Jeanette M. Wallin,[1] M.P.H.; Cydne L. Holt,[1] Ph.D.; Katherine D. Lazaruk,[1] Ph.D.; Theresa H. Nguyen,[1] B.S.; and P. Sean Walsh,[1] M.P.H.

Constructing Universal Multiplex PCR Systems for Comparative Genotyping

REFERENCE: Wallin JM, Holt CL, Lazaruk KD, Nguyen TH, Walsh PS. Constructing universal multiplex PCR systems for comparative genotyping. J Forensic Sci 2002;47(1):52-65.

ABSTRACT: Analysis of length polymorphisms at STR loci in the human genome has become a standard approach for comparative genotyping in many areas including disease research and diagnostics, parentage assessment, investigations of human diversity, and forensic science. The simultaneous analysis of multiple STR loci through multiplex PCR and multicolor fluorescence detection offers sample conservation, high throughput, and automated genetic analysis. Careful design and optimization of tetranucleotide STR multiplexes has led to reliable, standardized systems that powerfully differentiate and distinguish individual human DNA profiles. The development of these multiplex systems involved a rigorous experimental strategy that included careful selection of PCR primer sequences (for yield, specificity, and multiplex compatability), along with optimization of PCR component concentrations, thermal cycling parameters, and fluorescence detection conditions. This developmental approach rendered well-characterized DNA typing systems that are high performing (sensitive, specific, and balanced), optimized to universal parameters (same reaction conditions), resilient to fluctuations in reaction conditions, and simple to implement and use routinely.

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

Prevalent throughout eukaryotic chromosomal DNA, polymorphic short tandem repeat (STR) loci are key tools for rapid gene discovery, disease locus mapping, carrier diagnosis of disease states, linkage analyses, agricultural genetics, parentage assessment, and population diversity studies. The ability to detect genetic differences between individuals increases when DNA typing information at multiple polymorphic STR loci is combined. For example, the combined average probability that two unrelated individuals (U.S. Caucasian and African American population groups), chosen at random, have identical profiles (P_I) at 15 STR loci discussed in this report is ~1 in 10^{15} (see Table 1; 18,47). As P_I decreases, the opportunity to distinguish unshared alleles between individuals is likely to increase, thus enhancing informativeness of mixed DNA samples encountered in comparative genotyping, such as in bone marrow transplant monitoring (chimaerism) or forensic casework.

Biological specimen collection technique, sample storage conditions, DNA quality and quantity, and resilience of STR typing systems can influence ability to recover genetic information. Due to potential exposure to a virtually unlimited number of uncontrolled variables, forensic casework specimens may result in particularly challenging polymerase chain reaction (PCR) templates. Critical attributes mandatory for human identification studies are exemplified in the Amplification of Fluorescent-labeled STRs (AmpFℓSTR®) PCR Amplification Kits (see Table 1). Optimized reagents direct simultaneous PCR amplification (i.e., multiplex) of different combinations of 15 polymorphic tetranucleotide STR loci in a single reaction tube. Thirteen of these loci represent the core group of STR markers, carefully chosen by the Technical Working Group on DNA Analysis Methods (TWGDAM; currently referred to as SWGDAM) STR Working Group, for inclusion in the United States COmbined DNA Index System (CODIS) DNA database (refer to Table 1). Resident on 14 different human chromosomes, each of the 15 loci has undergone intensive study. Physical linkage and mendelian inheritance data for most loci are provided by the Cooperative Human Linkage Center (CHLC) or have been published previously (1,2,7,10,15,16,21,22,24,27,29,31,32,34,43,49,50,53,54,55,56,63). Mendelian inheritance at each locus was additionally confirmed in our laboratory (18). Issues pertinent to population genetics, such as approximation to Hardy-Weinberg proportions and independent inheritance between STR markers, have been addressed in separate studies (5,11,13,18,23,65).

Each STR allele amplified with AmpFℓSTR PCR primers is concomitantly labeled with a fluorescent tag, detected as either blue, green, or yellow on the ABI PRISM™ 377 DNA Sequencer or the ABI PRISM 310 Genetic Analyzer. This scheme allows analysis of multiple small DNA fragments (~100 to 350 base pairs) following a single electrophoretic separation. Discrete allele recognition is achieved through amplification from locus-specific PCR primers that generate fragments within distinct size ranges. Sample alleles are identified by comparison to an allelic ladder in accordance with recommendations of the DNA Commission of the International Society for Forensic Haemogenetics (9). Sizes of sample alleles and allelic ladders are measured using internal lane size standards (30). Alleles differing by one, two, or three base pairs are differentiated by virtue of routinely obtained single base precision (25).

Following a rigorous developmental scheme, optimal parameters for amplification and fluorescence detection were determined empirically for AmpFℓSTR Blue loci (D3S1358, FGA, and vWA), the founding multiplex. PCR reaction component concentrations and thermal cycling parameters that provided appropriate sensitivity, specificity, and signal balance important for forensic DNA typing were identified (59). These amplification and fragment detection conditions were fixed to serve as framework for expanding and building additional multiplex systems (see Table 1).

Critical for incorporation of 15 STR loci into a common proto-

[1] Human Identification Group, PE Biosystems, Foster City, CA.

Received 10 July 2000; and in revised form 25 Jan. 2001; accepted 12 April 2001.

TABLE 1—*AmpFℓSTR PCR Amplification kits.*

Locus	AmpFℓSTR Blue	AmpFℓSTR Green I	AmpFℓSTR Profiler™	AmpFℓSTR Profiler Plus™	AmpFℓSTR COfiler™	AmpFℓSTR SGM Plus™
D3S1358*	X		X	X	X	X
vWA*	X		X	X		X
FGA*	X		X	X		X
TH01*		X	X		X	X
TPOX*		X	X		X	
CSF1PO*		X	X		X	
D5S818*			X	X		
D13S317*			X	X		
D7S820*			X	X	X	
D8S1179*				X		X
D21S11*				X		X
D18S51*				X		X
D16S539*					X	X
D2S1338						X
D19S433						X
Amelogenin		X	X	X	X	X

* 13 CODIS Core Loci.

TABLE 1—*Fifteen STR loci of six AmpFℓSTR PCR amplification kits. D3S1358, vWA, FGA, D16S539, and D2S1338 are labeled with 5FAM dye and detected as blue using virtual filter set F on the ABI PRISM instruments. TH01, TPOX, CSF1PO, D8S1179, D21S11, D18S51, and Amelogenin are labeled with JOE dye and detected as green. D5S818, D13S317, D7S820, and D19S433 are labeled with NED dye and detected as yellow. TH01 and FGA primers in the AmpFℓSTR SGM Plus kit are identical in sequence to those in the other multiplexes but are labeled with NED.*

col was extensive screening and selection of PCR primers. Primer selection, PCR optimization, and fluorescent allele detection conditions are explored as primary focus points.

Materials and Methods

Sample Source and Extraction Protocols

Human DNA samples from bloodstains, hair, and buccal cell swabbings were extracted using either a phenol/chloroform organic method (Serological Research Institute, Richmond, CA; 46) or Chelex™ (PE Biosystems, Foster City, CA; 42,60); this sample set is used in all experiments, unless stated otherwise. Nonhuman DNA samples (BIOS Laboratories, Inc., New Haven, CT) included primates (gorilla, chimpanzee, orangutan, and macaque) and nonprimates (horse, cow, chicken, pig, rabbit, dog, cat, fish, hamster, mouse, rat), fungi (*Candida albicans, Saccharomyces cerevisiae*), and bacteria (*Rhodotorula rubra, Legionella pneumophilia, Escherichia coli, Listeria monocytogenes, Neisseria lactamica, Vibrio mimicus, Citrobacter freundii, Salmonella typhimurium*). DNA extracts were quantitated using the QuantiBlot® Human DNA Quantitation Kit (Perkin Elmer, Norwalk, CT; 61), on agarose gels, and/or by ultraviolet spectrophotometry.

Population database DNA samples were extracted from liquid blood of unrelated individuals, as follows: 459 African Americans, 200 U.S. Caucasians, and 113 U.S. West Coast Hispanics. All Caucasian and 195 African American DNA samples were provided by Laboratory Corporation of America (Research Triangle Park, NC; 18). Liquid blood was collected from all Hispanic individuals and the remaining 264 African Americans by the California Department of Justice DNA Laboratory (Berkeley, CA). DNA was extracted by the phenol/chloroform method, concentrated by Centricon® Centrifugal Filter Devices (model YM-100; Amicon Inc., Beverly, MA), and quantitated using PicoGreen® (Molecular Probes Inc., Eugene, OR) and an SLT Fluostar plate reader (TECAN, Research Triangle Park, NC) or by QuantiBlot.

PCR Amplification

Unless otherwise stated, 1.0 to 2.5 ng of genomic DNA was amplified in 50-μL reaction volumes using AmpFℓSTR PCR amplification reagents and suggested protocols (PE Biosystems; 35–38,40,41). Kit reagents included PCR reaction mix, AmpliTaq Gold™ DNA Polymerase (3), primer cocktail for relevant multiplex, positive control DNA 9947A (12), allelic ladder, and mineral oil. PCR reaction mix final concentrations are 10 mM Tris·HCl (pH 8.3), 50 mM potassium chloride (KCl), 1.25 mM magnesium chloride ($MgCl_2$), 800 μM blended deoxynucleotide triphosphates (dNTPs), and 0.16μg/μL bovine serum albumin (BSA). Four and a half units of AmpliTaq Gold DNA Polymerase were used in each 50-μL PCR reaction.

