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ORIGINAL ARTICLE

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Development of the AmpFℓSTR SEfiler PCR amplification kit: a new multiplex containing the highly discriminating ACTBP2 (SE33) locus

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Abstract The AmpFℓSTR SEfiler kit co-amplifies 11 short tandem repeat loci including SE33 in a single multiplex. After establishing the optimum in primer titration studies, the primer concentrations of all loci in the multiplex were chosen such that the heterozygote peak height ratios of each of the loci were balanced. The combined primer set was then tested to determine the robustness of the multiplex under various conditions. Different $MgCl_2$ concentrations were evaluated to establish the optimum concentration for the multiplex. The amplification of the various loci in the multiplex was tested at several annealing temperatures (55–63°C). Additionally, DNA from primates, non-primates and microorganisms were amplified to investigate the specificity of the kit. The stability of the AmpFℓSTR SEfiler kit was determined by addition of hematin, to simulate inhibition, and the use of degraded DNA. Population studies revealed a probability of identity of 6.47×10^{-15} for African Americans and 7.46×10^{-14} for US Caucasians. To assess the ability of the multiplex to analyze forensic samples, testing on blood, oral swabs and mixtures was performed. Based on the various studies, it was determined that the AmpFℓSTR SEfiler PCR amplification kit can be used to successfully analyze a variety of forensic, databasing and paternity samples.

Keywords SE33 · PCR · Multiplex · Development · Forensic samples

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Introduction

In the past decade, fluorescence-based DNA detection systems have been widely used in forensic DNA analysis. These methods have greatly aided the sensitivity and ease of measurement of PCR amplified short tandem repeat (STR) alleles. In the multiplexed STR genotyping kits, fluorescent dyes are covalently coupled to primers for each locus. Earlier amplification kits from Applied Biosystems (AmpFℓSTR kits) used PCR labeled primers with 5-FAM, JOE or NED and ROX dyes with emission spectra ranging from 522 nm to 607 nm (Wallin et al. 2002; Holt et al. 2002). More recently, Applied Biosystems has developed a novel five-dye chemistry for automated DNA fragment analysis.

The introduction of the 5-dye chemistry involves replacement of 5-FAM dye with 6-FAM, JOE with VIC, ROX with LIZ dyes and incorporation of the new PET dye into the existing system. The five dyes expand the spectral detection range to 660 nm thereby enabling more loci to be multiplexed into a single PCR amplification. The ability to multiplex more loci increases genotyping throughput significantly, since more loci can be analyzed simultaneously in a single lane or capillary. The AmpFℓSTR SEfiler PCR amplification kit utilizes the new 5-dye chemistry to co-amplify the gender specific amelogenin locus with the following 11 short tandem repeat loci: D2S1338, D3S1358, D8S1179, D16S539, D18S51, D19S433, D21S11, FGA, SE33, TH01, vWA. The AmpFℓSTR SEfiler kit contains the locus ACTBP2 (SE33), not present in previous AmpFℓSTR kits.

The human beta-actin-related pseudogene H-beta-Acpsi-2 (ACTBP2) or the SE33 locus is one of the most in-

formative tetranucleotide short tandem repeat loci used for human identification and paternity testing (Rolf et al. 1997; Wiegand et al. 1993). The SE33 locus is highly polymorphic and exhibits both structural variations as well as complex length and sequence polymorphisms, with some alleles differing by only 1 bp (Möller et al. 1995). Among the sequence polymorphisms, type I contains the known regular 4 bp repeat AAAG, while type II has a further hexanucleotide unit AAAAAG (Schneider et al. 1998; Gill et al. 1997). The presence of numerous interalleles in SE33, however, makes high demands on the resolving capacity of the electrophoresis device used for its analysis (Gill et al. 1994). More recently capillary electrophoresis has been shown to provide better resolving capacity for the SE33 locus (Rothämel et al. 2000; Dimo-Simonin et al. 1998).

Due to its extensive polymorphism, the Federal Criminal Police Office of Germany (BKA) has included SE33 as one of the eight core genetic loci with which to establish a database and multiplexes are being developed to include this locus (Junge et al. 2003). The seven other essential genetic loci of this database are D3S1358, D8S1179, D18S51, TH01, vWA, FGA and D21S11. To provide a multiplex containing the eight essential loci, the SEfiler multiplex has been developed. Additionally, the AmpFℓSTR SEfiler also includes the seven core STR loci recommended by ENFSI (European Network of Forensic Science Institutes) as well as all the loci for the Second Generation Multiplex (Sparkes et al. 1996) and the AmpFℓSTR SGM Plus PCR Amplification Kit. The experimental design described here was based on the recommendations of the DNA Advisory board guidelines for "Quality Assurance Standards for forensic DNA testing laboratories" (DNA Advisory Board 2000). Developmental studies included an evaluation of critical reagents, species specificity, sensitivity, stability, mixture studies and population data. In summary the new multiplex provides a rapid, reproducible, and sensitive DNA typing method for databasing, forensic and paternity testing.

Methods

DNA extraction and purification

Human DNA samples from bloodstains and buccal cell swabbings were extracted using Chelex (BioRad, Hercules, CA). Non-human

