR multiplex system. Through the incorporation of five-dye technology and non-nucleotide linkers, the AmpFℓSTR Identifiler PCR Amplification kit conserves AmpFℓSTR primer sequences and reaction conditions, ensuring robust, reliable generation of full CODIS, D2S1338, and D19S433 profiles from a single amplification.

The studies described here are appropriate for a reagent manufacturer but do not act as a substitute for a laboratory's internal validation. It is recommended that the laboratory conduct internal validation according to Section 8.1.3 of the DAB guidelines. Additionally, while the conditions recommended here are suitable for a wide range of sample types, several studies indicate that modified thermal cycling parameters and reaction conditions may extend the performance of the Identifiler kit to address severely compromised samples (32,33).

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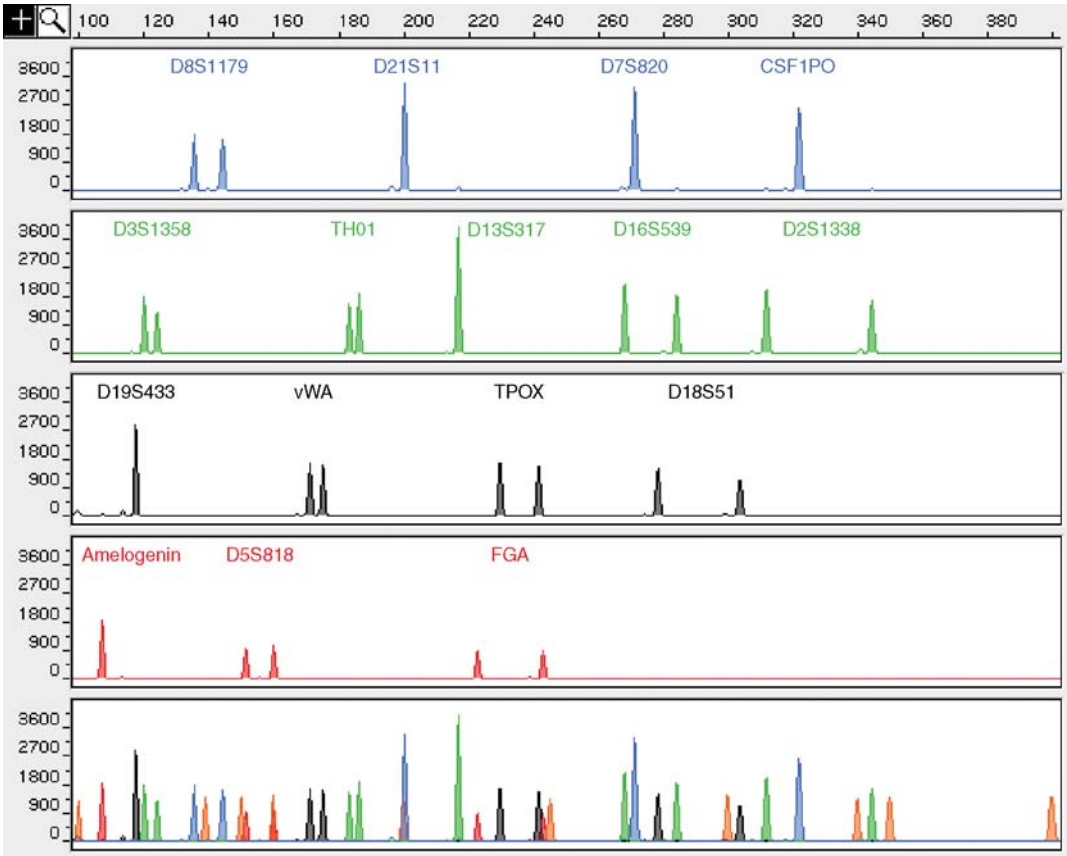

FIG. 7—*One ng amplification of a genomic sample. Amplification and analysis were performed according to protocols provided in the Identifiler kit User's Manual. Minimum heterozygote peak height ratio was 74% at D3S1358. Intracolor balance was 75% for blue, 76% for green, 79% for yellow, and 90% for red. Intercolor balance was 46%.*

allele frequency distributions and for concordance studies. We are grateful to Cydne Holt (San Francisco City and County Crime Laboratory, San Francisco, CA) for extensive and insightful comments on the manuscript. We also recognize members of the Human Identification R&D, Marketing and Manufacturing groups (Applied Biosystems) for technical assistance and valuable comments on this work.

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Additional information and reprint requests:
Lori K. Hennessy
850 Lincoln Centre Dr.
Foster City, CA 94404
Phone: (650) 554-2573
Fax: (650) 638-6393
E-mail: henneslk@appliedbiosystems.com