Samples were amplified in either GeneAmp® Thin-Walled Reaction Tubes in the DNA Thermal Cycler 480 or MicroAmp® Reaction Tubes with Caps in the GeneAmp PCR Systems 2400 or 9600 (Perkin Elmer). The recommended protocol is identical between these thermal cyclers for a given multiplex and the same between all six kits, with noted exceptions, as follows: enzyme activation at 95°C for 11 min; followed by 28 cycles (29 cycles for AmpFℓSTR Green I) of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min; and a final extension at 60°C for 45 min (30 min for AmpFℓSTR Blue and AmpFℓSTR Green I). Note that enzyme activation is necessary when using AmpliTaq Gold DNA Polymerase.

Sample Electrophoresis and Data Analysis

Amplification products were electrophoresed on either the ABI PRISM 377 DNA Sequencer (vertical slab gel) or the ABI PRISM 310 Genetic Analyzer (capillary electrophoresis (CE)). Briefly, for the 377 DNA Sequencer protocol (35–38,40,41), 4 μL of amplicons (2 μL for AmpFℓSTR Blue and AmpFℓSTR Green I) and 0.5 μL of GeneScan™-500 (or GeneScan™-350) [ROX] Internal Lane Size Standard (PE Biosystems) were added to 4.5 μL of load-

ing buffer (dextran blue dye, deionized formamide) and denatured at 95°C for 2 min. One and a half μL was loaded on a 5% LongRanger gel (Pharmacia Biotech Inc., Piscataway, NJ) in 36 cm well-to-read plates (PE Biosystems), and run for 2.25 h at 3 kV (full-scan mode; see AmpFℓSTR User's Manuals for 377-XL upgrade protocol). For the ABI PRISM 310 Genetic Analyzer (35–38,40,41), 1.5 μL of amplicons (1 μL for AmpFℓSTR Blue and AmpFℓSTR Green I) and 1 μL of GeneScan-500 (or GeneScan-350) [ROX] Internal Lane Size Standard were added to 24 μL of deionized formamide (<5 μS conductivity) and denatured at 95°C for 3 min. The PCR products were injected (5 s) and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4™). Data were collected using either the ABI PRISM 377 Collection software application, primarily with run module "GS Run 36F-2400," or the ABI PRISM 310 Collection software application with run module "GS STR POP4 (1 mL) F." Results were analyzed using the GeneScan Analysis® software application.

Results

Primer Selection

Multiplex PCR targets multiple loci simultaneously in a single reaction utilizing a cocktail of primer pairs. Without optimization, coamplification of several loci may introduce challenges for signal strength and amplification specificity. These challenges are due to potential competition for PCR building blocks and undesired complementarity both between primers (primer dimer) and between primers and genomic DNA template (mispriming). Consequently, development of a robust multiplex PCR environment commands careful optimization, particularly for automated analyses or human identification applications.

Signal Strength and Nontemplate Base Addition in Single Locus PCR Reactions—In the development of each AmpFℓSTR multiplex PCR system, oligonucleotide primers were designed to yield amplicons within specific size ranges and to maximize signal strength using a single amplification protocol (17,45,64). Hundreds of candidate primers were first screened for signal strength in singleplex reactions at each locus, using AmpFℓSTR PCR reaction mix and thermal cycling parameters. Of those yielding the highest signal, a range of concentrations was tested to determine signal strength plateau, between 0.2 to 0.4 μM for most primer pairs. Optimal primer concentrations were defined as approximately 20% greater than the concentration at the point of plateau. This provided a performance window within which signal intensity was consistent.

Primer selection strategy additionally addressed the terminal transferase-like activity of DNA polymerases (8,20). Significant amounts of amplicons (−N products), relative to products that have undergone nontemplate 3′ terminal addition (+N products), can compromise signal strength and may complicate data interpretation in certain circumstances (see *Thermal Cycling Temperatures and Times*). Thus, the strategy was to drive the reaction to produce a majority of +N products at the levels of primer selection and thermal cycling parameters. For each primer, the particular sequence at the 3′ end of each dye-labeled amplicon was considered (4,28,44) and efficiency of nontemplate base addition, using AmpFℓSTR PCR mix and conditions, was evaluated.

Signal Strength in Multiplex PCR Reactions—Candidate primer pairs were subsequently multiplexed together, using a PCR annealing temperature (T_A) of 59°C, to ascertain interlocus signal strength. Additionally, signal strength using a 61°C T_A assessed primer binding stability at higher stringency. Of particular significance in these studies was relative fluorescent intensity between loci detected with the same dye label in context of each multiplex. Signal strength between the three fluorescent dyes (5FAM, JOE, NED) of the AmpFℓSTR kits, detected on the ABI PRISM instruments, varies due to specific chemical characteristics of each dye (39). By maximizing signal strength at each locus, similar peak heights (relative fluorescence units, RFU) were obtained routinely for loci with the same dye label ("balanced"). When peak heights from high-quality, nondegraded DNA profiles are relatively balanced between loci, the potential to recover data from every locus in the typing system is enhanced. An additional benefit is seen when challenging samples are processed; the potential of data loss due to weak signal at particular loci is decreased. When DNA degradation and PCR inhibition are observed, obvious profile trends (e.g., loss of or weak higher molecular weight loci) allow these conditions to be identified more readily and addressed appropriately.

Amplification Specificity—Candidate primers which passed the above described tests were scrutinized for amplification specificity in several assays. Primers were tested together in multiplex using conditions of reduced stringency. In each assay, PCR specificity was challenged by evaluating the effects of one or more of the following: 1. amplification with AmpliTaq® DNA polymerase, 2. reduced T_A, 3. increased DNA template, 4. Chelex-extracted (single-stranded) DNA template, and 5. increased $MgCl_2$ concentration. AmpliTaq, as opposed to AmpliTaq Gold, can reduce PCR stringency as the enzyme may begin extending primers which have annealed during PCR setup or while the thermal cycler is ramping to 95°C, in absence of a manual hot start. Multiple amplifications were performed on approximately 15 organic and Chelex extracts over the course of multiplex development (including final versions). Candidate primer pairs which produced nonspecific PCR products under these less stringent conditions, particularly between 75 to 400 base pairs (bp), were rejected from further consideration.

Excessive $MgCl_2$ can result in spurious peaks during PCR. A representative example of AmpFℓSTR specificity at high $MgCl_2$ (3.0 m*M*) is shown in the last panel of Fig. 1. Nonspecific PCR products were not observed at any of the following concentrations tested: 0.6, 0.7, 0.8, 0.85, 0.9, 0.95, 1.0, 1.05, 1.10, 1.15, 1.20, 1.25, 1.30, 1.35, 1.40, 1.45, 1.50, 1.75, 2.0, 2.5, 3.0 m*M* (a subset is shown in Fig. 1).

Specificity was additionally examined with regard to species using nonhuman DNA extracts. DNA samples from each primate (2.5 ng) and nonprimate (50 ng) were examined; fungal and bacterial DNA copy numbers were equivalent to approximately 50 ng of human DNA. As expected, amplicons were generated from the primate DNA samples at most loci (data not shown; 26,59). Nonprimate DNA samples did not produce any amplicons, with the exception of a previously reported monomorphic product in the cow, pig, and dog DNA samples generated by amelogenin primers (6).

Primer Binding Site Mutations—Candidate primers meeting the specificity requirements were subjected to database screens in search of primer binding site (pbs) mutations. A mutation in the pbs region of the target sequence may compromise amplification efficiency of that allele, particularly when the position of the mutation is close enough to the 3′ end of the primer to destabilize annealing (26,44,51,62, unpublished data). Because the intensity of alleles at a locus can be informative in the interpretation of DNA mixtures,