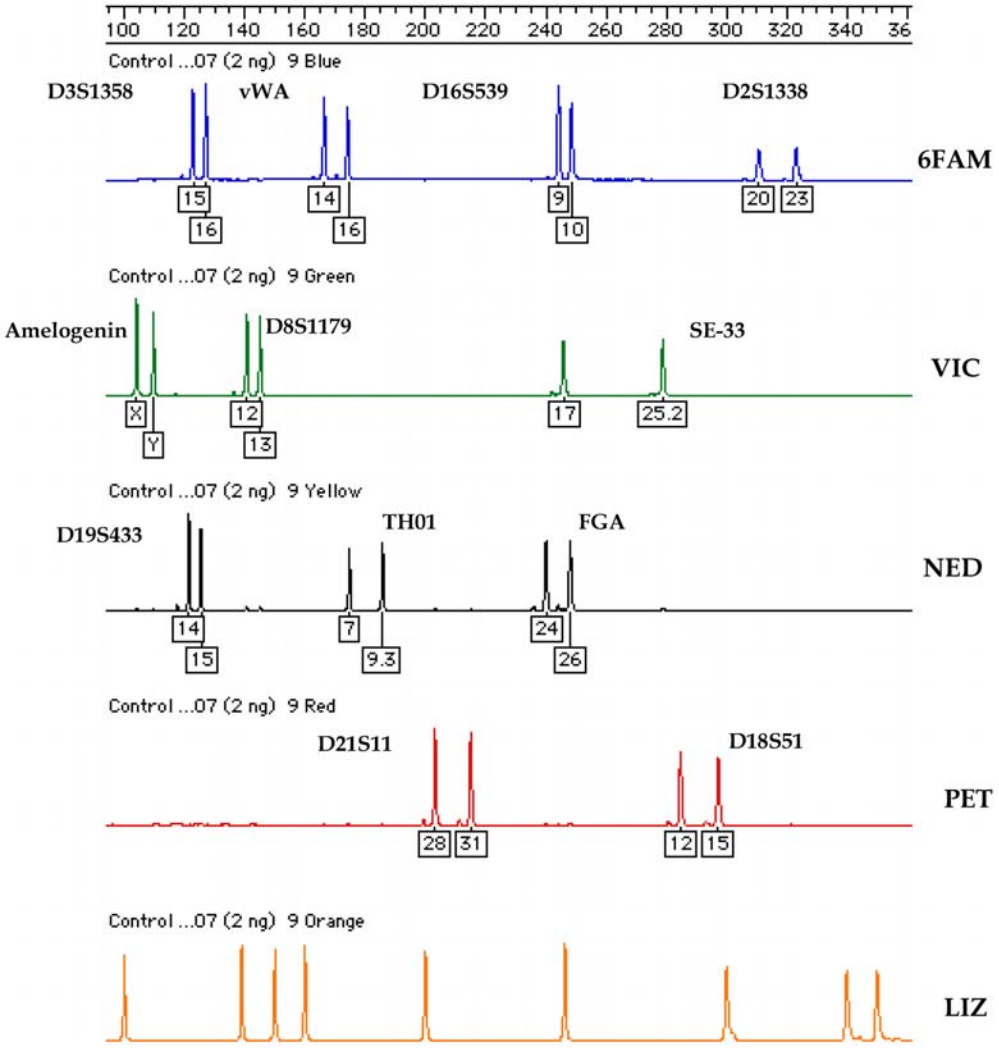

Fig. 1 Electropherogram showing genotype profile of 2 ng AmpFℓSTR control DNA 007 amplified with SEfiler primers

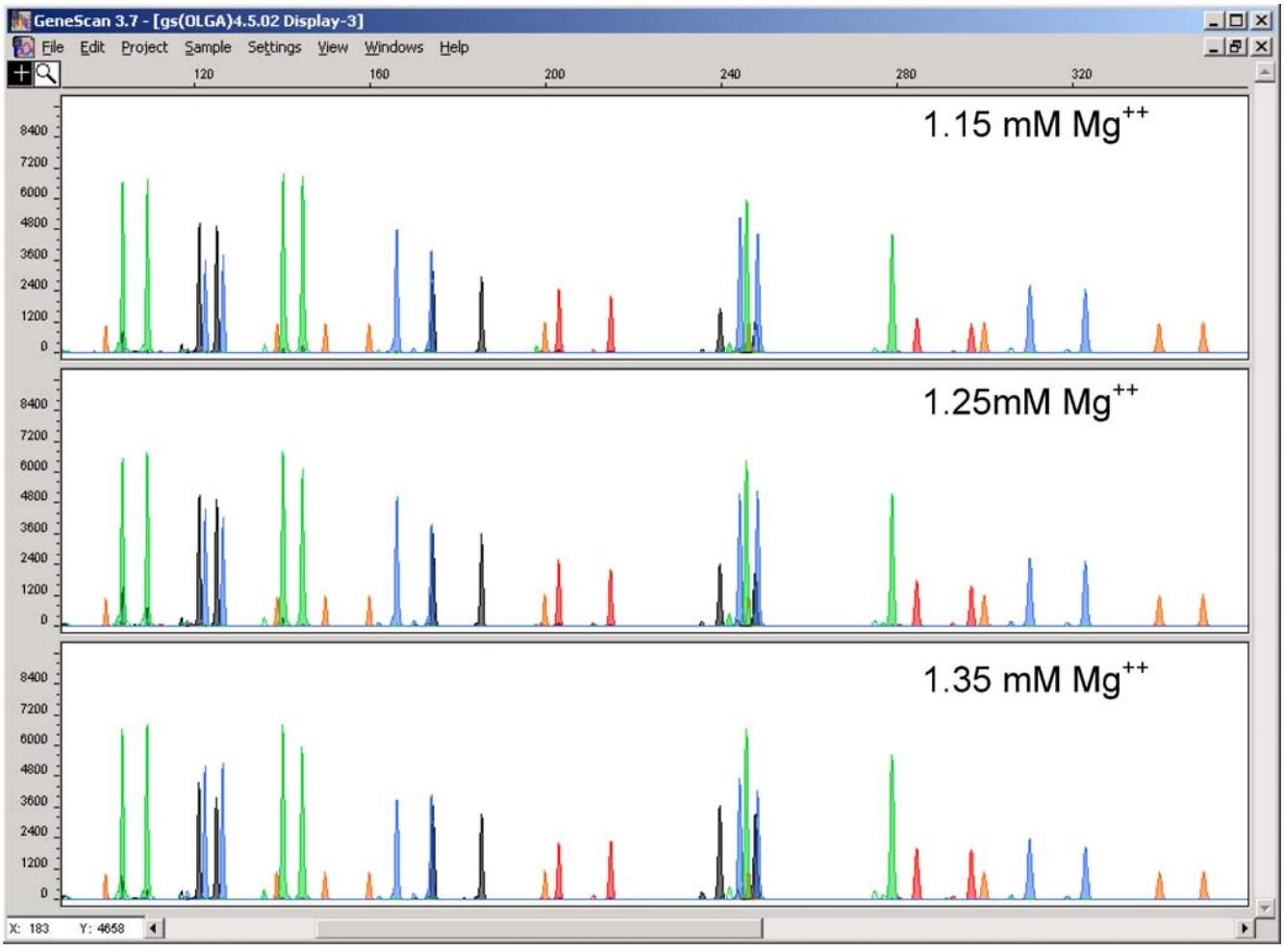

Fig. 2 Effect of varying $MgCl_2$ concentrations. Samples were separated on an ABI PRISM 310 Genetic Analyzer and analyzed using the GeneScan software on Windows NT platform

DNA samples and Center d'Etude du Polymorphisme Humain (CEPH) family DNA samples were obtained from and quantitated by BIOS laboratories, Inc. (New Haven, CT). For the inhibition study, hematin (Sigma, St. Louis, MO) was diluted to 1 mM in 0.1 N NaOH and added to the reaction at the indicated concentration. Degraded DNA was prepared as previously described (Wallin et al. 1998) using 0.005 units/µl of deoxyribonulease I (DNAse I, Gibco BRL, Gaithersberg, MD). The source and sample preparation for the population samples has been described previously (Budowle et al. 2001).

PCR amplification

The primer sequences for ACTBP2 (SE33) locus are the same as given on the STRbase web site (http://www.cstl.nist.gov/div831/strbase//images/se33.jpg). We are aware of primer binding site mutations that have been reported in this region at a rate of 0.2% of the population (Hering et al. 2002). To avoid discrepant results due to primer binding site mutations we recommend that evidence and reference samples be typed with the same primer set. All optimization experiments were performed using the AmpFℓSTR PCR amplification kit reagents and protocols. Target DNA containing 1–2.5 ng, were amplified in 50 µl reactions volumes. Samples were amplified in GeneAmp thin walled reaction tubes (Applied Biosystems, Foster City, CA) in the DNA thermal Cycler 480 or MicroAmp (Applied Biosystems) reaction tubes with caps in the GeneAmp PCR Systems 9600 and 9700 (silver block). Thermal cycling conditions for all of the PCR reactions are as described in the AmpFℓSTR SEfiler kit user's manual: enzyme activation at 95°C for 11 min, followed by 28 cycles of denturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 60°C for 45 min.

Sample electrophoresis and data analysis

Amplification products were separated on the ABI PRISM 310 Genetic Analyzer, 377 Sequencer and 3100 Genetic Analyzer (Applied Biosystems). Briefly, preparation of the amplified product of electrophoresis for the ABI PRISM 310 analyzer is as follows: 1.5 µl of amplified product/ladder and 0.5 µl of GeneScan-500 LIZ Internal lane size standard were added to 24.5 µl of deionized Hi-Di formamide (Applied Biosystems) and denatured at 95°C for 3 min and chilled on ice for 3 min. The PCR products were electokinetically injected for 5 s and electophoresed at 15 kV in POP-4 performance optimized polymer (Applied Biosystems). Data was collected using the ABI PRISM 310 collection software application version 1.0.2 with the GeneScan software run module GS POP4 (1 mL) G5 (virtual filter set G5). The results were analyzed using the 310 GeneScan Analysis software application version 3.1.2 (for Macintosh OS) or GeneScan version 3.7.1(for Windows NT OS). Some of the studies were also performed on the ABI PRISM 377 sequencer or ABI PRISM 3100 genetic analyzer. The ABI PRISM 377 DNA instrument sequencing protocol as well as the ABI PRISM 3100 instrument protocol is outlined in the user's

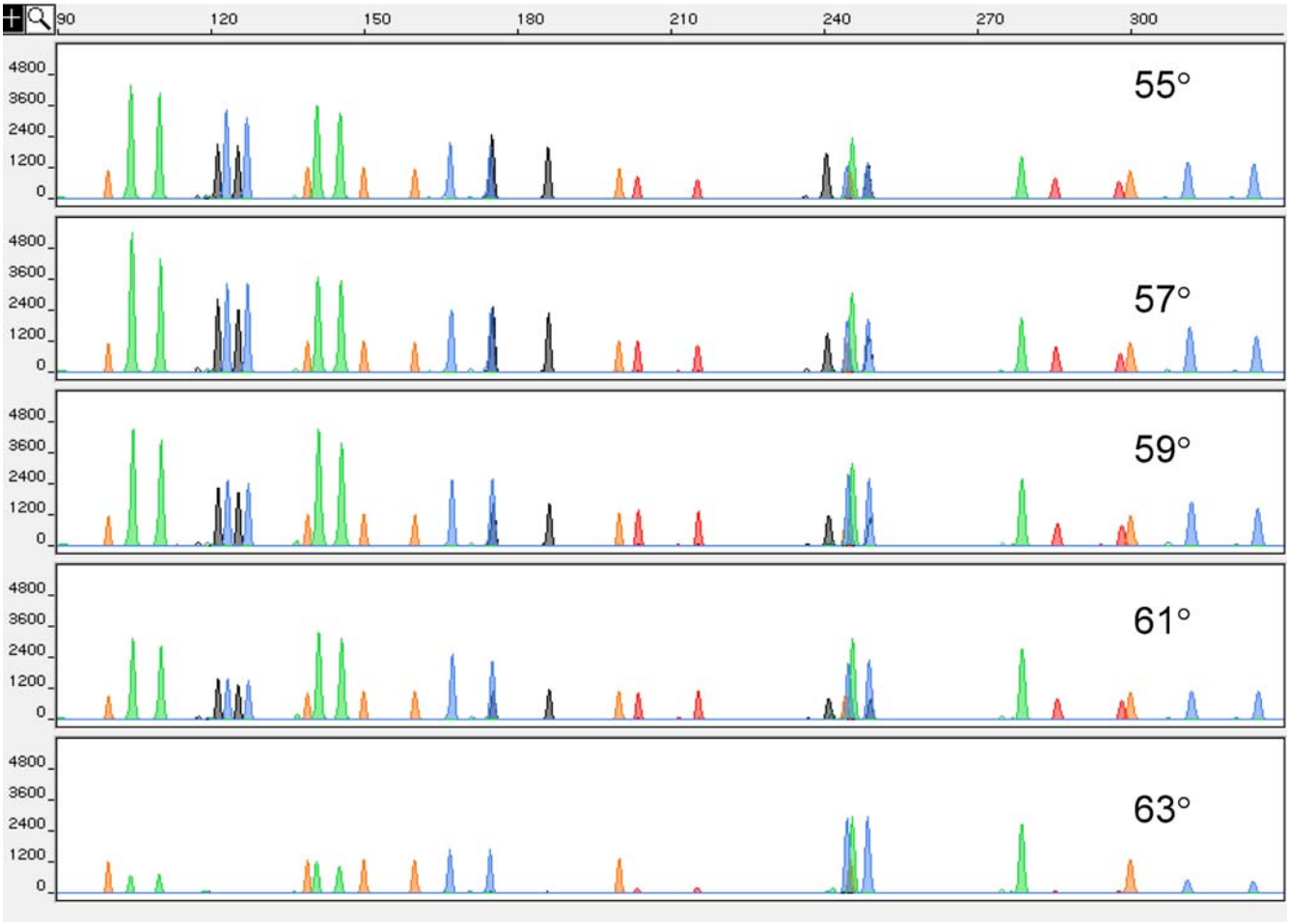

Fig. 3 Effect of varying annealing temperature. AmpFℓSTR control DNA 007 amplified with SEfiler primers at different annealing temperature

manual (AmpFℓSTR SEfiler PCR amplification kit user's manual). Data on accuracy, stutter, sizing and precision on the various ABI PRISM instruments using the SEfiler kit are also documented in the SEfiler kit user's manual. A ladder containing SE33 alleles including 1 bp variants (21, 21.1 and 21.2) has been included in the kit to accurately genotype population samples. Genotypes were assigned using the Genotyper 3.7 software on Windows NT OS (Applied Biosystems) or Genotyper software v2.5.2 on Mac OS (Applied Biosystems).