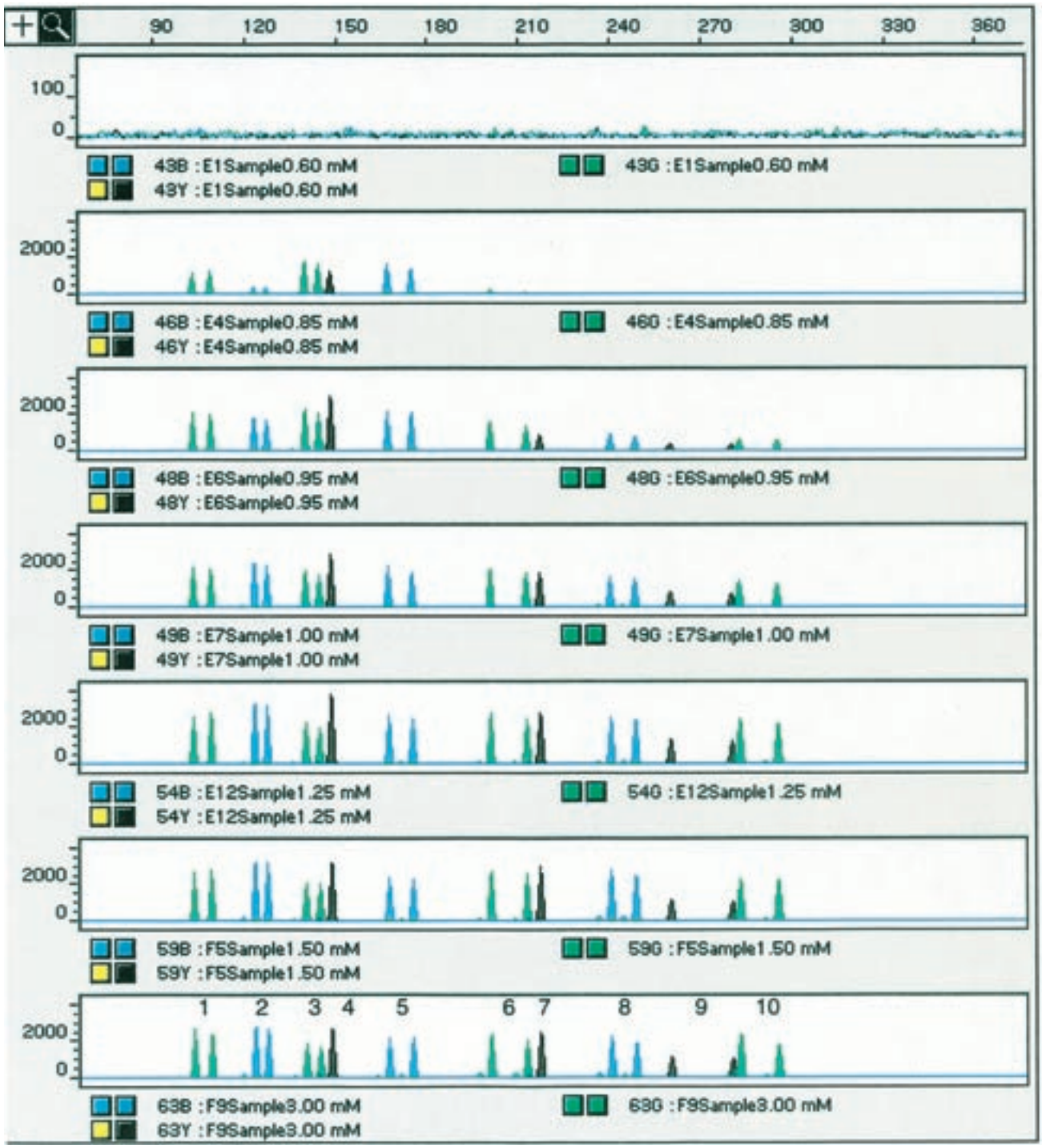

FIG. 1—*PCR specificity at different concentrations of $MgCl_2$: 0.6, 0.85, 0.95, 1.0, 1.25, 1.5, 3.0 mM. Triplicate amplifications were performed; one set of representative results are shown. The X-axes indicate base pair size and the y-axes indicate signal strength (RFU). Results were acquired using recommended conditions for AmpFℓSTR Profiler Plus and the 310 Genetic Analyzer. Locus key: 1. Amelogenin, 2. D3S1358, 3. D8S1179, 4. D5S818, 5. vWA, 6. D21S11, 7. D13S317, 8. FGA, 9. D7S820 and 10. D18S51.*

pbs mutations were avoided by designing primers in conserved sequence regions flanking STR motifs. Since it is not possible to select a primer in a region in which every base is conserved in every individual of all populations, pbs mutations were at least minimized by implementing a peak height ratio (PHR) assay. This involved screening candidate primers in population database samples for pbs mutation detection. Database samples supplied many genotypes, comprised of DNA from individuals of African American, U.S. Caucasian, and U.S. West Coast Hispanic descent.

Screens for pbs mutants relied upon attributes of heterozygous and homozygous profiles. Using AmpFℓSTR PCR amplification and detection conditions (including input DNA concentration of 1

to 2.5 ng), heterozygous alleles at each locus were found to amplify with relatively the same efficiency and, therefore, had similar within-locus peak heights. Peak height ratios (peak height of the lower intensity allele divided by peak height of the higher intensity allele, expressed as a percentage) of ≥70% at all 16 loci were typically observed under these conditions (35–38,40,41,59). A mutation in the primer binding region may result in less efficient amplification, causing a reproducible drop of the observed PHR to below 70% (i.e., imbalanced).

While evaluating heterozygous profiles, samples suspected of containing a mutant sequence due to reproducible imbalance at one or more loci were reamplified at lower (≤57°C) and higher (≥61°C) T_A. Since primer annealing stringency can alter amplification efficiency (particularly in the presence of a primer/template mismatch), PHRs between mutant and consensus alleles were generally >70% at lower T_A and <<70% at higher T_A. Mutations were confirmed by DNA sequencing using primers flanking the candidate PCR primer binding region. Figure 2*A* illustrates a reproducibly imbalanced profile at the D16S539 locus identified in a population database sample containing a point mutation (transversion) under the reverse PCR primer (Fig. 2*B*). Interestingly, in each sample containing this mutation (4 total), two other single nucleotide point mutations (1 transversion and 1 transition) were identified upstream of the repeat region, bracketing the final forward primer. In general, several of the 16 STR markers examined displayed single nucleotide transition and transversion events in the flanking regions of the repeat polymorphisms; mutations involving insertions or deletions were not observed.

Homozygous loci were also investigated in pbs mutant screens. Using AmpFℓSTR conditions, homozygous peaks were observed to be approximately two-fold higher in peak height than heterozygous peaks at a given locus, as expected. Potential pbs mutations were detected in homozygous profiles by comparing peak heights to peaks of other loci with the same dye label. Cases in which the apparent homozygous peak was of similar peak height to heterozygous profiles were reexamined. As described for heterozygous genotypes, a potential mutation in a homozygous profile was tested for reproducibility at varied annealing temperatures and confirmed by DNA sequencing (data not shown).

Pbs mutation(s) that destabilize primer binding enough to render a very weak or undetected (null) allele are potentially flagged by artificially inflated population homozygosity levels. In statistical testing of random population database samples, amplified with final primer sequences, excess homozygosity was not detected (18,33).

Upon confirmation of a destabilizing pbs mutation, an improved primer was selected by shifting the priming region to permit efficient amplification of both consensus and mutant sequences (data not shown). Alternatively, a degenerate primer, containing the complementary mutant nucleotide sequence, was added to the consensus primer pair. Because complementarity dictates competitive binding, efficient amplification of the mutant allele was facilitated without affecting amplification efficiency of nonmutant alleles (14). This strategy accommodated pbs point mutations at both D16S539 (see Fig. 2*C*) and vWA (26). The reported databases in Holt et al. (18) contained no detectable pbs mutations at any loci, using finalized primer cocktails.

PCR Reaction Mix Components

Performance of candidate primers was interrogated in various formulations of the AmpFℓSTR PCR reaction mix. This identified reasonable windows in which results did not vary significantly. A range of concentrations of each component was tested individually (±15 to 20% of the standard PCR reaction mix concentration). Evaluation of more extreme concentrations (e.g., ±50%) ensured maximal signal strength of all loci and defined points and characteristics where system performance began to deteriorate.

Components tested in dilution series were Tris·HCl (pH 8.3), KCl, $MgCl_2$, dNTPs, and BSA; varying amounts of AmpliTaq Gold and relevant primer cocktail were additionally tested. An example of effects of KCl concentration on AmpFℓSTR COfiler multiplex reactions is shown in Fig. 3. Concentrations of KCl tested were 50 m*M* (standard concentration), ±20% of 50 m*M* (40 m*M,* 60 m*M*), and ±50% of 50 m*M* (25 m*M,* 75 m*M*). Peak heights at 40, 50, and 60 m*M* KCl were similar for each locus. At 25 m*M* KCl, peak heights were approximately 50% of those at 50 m*M;* at 75 m*M,* lower peak height intensities were seen in this sample and correlated with amplicon size. Such experiments were performed to test each component on approximately ten organic and Chelex extracts during multiplex system development (including final versions). Comparable results were observed for each DNA sample and each multiplex system at 25 to 60 m*M.* Variation in peak height at 75 m*M* KCl, depending on the sample, was noted. In every case, genotyping results remained unchanged.

Optimal $MgCl_2$ concentration and performance windows were determined similarly. Figure 1 shows results using AmpFℓSTR PCR reaction mix at $MgCl_2$ concentrations of 1.25 m*M,* ± 20% (1.0 m*M,* 1.5 m*M*), and incremental concentrations to in excess of ± 50% (3.0 m*M*). At 0.95 m*M* $MgCl_2$, decreased amplification efficiency was evidenced most obviously by lower peak heights at the larger loci (FGA, D18S51, D7S820). Signal intensity progressively deteriorated with decreasing concentrations of $MgCl_2$ until finally, at 0.6 m*M,* no loci were detectable. Significant differences in signal strength were not observed from 1.0 m*M* to 3.0 m*M* $MgCl_2$. Genotyping results remained unchanged regardless of $MgCl_2$ concentration. The optimal concentration was defined at 1.25 m*M* to maintain maximum PCR specificity and because slight increases in the proportion of stutter/slippage PCR products were sometimes observed with increasing $MgCl_2$ (unpublished observations). This optimum created a robust ±20% performance window.

Thermal Cycling Temperatures and Times

Effects of a range of thermal cycling temperatures and times, bracketing the standard AmpFℓSTR parameters, on STR multilocus profile results were examined from the DNA Thermal Cycler 480 and GeneAmp 2400 and 9600 PCR systems. Denaturation and annealing temperature optima were determined by testing those of the final recommended protocols (94 and 59°C, respectively) and ±2°C (TC480) or ±1.5°C (GeneAmp 2400, 9600) to verify reproducible performance within a temperature window. Temperature variation specifications on each thermal cycler, ±1.5°C (TC480) and ±1.25°C (GeneAmp 2400, 9600), were thus adequately addressed. For each multiplex, approximately three to five organic and Chelex extracts were tested several times in final kit configuration. No significant differences in peak height intensities or balance between loci were observed over the denaturation and annealing temperature ranges or between the different thermal cyclers (data not shown).