Results and discussion

PCR reaction components

For reliable locus-specific amplification, the primers for the AmpFℓSTR SEfiler kit were tested. One primer of each locus-specific primer pair was labeled with the 6FAM, VIC, NED and PET dyes detectable as blue, green, yellow and red, respectively on the ABI PRISM instruments. Additionally, the LIZ dye, detected as orange, was used to label an in-lane size standard. To optimize the primer concentrations in the SEfiler kit, the primers were initially titrated individually and evaluated for robustness in a singleplex reaction. These primers were subsequently combined in a multiplex to determine interlocus signal intensity. As with previous kits, the AmpFℓSTR primers were developed to obtain amplified products with robust signal intensity as well as balanced peak heights for single source DNA samples. Data depicting the genotypes of the control DNA 007 amplified using the AmpFℓSTR SEfiler PCR amplification kit is shown in Fig. 1.

One of the critical PCR reaction components present in the SEfiler kit is $MgCl_2$. To determine the $MgCl_2$ concentration optimal for the multiplex, several PCR reaction buffers were formulated containing different concentrations of $MgCl_2$ (0.5 mM–3.0 mM). As shown with previous multiplexes, at 0.5 mM $MgCl_2$, none of the SEfiler loci were amplified. At 1.0 mM $MgCl_2$ only the smaller loci were clearly visible, the larger loci including SE33 and FGA showed diminished peak heights. In contrast at 1.15 mM $MgCl_2$ and higher $MgCl_2$ concentrations all the SEfiler kit loci were evenly amplified. Robust and reliable amplification was consistently observed at 1.25 mM $MgCl_2$ and within the ±0.1 mM performance window (Fig. 2). The optimal magnesium chloride concentration was therefore set at 1.25 mM to obtain maximum signal strength and PCR specificity.

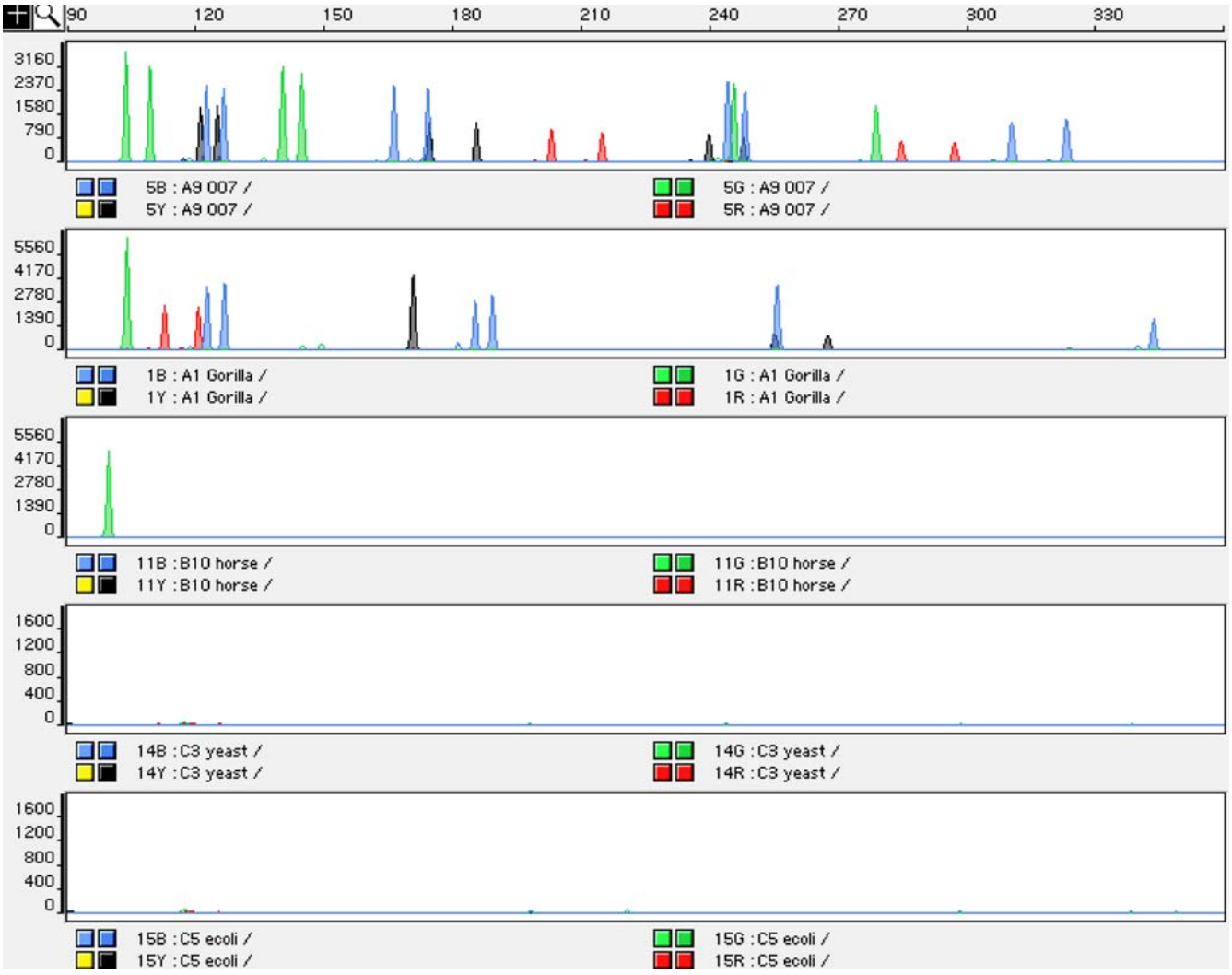

Fig. 4 Species specificity: electropherogram depicting profile obtained from AmpFℓSTR control DNA 007 (human), primate (Gorilla), non-primate (horse), and microorganisms (yeast and *E.coli*)

Thermal cycling parameters

The effect of a range of PCR cycling conditions (time and temperature) was evaluated using the DNA thermal cycler 480, GeneAmp 9600 and GeneAmp 9700. The optimal thermal cycling parameters were determined to be in the middle of a window that meets performance requirements for specificity and sensitivity. The thermal cycling conditions tested included cycle number (27, 28, 29, and 30 cycles), denaturation temperature (92.5°C, 94°C and 95.5°C) and enzyme activation time (5, 8, 11, 14 and 17 min). The optimal cycle number was determined to be 28 cycles, since low amounts of DNA (31 pg) will not generate a full profile providing the optimal sensitivity (data not shown). In similar experiments the optimal activation temperature and time were determined to be 95°C and 11 min, respectively (data not shown).

An important reaction condition for the amplification of a multiplex is annealing temperature. To determine the optimum annealing temperature for the multiplex, different annealing temperatures (55°C–63°C) were evaluated. Peak heights were visible for all loci at 55°C, 57°C and 59°C and 61°C (Fig. 3). However, a decrease in peak heights was observed at 63°C. Specifically D3S1358 and D19S433 showed complete dropout, while most other loci showed reduced peak heights. The locus SE33 however, showed strong amplification even at 63°C. These experiments verified that a 2°C window around the set point (59°C) for the multiplex yields specific PCR products with the desired sensitivity for the DNA samples tested.

Species specificity

In forensic casework samples, non-human DNA may occasionally be present. To investigate the interpretation of SEFiler results from non-human sources, DNA samples previously extracted from primates (1 ng each from gorilla, chimpanzee and orangutan) and non-primates (2 ng each of mouse, dog, cat, pig, horse, chicken, yeast and bacteria) were amplified using the AmpFℓSTR SEfiler primers. Results were analyzed using the ABI PRISM 310 Genetic Analyzer with GeneScan Analysis software.

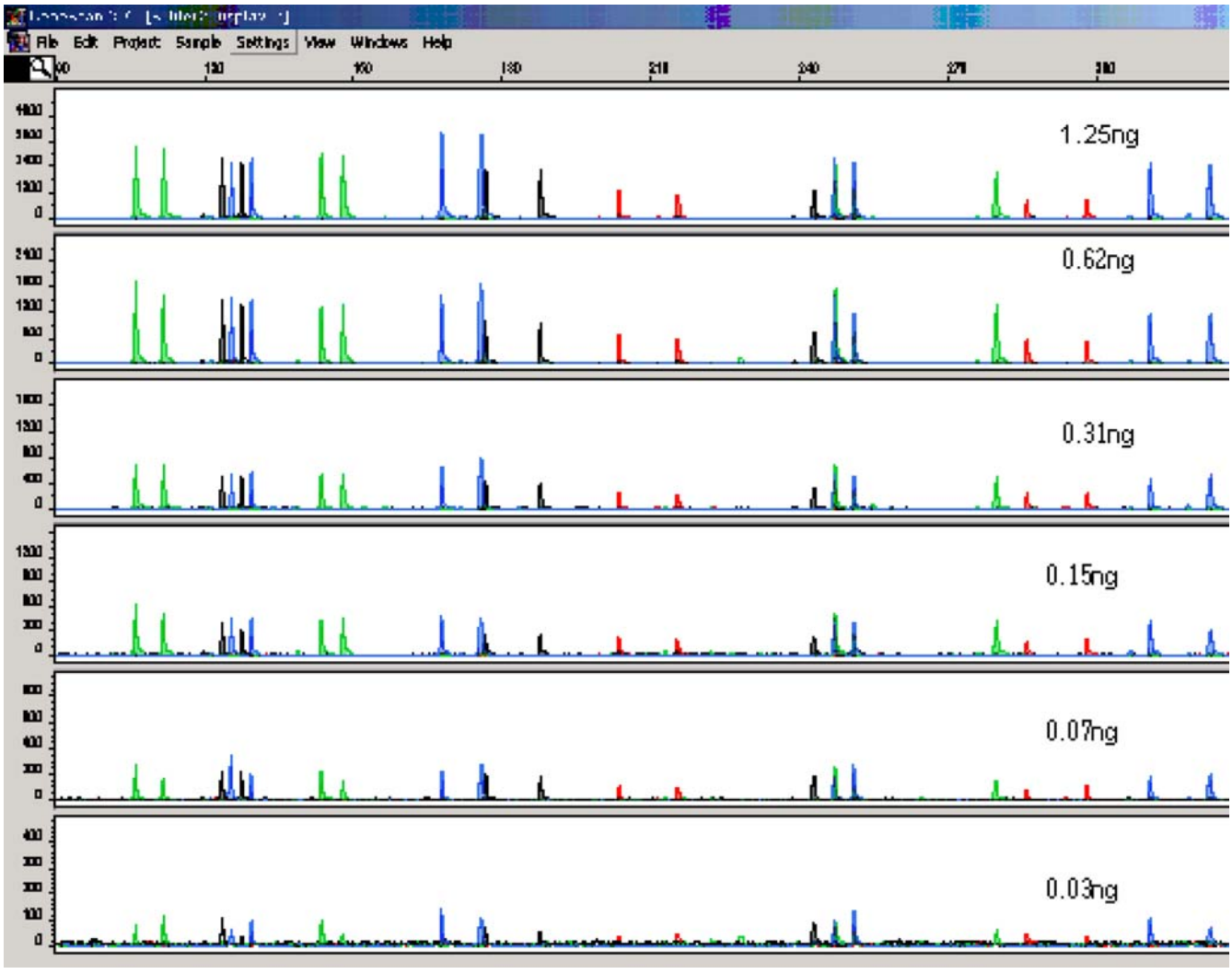

Fig. 5 Electropherogram depicting AmpFℓSTR control DNA 007 at various concentrations electrophoresed on an ABI PRISM 377 instrument with Windows NT platform

The primate DNA samples all amplified producing fragments within the 100–400 base pair region (Fig. 4). However, these allele sizes are usually not observed in human DNA profiles. The alleles of some of the loci from the primate samples have previously been sequenced by Applied Biosystems and the sequences show significant homology between the primate and human DNA (Lazaruk et al. 2001).

Microbial samples tested did not yield any detectable product (Fig. 4). For the amelogenin locus, the dog, pig and horse samples (Fig. 4) produced a fragment 4 bp shorter than the primate X-specific product. Similar results have been reported for previous AmpFℓSTR kits (AmpFℓSTR Identifiler PCR amplification kit user's manual, AmpFℓSTR Profiler Plus PCR amplification kit user's manual, AmpFℓSTR SGM Plus PCR amplification kit user's manual).