Activation time for AmpliTaq Gold DNA polymerase was determined at a fixed activation temperature of 95°C. This optimal temperature was determined previously by enzyme activation assays (3) and confirmed as optimal with the STR multiplexes after

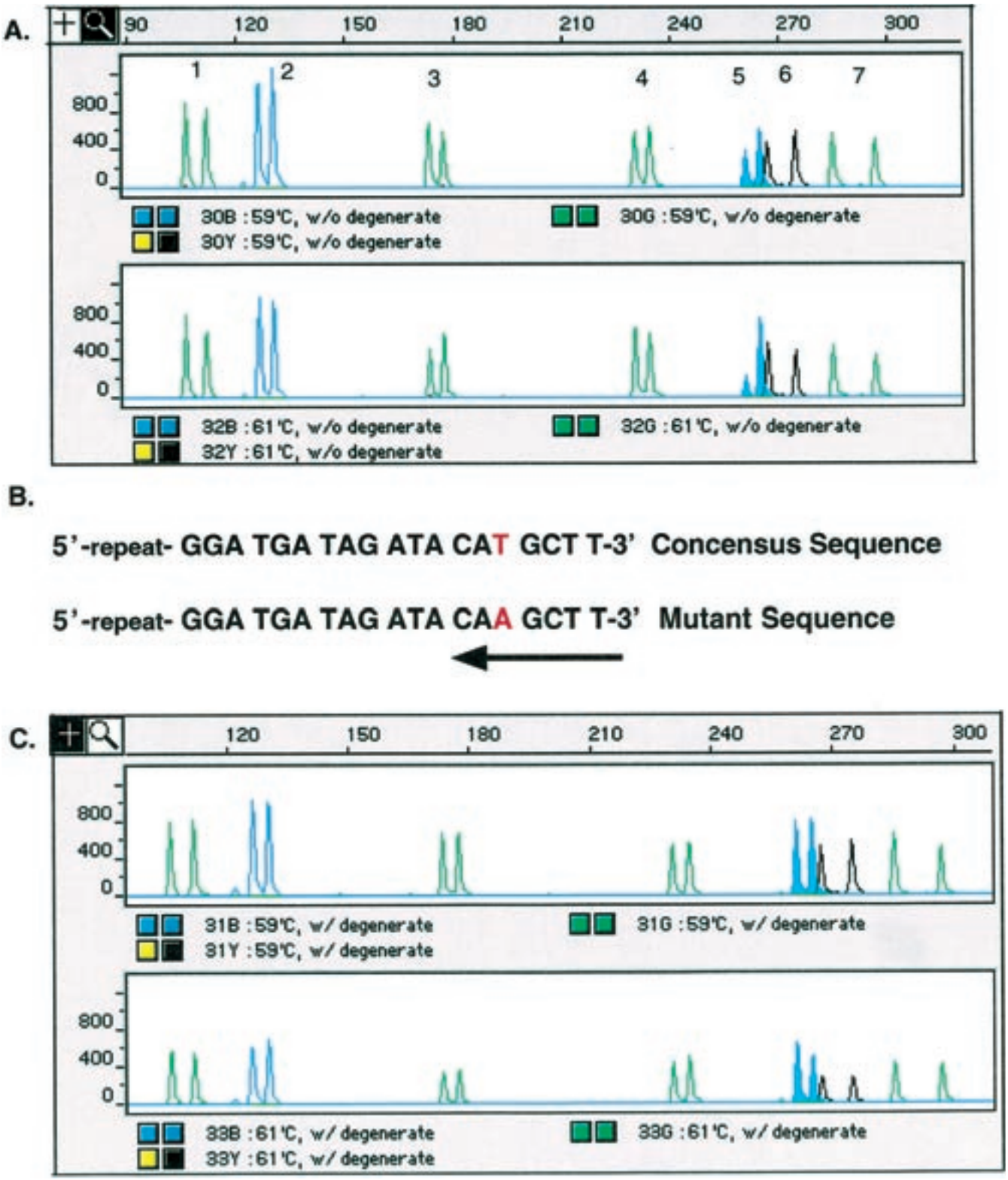

FIG. 2—*Degenerate primer strategy to address D16S539 point mutation. A. D16S539 amplified at annealing temperatures of 59°C (standard; Panel 1) and 61°C (Panel 2) using AmpFℓSTR COfiler primer set excluding the degenerate primer. The peak height ratios are 63% (Panel 1) and 27% (Panel 2). B. D16S539 sequence base change (T→A transversion) and position under reverse primer (3rd nucleotide from 3′ end) in consensus and mutant DNA sequence. C. D16S539 amplified at annealing temperatures of 59°C (standard; Panel 1) and 61°C (Panel 2) using AmpFℓSTR COfiler primer set, including the degenerate primer. The peak height ratios are 99% (Panel 1) and 78% (Panel 2). The X-axes indicate base pair size and the Y-axes indicate signal strength (RFU). Amplicons were detected on the 377 DNA Sequencer. Locus key: 1. Amelogenin, 2. D3S1358, 3. TH01, 4. TPOX, 5. D16S539, 6. D7S820, and 7. CSF1PO.*

evaluating results at 94, 95, and 96°C (data not shown). Enzyme activation was tested over different lengths of time to measure the point at which signal strength plateaued. In each multiplex, as exemplified by AmpFℓSTR Profiler in Fig. 4, maximum yield of amplicons was obtained after 8 min. An optimal activation time of 11 min provided a window of ±20% (8.8 to 13.2 min) for reproducible performance.

As described earlier, one design goal concerning nontemplate nucleotide addition was to drive reactions to predominantly +N products to enhance signal strength and simplify data interpretation. Signal strength was reduced when both +N and −N products were present due to division of two different amplicon lengths into two differently sized peaks. As follows, when all amplicons were either −N or +N products, all signal contributed to a single peak. This effect is evident when relative signal and number of peaks per allele are compared in the first two electropherograms of Fig. 5. If approximately equal proportions of +N and −N products are present, there is some potential to complicate allele assignment, unless additional information across the entire profile is considered. For instance, when interpreted in isolation, the TH01, 10, 10 genotype, intentionally generated under suboptimal conditions (Fig. 5, panel 1 inset), resembles the TH01 9.3, 10 heterozygote in Panel 3.

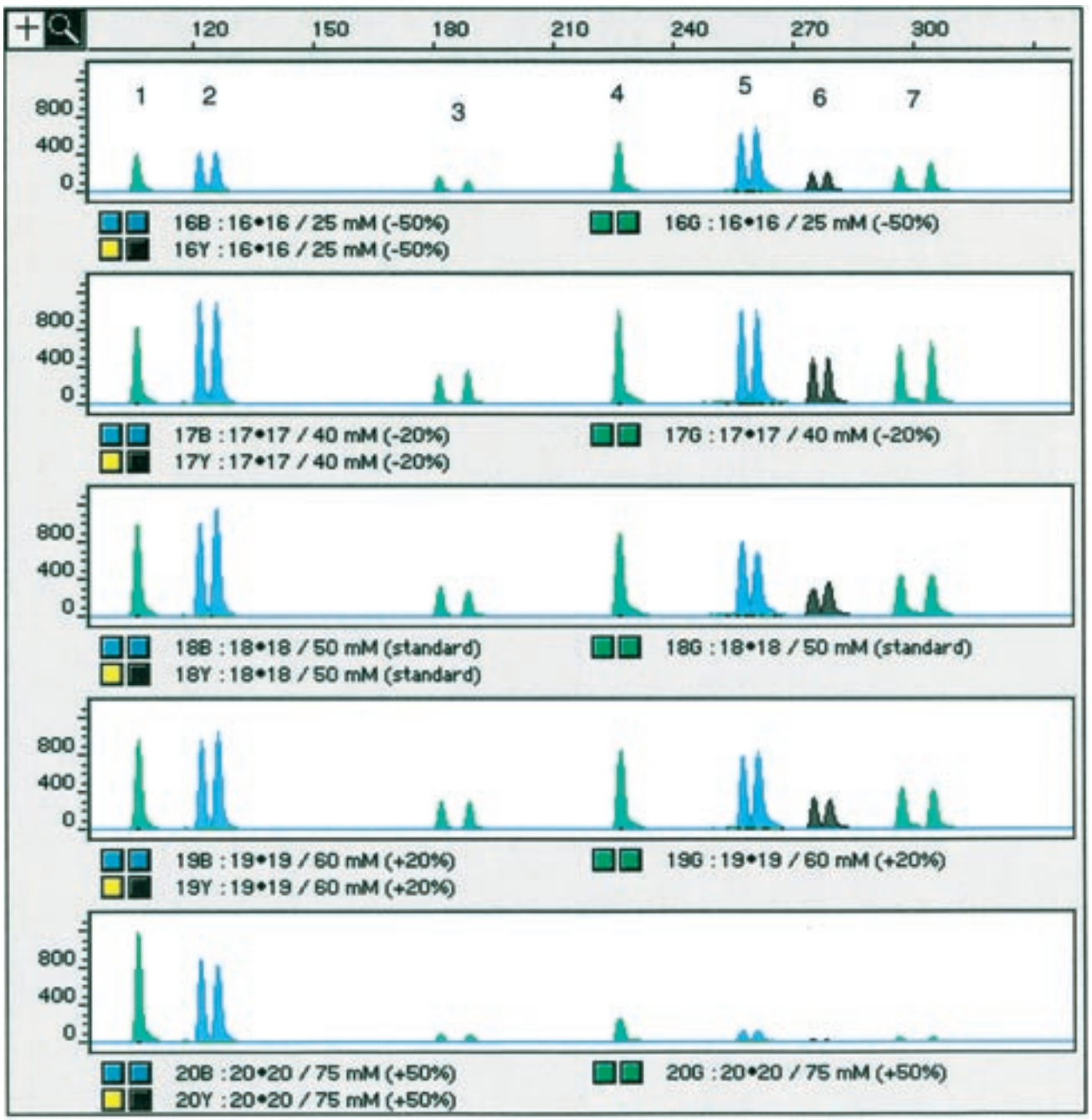

FIG. 3—*Amplification results of AmpFℓSTR control DNA 9947A at different concentrations of KCl: 25, 40, 50, 60, 75 m*M. *One of three different DNA extracts is shown. The X-axes indicate base pair size and the Y-axes indicate signal strength (RFU). Results were acquired using recommended conditions for AmpFℓSTR COfiler and the 377 DNA Sequencer. Locus key: 1. Amelogenin, 2. D3S1358, 3. TH01, 4. TPOX, 5. D16S539, 6. D7S820, and 7. CSF1PO.*