Sensitivity

To determine the sensitivity of the amplification kit different DNA concentrations were amplified with the SEfiler primer set. Serial dilutions of AmpFℓSTR control DNA 007 were prepared and amplified using the primers and separated on the ABI PRISM 377 (Fig. 5). The data indicates that an input DNA of 1 and 2 ng produces robust and reliable peak heights. Additionally, full profiles were obtained with good signal intensity were obtained when approximately 0.3–0.6 ng of DNA was added to the PCR amplification reaction. At 0.15 ng, full profiles were obtained. However at 0.03 ng, peaks were extremely low or undetectable, thus amounts less then 30 pg did not produce a typeable result. Similar results were obtained with the ABI PRISM 310 instrument. It is recommended however, that individual laboratories using the SEfiler kit determine the dynamic range for the minimum peak height interpretational threshold based on their own results. Additional DNA may be required if the DNA is degraded. Some minor artifacts may be observed due to the presence of the dyes that could complicate interpretation of low levels of DNA (AmpFℓSTR Identifiler PCR amplification

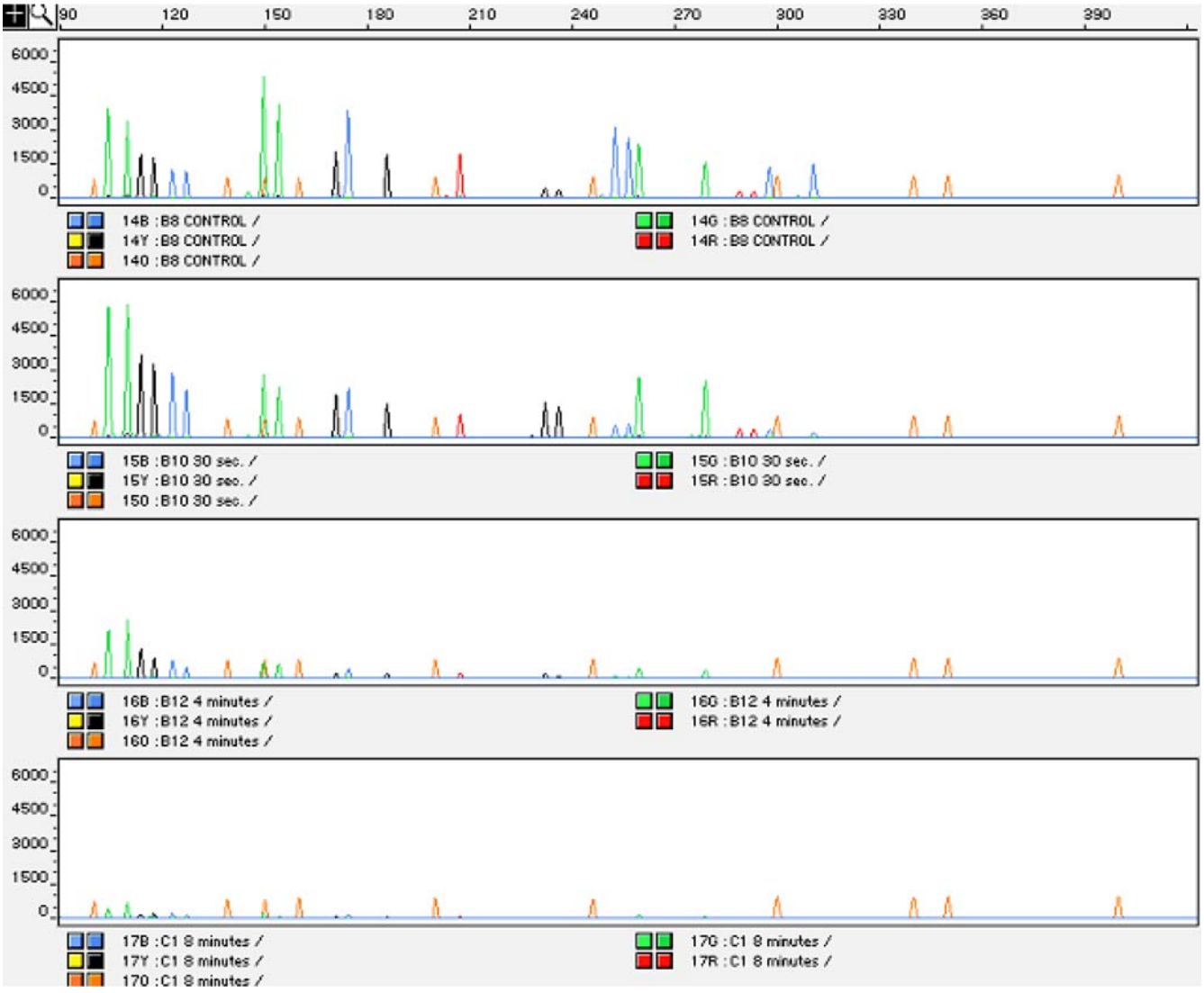

Fig. 6 Analysis of degraded DNA: AmpFℓSTR control DNA in the absence of DNase 1 (*panel 1*), incubated for 30 s (*panel 2*) and incubated for 4 min (*panel 3*)

kit user's manual, AmpFℓSTR SEfiler PCR amplification kit user's manual). These artifacts can however be distinguished by using appropriate negative controls and should therefore not compromise the accurate typing of samples, even at low DNA levels.

Degraded DNA

Forensic samples often contain degraded DNA, since environmental exposure degrades DNA randomly into smaller fragments. The ability of the SEfiler primers to amplify degraded DNA was investigated by amplifying high molecular weight genomic DNA (8 µg) incubated with DNAse I for several time periods as described previously. Undegraded DNA (1.5 ng, time 0 min) and 4 ng of degraded DNA from each time point were then amplified using the amplification kit.

As shown in Fig. 6, with increasing exposure to DNAse I, there was a loss in PCR product yield at every locus. At the 30 s time point although all loci were visible, there was significant loss of signal. At 4 min differential amplification was observed as the large loci (D2S1338, D18S51, FGA and SE33) exhibited diminished peak heights (Fig. 6). At longer time points none of the loci were detectable. These results are consistent with the extent of degradation observed in an agarose gel (data not shown). This data indicates that there is an inverse relationship between the size of the locus and the amplification ability of the SEfiler primers when used on degraded DNA samples.

PCR inhibition

DNA samples from crime scenes frequently contain inhibitors that can affect the successful amplification of DNA samples. A common inhibitor associated with DNA is

Fig. 7 Effect of hematin: AmpFℓSTR control DNA 007 amplified with the SEfiler kit in the presence of varying concentrations of hematin, analyzed on the ABI PRISM 310 Genetic Analyzer

hematin, present in hemoglobin derived from red blood cells. To examine the ability of SEfiler primers to amplify in the presence of inhibitors, varying concentrations of hematin (0–30 µM) were added to the PCR reaction. Differential amplification, defined as increased amplification of some loci as compared to other loci, was observed with increasing hematin concentration. Specifically as the hematin concentration was increased amplification of larger loci were inhibited (Fig. 7). At 20 µM substantial inhibition of D2S1338, SE33, FGA and D21S11 loci was observed. In contrast at 26 µM complete inhibition of amplification of all loci of the control DNA was observed. These results show similarity to the results obtained from the degraded DNA study since PCR inhibition also results in the loss of the larger sized STR loci for the SEfiler kit.

Mixture studies

A mixture is generally identified by the presence at one or more loci of three or more bands, high stutter values or imbalanced heterozygous peaks. Stutter data and minimum/maximum heterozygote data on all loci is available in the SEfiler user's manual (AmpFℓSTR SEfiler PCR amplification kit user's manual). Among the functional studies to assess the ability of detection systems to recognize mixtures is the examination of mixtures of purified DNA in defined ratios. Several DNA mixtures of two purified DNAs in various defined ratios (1:1, 1:3 and 1:9) were prepared while holding the total input DNA concentration to 2 ng. These DNA mixtures were then amplified using the AmpFℓSTR SEfiler kit primer set and analyzed on an ABI PRISM 310 Genetic Analyzer. As shown in Fig. 8 (data shown for the SE33 locus only), the ratio of the peak height of the major:minor component generally reflected the ratios of the input genomic DNAs. Additionally based on the ratios, the minor component of the mixture was not detected below the 10% or at the 1:10 level.

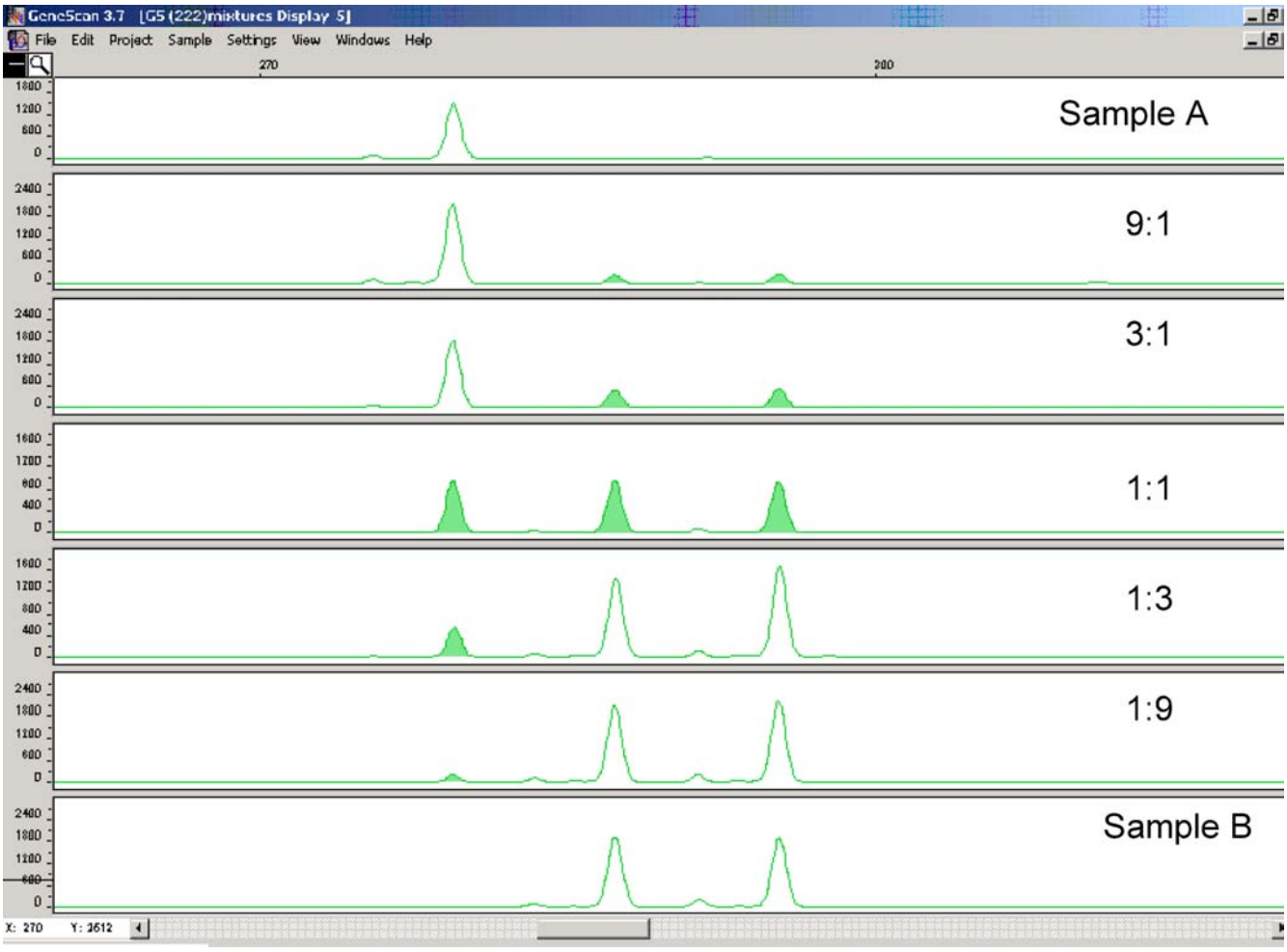

Fig. 8 Mixture studies: data for two DNA samples mixed together at defined ratios and amplified with the AmpFℓSTR SEfiler PCR amplification kit for the SE33 locus. Samples A and B are male and female, respectively. The ratios shown in the panels are 1:10, 1:3 and 1:1. The minor component allele is highlighted in panels 2, 3, 5 and 6. All alleles are highlighted in panel 4