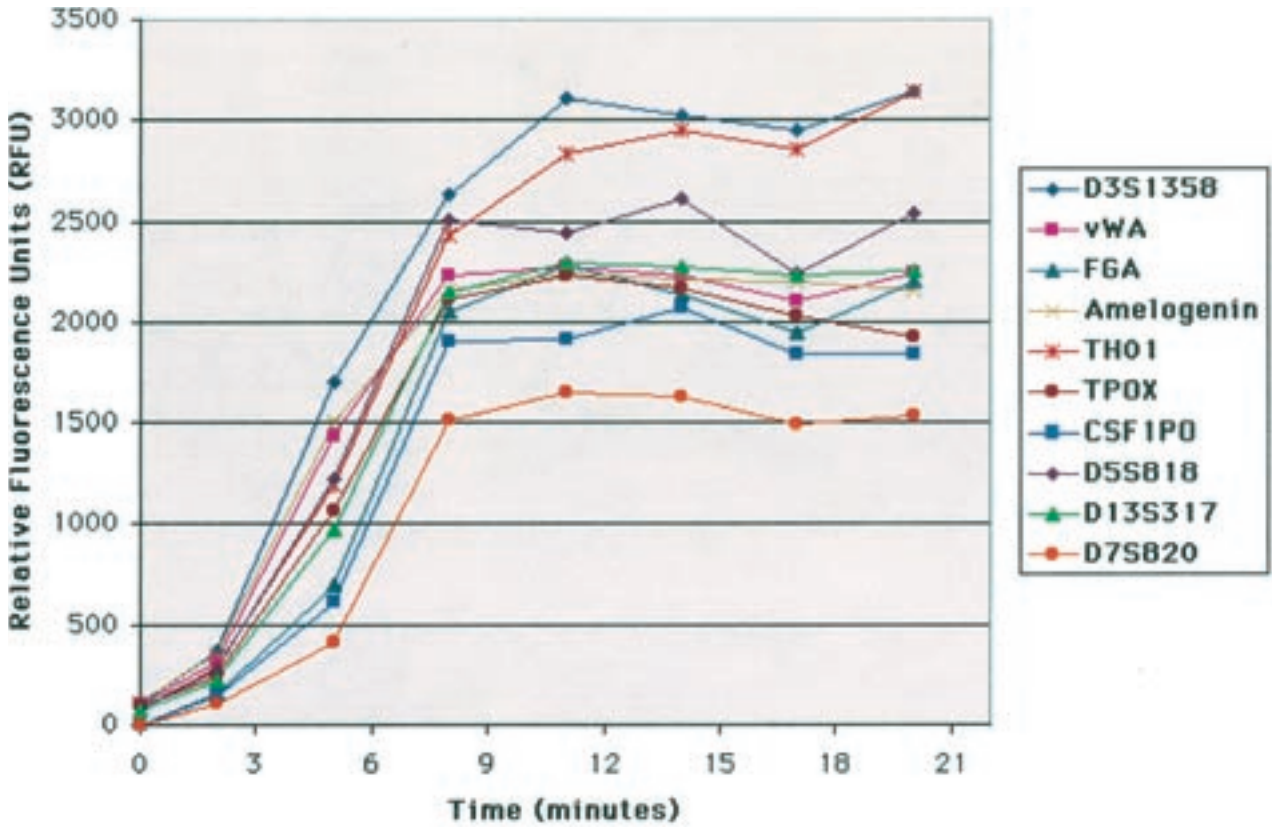

FIG. 4—*AmpliTaq Gold DNA polymerase activation at 95°C in a MicroAmp 9600 PCR system for different lengths of time: 2, 5, 8, 11, 14, 17, 20 min. Averaged peak heights of a DNA sample amplified in duplicate are shown. The X-axis indicates activation time and the Y-axis indicates signal strength (RFU). Results were acquired using recommended conditions for AmpFℓSTR Profiler and the 377 DNA Sequencer.*

While our primer design strategy promoted nontemplate nucleotide addition, this alone was insufficient for complete addition at all loci immediately following PCR cycling. A final post-PCR incubation step drove all, or nearly all, amplicons to +N length from as much as 5 ng of starting template genomic DNA, a two-fold excess of the maximum DNA quantity recommended for detection on the ABI PRISM instruments (see *Optimization for Amplicon Detection*). Amplification of 5 ng input genomic DNA and only a 30-min incubation was sufficient for complete amplicon extension in the triplex systems (AmpFℓSTR Blue, AmpFℓSTR Green I) but resulted in incomplete conversion to +N products of AmpFℓSTR Profiler amplicons (ten loci; Fig. 6*A,* panel 3). Final extension required lengthening to 45 min for this multipex (Fig. 6*A,* panel 4) as well as the other multiplexes of 7–11 loci. Among the 16 loci of the six multiplexes, least efficient addition of the nontemplated 3′ nucleotide was seen at D3S1358 and vWA. Figure 6*B* illustrates the dependence of input genomic DNA quantity, and therefore again the amount of total amplicons generated, on the proportion of +N products at a fixed extension time and temperature.

Optimization for Amplicon Detection

Optimization of the AmpFℓSTR multiplex systems on ABI PRISM laser-induced fluorescence (LIF) detection platforms was necessary to maximize signal strength and ensure reliable data interpretation. Signal strength may be altered by many parameters, including PCR input DNA concentration, number of PCR cycles, amount of sample loaded on a gel (or, for CE, time and voltage of electrokinetic injection), and intensity of fluorescent dye labels under the given detection conditions. To examine signal strength across the linear dynamic range of both the ABI PRISM 310 and 377 instruments, studies of these sensitivity parameters were performed using DNA samples amplified over a large range of concentrations. Results of multiple experiments performed on approximately three to five organic and Chelex extracts, using each multiplex, showed comparable sensitivity under optimized conditions. Furthermore, the 310 Genetic Analyzer and the 377 DNA Sequencer final protocols resulted in similar sensitivity.

Shown in Fig. 7 are representative sensitivity results, generated on a 377 DNA Sequencer. Amplifications covered a range of template DNA concentrations, from 10 pg to 10 ng, using AmpFℓSTR Profiler Plus. Examination of signal strength at the lower end of the dynamic range of both the ABI PRISM 310 and 377 instruments assessed factors that might be considered in implementing interpretation guidelines regarding minimum signal. With very low template copy number, PCR founder effects and stochastic allele sampling (48) may disable detection of one allele at a heterozygous locus or may result in an imbalanced heterozygote (<70% PHR). Consequently, AmpFℓSTR protocols were designed to produce little or no signal from approximately 35 pg (roughly ten copies of DNA template, or five diploid cells).

Virtually no peaks were detectable (<50 RFU) with less than ~20 to 40 pg template DNA (Fig. 7, lower panels). Stochastic sampling effects were sometimes observed when ≤250 to 300 pg DNA was amplified. For example, in Fig. 7, stochastic sampling was observed from amplification of ~300 pg at the D18S51 locus (63% PHR), and at some of the other loci using <300 pg. The average