Table 1 Population statistics for the SEfiler multiplex

Locus	Probability of identity		Probability of paternity exclusion	
	African American	U.S. Caucasian	African American	U.S. Caucasian
D2S1338	0.025	0.038	0.745	0.621
D3S1358	0.114	0.099	0.734	0.65
D8S1179	0.079	0.072	0.477	0.763
D16S539	0.074	0.085	0.67	0.42
D18S51	0.038	0.056	0.725	0.912
D19S433	0.045	0.126	0.632	0.516
D21S11	0.051	0.057	0.745	0.734
FGA	0.034	0.044	0.784	0.676
SE33	0.019	0.02	0.745	0.792
TH01	0.103	0.119	0.578	0.734
vWA	0.067	0.077	0.613	0.705
	6.47×10^{-15}	7.46×10^{-14}	0.999997	0.999998

Population and inheritance studies

To obtain population data using the SEfiler Kit, population samples from U.S. Caucasians (n=69) and African Americans (n=104) were genotyped. Concordant genotypes were obtained from common loci of identical populations samples using the AmpFℓSTR Identifiler kit. The database genotypes were used to generate allele frequency data and determine the probability of identity for the two populations (Table 1). The P_I values, the probability that two individuals selected at random will have an identical genotype, for all the loci was $1{:}1.54\times10^{14}$ (African American) and $1{:}1.34\times10^{13}$ (U.S. Caucasian). Thus the combination of STR loci present in the SEfiler kit is extremely informative.

Extensive population data has previously been reported for the SE33 locus (Bäßler et al. 1999; Brinkmann et al. 1996; Corte-Real et al. 1999; Klintschar and Neuhuber 1998; Lászik et al. 2001; Lessig et al. 2000; Liu et al. 1997; Shimada et al. 2002; Van Hoofstat et al. 1998). In the present study the highly polymorphic SE33 locus exhibited 34 different alleles showing a characteristic bimodal distribution (Table 2). Thus, the presence of the

Table 2 Frequency of the various alleles of the ACTBP2 locus represented in the population data

Alleles	African American (*n*=104)	Caucasians (*n*=69)
4.2	0.00	0.00
6.3	0.00	0.00
8	0.00	0.00
9	0.00	0.00
9.2	0.48	0.00
11	0.48	0.00
11.2	0.48	0.00
12	0.48	2.17
12.2	0.96	0.00
13	1.92	2.90
13.2	0.48	0.00
14	2.88	3.62
14.2	0.96	0.00
15	4.81	3.62
15.2	0.00	0.00
16	6.25	5.07
16.2	0.48	0.00
17	8.17	10.14
17.2	0.00	0.00
18	11.54	7.25
19	8.17	7.97
20	8.65	2.90
20.2	0.96	0.00
21	4.33	5.07
21.1	0.00	0.00
21.2	0.48	0.72
22	0.48	0.00
22.2	0.96	4.35
23	0.48	0.00
23.2	0.96	3.62
24.2	0.48	0.00
25.2	3.37	5.07
26.2	5.77	4.35
27.2	11.54	7.97
28.2	4.33	9.42
29.2	4.33	4.35
30.2	2.88	6.52
31.2	1.44	2.17
32.2	0.00	0.72
33.2	0.00	0.00
34.2	0.00	0.00
35	0.00	0.00
35.2	0.00	0.00
36	0.00	0.00
37	0.00	0.00

SE33 locus significantly increases the power of discrimination of the multiplex.

In addition inheritance was also examined for the SE33 locus using CEPH family DNA sets. Four CEPH family sets # 1331, 13291, 13292 and 13294 were examined using the SEfiler primer set. The genotype results confirmed independent assortment of all loci including SE33 thereby verifying Mendelian inheritance.

Finally to evaluate the performance of the SEfiler kit on forensic samples, liquid blood, bloodstains and buccal cell swabs were collected. DNA was extracted from these samples and successfully amplified using the AmpFℓSTR SEfiler primers (data not shown). The genotypes of the liquid specimens and dried specimens from the same donors were reproducible. In addition previous studies have shown the use of the ACTBP2 locus in case work DNA samples (Hochmeister et al 1998; Schmitter et al 1995).

Separation of closely spaced alleles in the ACTBP2 locus

The ACTBP2 locus is unique since it exhibits interalleles with 1 or 3 sequence and bp variants, which though rare do exist. To test the ability to resolve and detect alleles differing by 1 or 3 base pairs, the GEDNAP (German DNA profiling group) has recommended that a mixture of alleles containing the rare alleles at the ACTBP2 locus be run at least once at the beginning and the end of the run (Rand et al. 2002). We have provided two of these alleles (21 and 21.1) in the allelic ladder and recommend that the ladder be run every 8–10 injections interspersed with the samples (Fig. 9).

Conclusions

A new multiplex, AmpFℓSTR SEfiler has been developed utilizing the highly polymorphic SE33 locus. PCR components and cycling conditions have been optimized to obtain robust and reliable amplification of the loci under

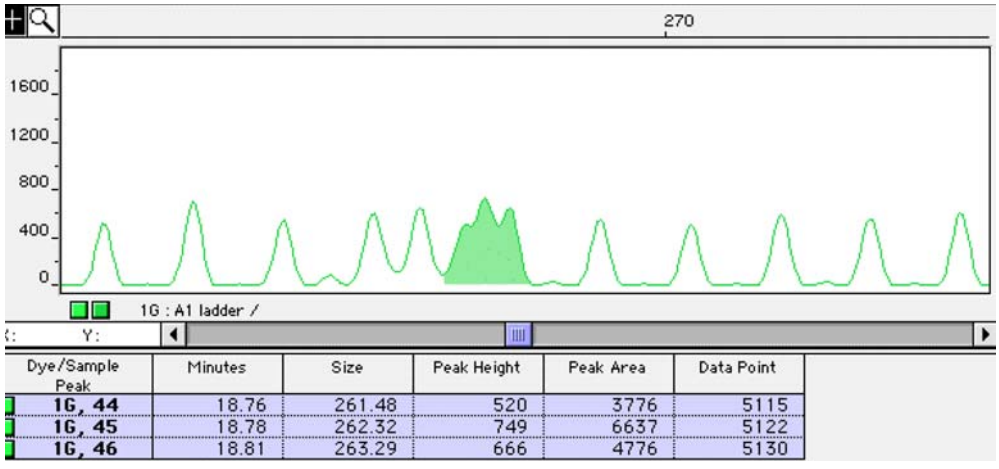

Fig. 9 Allelic ladder for the ACTBP2 locus depicting the closely spaced alleles

varying conditions. Species specificity, sensitivity, stability and mixture studies revealed that the SEfiler multiplex shows adequate sensitivity to simultaneously detect all the loci without loss in specificity. Population data showed a high discrimination power for the combined multiplex due to the inclusion of the informative SE33 locus.

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