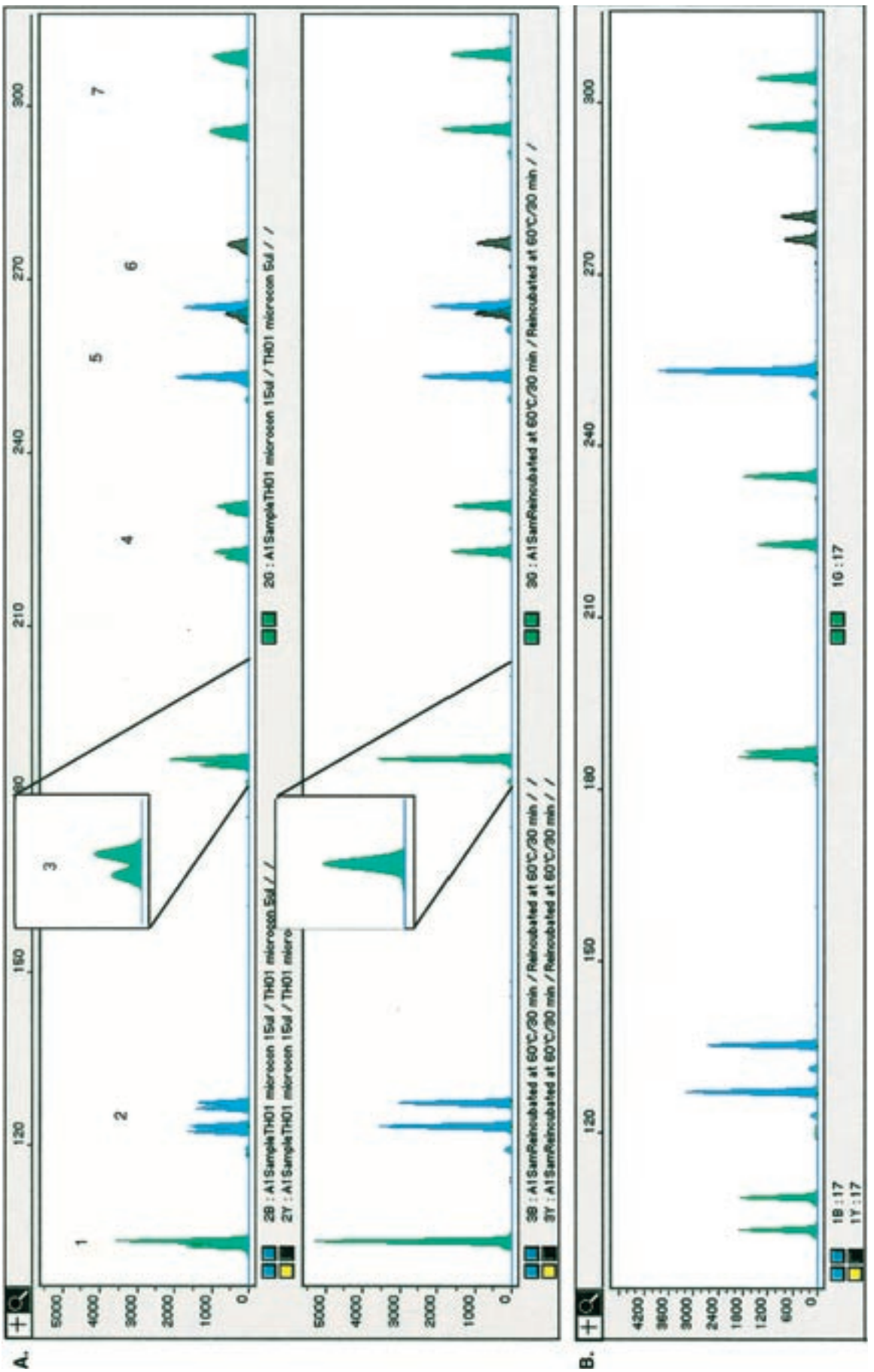

FIG. 5—*Effect of incomplete nontemplate nucleotide addition on signal strength and data interpretation. Panel 1 shows a TH01 10,10 genotype generated using 5 ng of DNA and by eliminating the final thermal cycling 60°C extension. After later incubating the sample for 30 min at 60°C, nontemplate nucleotide addition was complete (Panel 2). For comparison, Panel 3 shows a TH01 9.3,10 genotype generated using ~1.5 ng following recommended conditions. The X-axes indicate base pair size and the Y-axes indicate signal strength (RFU). Results were acquired using AmpFℓSTR COfiler and the 310 Genetic Analyzer. Locus key: 1. Amelogenin, 2. D3S1358, 3. TH01, 4. TPOX, 5. D16S539, 6. D7S820, and 7. CSF1PO.*

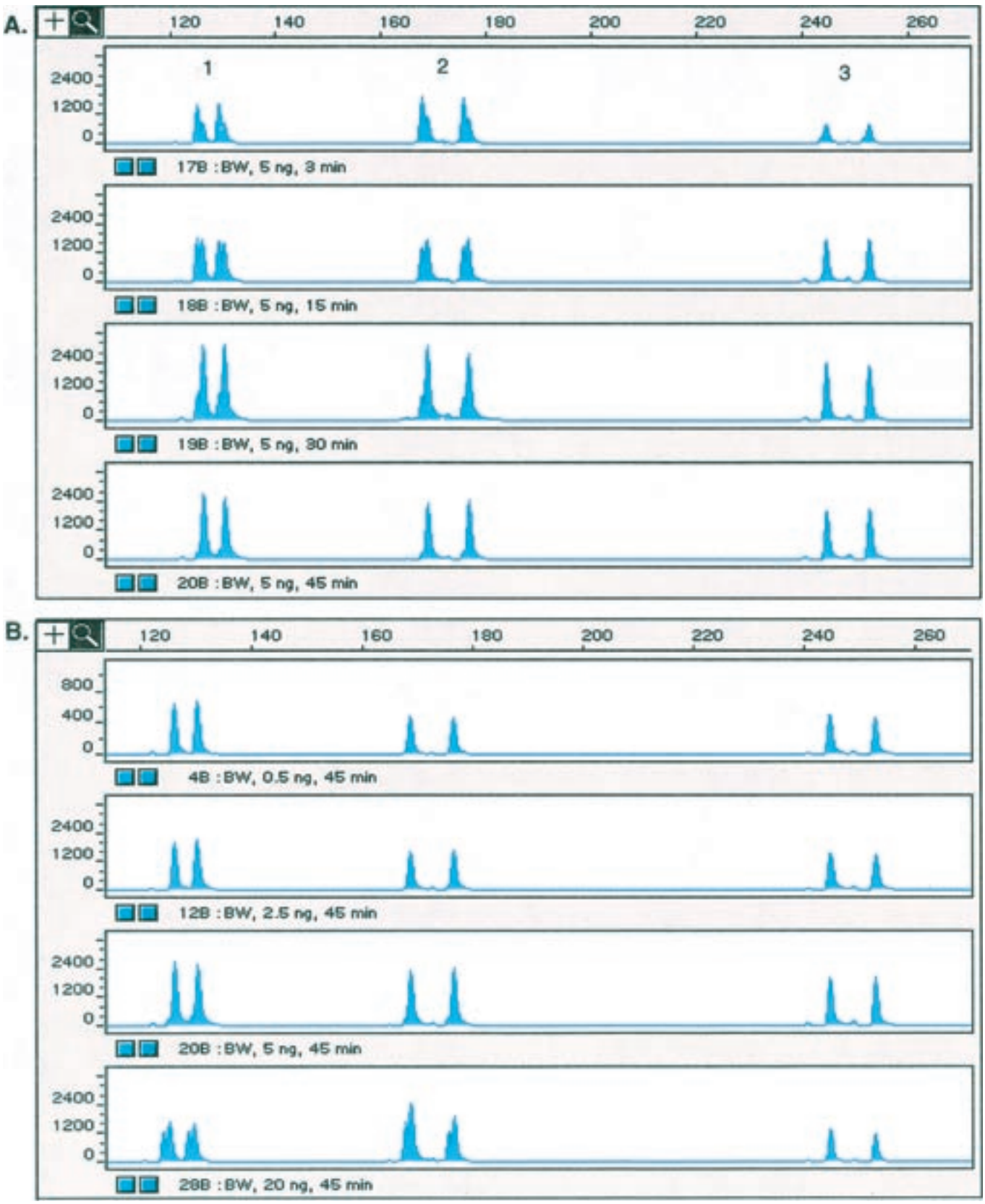

FIG. 6—*Efficiency of AmpliTaq Gold DNA polymerase in performing nontemplate nucleotide addition at the 3′ terminus of dye-labeled amplicons. A. Final PCR extension times shown are 3, 15, 30, and 45 min (Panels 1–4, respectively), using 5 ng DNA at 60°C. B. One half, 2.5, 5, and 20 ng (Panels 1–4, respectively) genomic DNA amplified using a final PCR extension time of 45 min at 60°C. The x-axes indicate base pair size and the y-axes indicate signal strength (RFU). Results were acquired using recommended conditions for AmpFℓSTR Profiler and the 377 DNA Sequencer; only the FAM-labeled loci are shown. Locus key: 1. D3S1358, 2. vWA, and 3. FGA.*

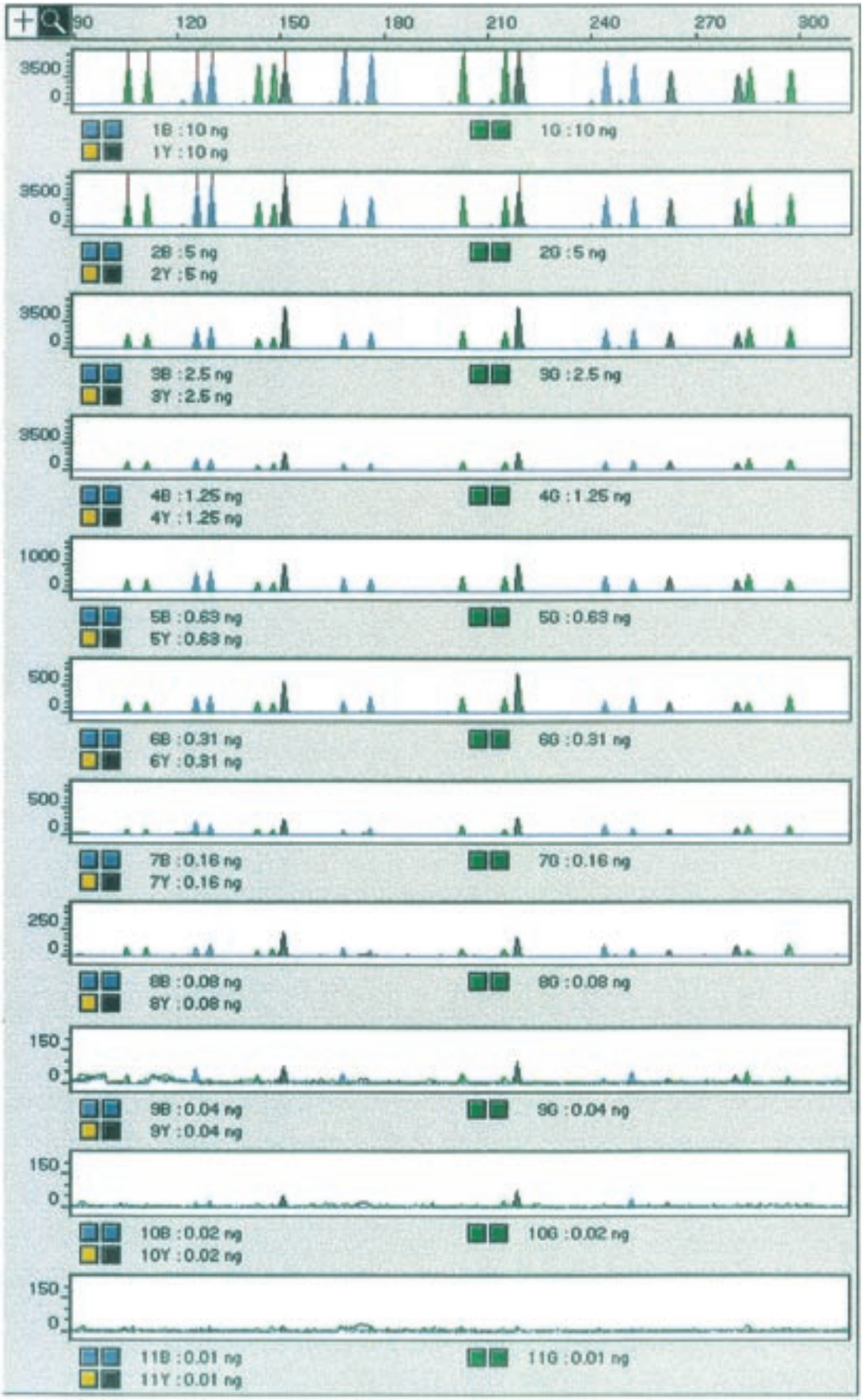

FIG. 7—*PCR amplification of varying DNA template concentrations: 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, 0.01 ng, respectively. Triplicate amplifications were performed; one set of representative results are shown. The x-axes indicate base pair size and the y-axes indicate signal strength (RFU). Red bars in Panels 1 and 2 indicate off-scale peaks. Results were acquired using recommended conditions for AmpF ℓSTR Profiler Plus and the 377 DNA Sequencer. Locus key: 1. Amelogenin, 2. D3S1358, 3. D8S1179, 4. D5S818, 5. vWA, 6. D21S11, 7. D13S317, 8. FGA, 9. D7S820 and 10. D18S51.*

peak height observed from ~300 pg at a heterozygous locus ranged from 75 to 150 RFU. Therefore, in all experiments, loci with single peaks ≤~150 RFU were interpreted with caution. Detection of only one allele at a truly heterozygous locus was not seen until fluorescent signal was less than 50 RFU (~40 pg template DNA). When considering the triplexes versus the larger multiplexes, there was no observed difference in the DNA concentration range that was potentially susceptible to unequal allele sampling.

Examinations of signal strength at the high end of the dynamic range of both the ABI PRISM 310 and 377 instruments assessed increasing amounts of input DNA template. Maximum signal on these instruments is 8191 RFU (unaveraged, raw data). Therefore, peak intensities of 8191 RFU may be quantitated inaccurately and are referred to as offscale. Offscale peaks may be identified in Genescan Analysis software, Version 3.X, by red bars in the electropherograms (see Panels 1 and 2 of Fig. 7). Among all six multiplex systems, peaks generally exceeded the dynamic range maximum at input genomic DNA concentrations greater than 2.5 ng, as illustrated in Fig. 7.

Discussion

Well-optimized PCR reactions were designed to produce reliable STR typing systems. Universal reaction conditions allowed a glimpse at human polymorphisms on 14 different chromosomes and provide a clear direction towards automation. In lieu of reliable means to predict primer characteristics *a priori,* success at fixed conditions was achieved by thorough empirical query of PCR primer performance. Selection and incorporation of highly competent, locus-specific primers maximized signal strength and specificity under both optimal and suboptimal amplification conditions. PCR primers that performed well within a range of thermal cycling parameters and PCR environments (performance windows) were selected deliberately so that the six described multiplex systems are tolerant to reaction condition alterations. Such robustness lends to standardized laboratory use and provides an appropriate environment to assay challenging DNA samples. Successful integration of key attributes (e.g., balanced fluorescent signal within each color, complete terminal nucleotide addition, absence of spurious peaks) into final validated protocols enhanced the straightforward nature of multilocus STR data interpretation. Furthermore, rigorous development led to a greater understanding of issues potentially affecting assay performance and data interpretation, including primer binding site (pbs) mutations, amplification efficiency, incomplete nontemplate nucleotide addition, and instrument dynamic range.

Due to the polymorphic nature of the human genome, potential for pbs mutations was considered and minimized by careful primer selection. Nonetheless, as comparative genotyping information expands, observations of such mutations, albeit rare, may be observed. In contrast to genotype comparisons performed within a laboratory that employs the same standardized typing platform(s), some degree of locus nonconcordance (i.e., homozygosity versus heterozygosity) may be seen in interlaboratory genotype comparisons of results generated using primers of differing sequences (62). Primer sequences for each locus are identical between all AmpFℓSTR multiplexes.

While DNA quality, PCR inhibitors, gross changes to PCR parameters, etc. may affect amplification efficiency, no consequence to reliability of STR genotype determination was encountered. Studies of exaggerated suboptimal PCR and detection conditions show that as peak intensities approach background noise levels, alleles simply become undetectable. As performance boundaries of the STR typing systems are reached, they are reflected as loss of information; no genotype result is obtained. Understanding conditions that contribute to low signal may be helpful for both data interpretation and troubleshooting of challenging samples.

Characterization of STR multiplex performance also delineates limitations regarding input DNA concentration at both lower and upper extremes. These studies suggest that care should be taken in interpreting peaks of low fluorescence signal, especially if chance allele sampling or PCR founder effects (e.g., an undetected allele) are a possibility. Intralaboratory determination of sensitivity levels may be instructional as slight differences in DNA quantitation methods, PCR amplification efficiency (e.g., quality of DNA extracts), and detection sensitivity may exist across laboratories. Although it is prudent to recognize a cautionary zone for signal strength, valuable information may be gleaned from data displaying low peak heights.

While mindful interpretation of peaks with a low signal-to-noise ratio is imperative, equally important are peaks of the opposite spectrum. When excess PCR product is generated, incomplete terminal nucleotide addition, offscale data, and/or fluorescence bleedthrough ("pull-up") may result. Incompletely extended PCR products (−N) are easily recognized; detection of −N products is probable at multiple loci, particularly D3S1358 and vWA. Moreover, reduced peak heights at the locus in question, the intralocus proportion of −N products, and the ability, or lack thereof, to drive +N products by additional incubation at 60°C all serve as interpretational aids.

Accurate quantitative interpretation of data requires on-scale fluorescence signal. When a peak is detected as offscale, the actual height of the peak is undetermined. Because the ABI PRISM 310 and 377 detectors quantitate raw signal no greater than 8191 RFU, this maximum value is the default peak height. Additionally, suboptimal multicomponent spectral separation may result, causing pull-up and possibly baseline problems in electropherograms (39,52,58). In general, amplicons labeled with 5FAM (blue dye) tend to exceed the ABI PRISM 310 and 377 dynamic range before the other dyes used for the AmpFℓSTR multiplexes (detected with Filter Set F). This is largely due to dye excitation efficiency. The closer the dye's excitation maximum is to the excitation source wavelength(s), the brighter the emitted fluorescence. The excitation maximum of 5FAM (493 nm) is near the primary wavelengths of the dual-line argon ion laser (488, 514.5 nm) whereas those of JOE (528 nm) and NED (553 nm) are further away (39).

The AmpFℓSTR multiplex systems satisfy stringent requirements for human genetic comparative applications, allowing for standardized approaches that facilitate accurate interlaboratory comparison. Collectively, components of this development process address developmental validation guidelines of both TWGDAM and the DNA Advisory Board, written to promote quality assurance in human genetic comparisons (57). Additional studies were performed during development of each multiplex PCR system to examine robustness with mock forensic samples. Such studies, including examination of DNA from adjudicated biological evidence, DNA coextracted and coamplified with PCR inhibitors, degraded DNA, and single locus versus multiplex amplifications, are topics of separate reports (19,35–38,40,41,59). Taken together, the studies described in this manuscript indicate that each AmpFℓSTR multiplex in its final version, along with associated procedures for PCR and detection provide robust, reliable results. In understanding the optimization of each parameter and limitations of the multiplex PCR systems, analysts are empowered to interpret results soundly and conservatively.

Acknowledgments

We are grateful to Laboratory Corporation of America (Research Triangle, NC), California Department of Justice DNA Laboratory (Berkeley, CA), and John Hartman (Orange County Coroner Department, Santa Ana, CA) for providing DNA samples. We thank Eileen Brown (PCR Technical Support, PE Corporation, Norwalk, CT) for generating AmpliTaq Gold activation time data and acknowledge technical contributions by Nicola Fildes (Human Identification Group, PE Biosystems), James Robertson (FBI Academy, Quantico, VA), and Nicola Oldroyd (Technical Support, PE Corporation, United Kingdom) to AmpFℓSTR Blue, AmpFℓSTR Green I, and AmpFℓSTR SGM Plus multiplex development, respectively.

References

1. Anker R, Steinbrueck T, Donis-Keller H. Tetranucleotide repeat polymorphism at the human thyroid peroxidase (hTPO) locus. Hum Mol Genet 1992;1:137.
2. Barber, Parkin. Sequence analysis and allelic designation of the two short tandem repeat loci D18S51 and D8S1179. Int J Legal Med 1996; 109:62–5.
3. Birch DE, Kolmodin L, Laird WJ, McKinney N, Wong J, Young KY, et al. Simplified hot start PCR. Nature 1996;381:445–6.
4. Brownstein MJ, Carpten JD, Smith JR Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques 1996;20:1004–10.
5. Chakraborty R, Stivers DN, Su B, Zhong Y, and Budowle B. The utility of short tandem repeat loci beyond human identification: Implications for development of new DNA typing systems. Electrophoresis 1999;20: 1682–96.
6. Buel E, Wang G, Schwartz M. PCR amplification of animal DNA with human X-Y amelogenin primers used in gender determination. J Forensic Sci 1995;40:641–4.
7. CHLC (Cooperative Human Linkage Center). http://www.chlc.org, University of Iowa, #140 EMRB, Iowa City, IA, 52242.
8. Clark J. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucleic Acids Res 1988; 16(20):9677–86.
9. DNA Recommendations. Report concerning further recommendations of the DNA commission of the ISFH regarding PCR-based polymorphisms in STR (short tandem repeat) systems. Int J Leg Med 1994;107: 159–60.
10. Edwards A, Hammond H, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tetrameric repeat loci in four human population groups. Genomics 1992;12:241–53.
11. Entrala C, Lorente M, Lorente JA, Alvarez JC, Moretti T, Budowle B, et al. Fluorescent multiplex analysis of nine STR loci: Spanish population data. Forensic Sci Int 1998;98:179–83.
12. Fregeau CJ, Aubin RA, Elliott JC, Gill SS, Fourney RM. Characterization of human lymphoid cell lines GM9947 and GM9948 as intra- and interlaboratory reference standards for DNA typing. Genomics 1995;28: 184–97.
13. Garofano L, Pizzamiglio M, Vecchio C, Lago G, Floris T, D'Errico G, et al. Italian population data on thirteen short tandem repeat loci: HUMTH01, D21S11, D18S51, HUMVWFA31, HUMFIBRA, D8S1179, HUMTPOX, HUMCSF1PO, D16S539, D7S820, D13S317, D5S818, D3S1358. Forensic Sci Intl 1998;97:53–60.
14. Gibbs RA, Nguyen PN, and Caskey CT. Detection of single DNA base differences by competitive oligonucleotide priming. Nucleic Acids Res 1989;17(7):2437–48.
15. Green ED, Mohr RM, Idol JR, Jones M, Buckingham JM, Deaven LL, et al. Systematic generation of sequence-tagged sites for physical mapping of human chromosomes: application to the mapping of human chromosome 7 using yeast artificial chromosomes. Genomics 1991;11:548–64.
16. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am J Hum Genet 1994;55:175–89.
17. He Q, Marjamaki M, Soini H, Mertsola J, Viljanen MK. Primers are decisive for sensitivity of PCR. BoiTechniques 1994;17:82–6.
18. Holt C, Stauffer C, Wallin J, Lazaruk K, Nguyen T, Budowle B, et al. Practical applications of genotypic surveys for forensic STR testing. For Sci Int 2000;112:91–109.
19. Holt C, Buoncristiani M, Wallin J, Nguyen T, Lazaruk K, Walsh S. TWGDAM validation of the AmpFℓSTR PCR amplification kits for forensic DNA casework. J Forensic Sci 2002;47(1).
20. Hu G. DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3′ end of a DNA fragment. DNA Cell Biol 1993;12(8): 763–70.
21. Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, et al. An STR-based map of the human genome. Science 1995;270:1945–53.
22. Kimpton C, Walton A, Gill P. A further tetranucleotide repeat polymorphism in the vWF gene. Hum Mol Genet 1992;1:287.
23. Kupferschmidt K, Calicchio T, Budowle B. Maine caucasian population DNA database using twelve short tandem repeat loci. J Forensic Sci 1999;44(2):392–5.
24. Lareu M-V, Barral S, Salas A, Rodriguez M, Pestoni C, Carracedo A. Further exploration of new STRs of interest for forensic genetic analysis. In: Olaisen B, Brinkmann B, Lincoln PJ, editors. Progress in Forensic Genetics 7: Proceedings of the 17th International ISFH Congress, Oslo 2–6 Sept 1997. Elsevier: Amsterdam, 1998;195–200.
25. Lazaruk KD, Walsh PS, Oaks F, Gilbert D, Rosenblum B, Menchen S, et al. Genotyping of forensic STR systems based on excellent sizing precision in a capillary electrophoresis instrument. Electrophoresis; 1998; 19:86–93.
26. Lazaruk KD, Wallin JM, Holt CL, Nguyen T, Walsh PS. Sequence variation in humans and other primates at six short tandem repeat loci used in forensic identity testing. For Sci Int. 2001;119(1):1–10.
27. Li H, Schmidt L, Wei M-H, Hustad T, Lerman MI, Zbar B, et al. Three tetranucleotide polymorphisms for loci: D3S1352; D3S1358; D3S1359. Hum Mol Genet 1993;2(8):1327.
28. Magnuson VL, Ally DS, Nylund SJ, Karanjawala ZE, Rayman JB, Knapp JI, et al. Substrate nucleotide-determined non-templated addition of adenine by Taq DNA Polymerase: Implications for PCR-based genotyping and cloning. Biotechniques 1996;21:700–9.
29. Mancuso D, Tuley E, Westfield L, Worrall N, Shelton-Inloes B, Sorace J, et al. Structure of the gene for human von Willebrand factor. J Biol Chem 1989;264:19514–27.
30. Mayrand PE, Corcoran KP, Ziegle JS, Robertson JM, Hoff LB, Kronick MN. The use of fluorescence detection and internal lane standards to size PCR products automatically. Appl Theor Electrophor 1992;3:1–11.
31. Mills KA, Even D, Murray JC. Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). Hum Mol Genet 1992;1:779.
32. Nakahori Y, Takenaka O, Nakagome Y. A human X-Y homologous region encodes amelogenin. Genomics 1991;9:264–9.
33. Nei M, Roychoudhury AK. Sampling variances of heterozygosity and genetic distance. Genetics 1974;76:379–90.
34. Oldroyd NJ, Urquhart AJ, Kimpton CP, Millican, ES, Watson, SK, Downes T, et al. A highly discriminating octoplex short tandem repeat polymerase chain reaction system suitable for human individual identification. Electrophoresis 1995;16:334–7.
35. PE Biosystems. AmpFℓSTR Blue User's Manual. Foster City; Version A, 1996.
36. PE Biosystems. AmpFℓSTR Green I User's Manual. Foster City; Version A, 1997.
37. PE Biosystems. AmpFℓSTR Profiler User's Manual. Foster City; Version A, 1997.
38. PE Biosystems. AmpFℓSTR Profiler Plus User's Manual. Foster City; Version A, 1997.
39. PE Biosystems. Genescan Reference Guide. Foster City; 1997.
40. PE Biosystems. AmpFℓSTR COfiler User Bulletin. Foster City; 1998.
41. PE Biosystems. AmpFℓSTR SGM Plus User's Manual. Foster City; 1999.
42. Perkin-Elmer Corporation. AmpliType User's Guide. Foster City; Version 2, 1993.
43. Polymeropoulos MH, Xiao H, Rath DS, Merril CR. Tetranucleotide repeat polymorphism at the human tyrosine hydrolase gene (TH). Nucleic Acids Res 1991;19:3753.
44. Robertson J, Walsh-Weller J. PCR primer design and optimization. In: Lincoln PJ, Thomson J, editors. Forensic DNA Profiling Protocols. New Jersey: Humana, 1998;126–7.
45. Saiki R. The design and optimization of the PCR. In: Erlich H, editor. PCR technology: Principles and Applications for DNA Amplification. New York: Stockton, 1989;7–16.
46. Sambrook J, Fritch EF, Maniatis T. Molecular cloning: a Laboratory Manual. New York: Cold Spring Harbor Laboratory, 1989.
47. Sensabaugh G. Biochemical markers of individualization. In: Saferstein R, editor. Forensic Science Handbook. New Jersey: Prentice-Hall, 1982; 338–415.

48. Sensabaugh GF, von Beroldingen C. The polymerase chain reaction: application to the analysis of biological evidence. In: Farley MA, Harrington JJ, editors. Forensic DNA Technology. Michigan: Lewis, 1991;63–82.
49. Sharma V, Litt M. Tetranucleotide repeat polymorphism at the D21S11 locus. Hum Mol Genet 1992;1:67.
50. Sheffield VC, Weber JL, Buetow KH, Murray JC, Even DA, Wiles K, et al. A collection of tri- and tetranucleotide repeat markers used to generate high quality, high resolution human genome-wide linkage maps. Hum Mol Genet 1995;4(10):1837–44.
51. Sommer R, Tautz D. Minimal homology requirements for PCR primers. Nucleic Acids Res 1989;17(16):6749.
52. Sparkes R, Kimpton C, Gilbard S, Carne P, Andersen J, Oldroyd N, et al. The validation of a 7-locus multiplex STR test for use in forensic casework: (II) Artefacts, casework studies, and success rates. Int J Legal Med 1996;109:195–204.
53. Straub RE, Speer MC, Luo Y, Rojas K, Overhauser J, Ott J, et al. A microsatellite genetic linkage map of the human chromosome 18. Genomics 1993;15:48–56.
54. Sullivan KM, Mannucci A, Kimpton CP, and Gill P. A rapid and quantitative sex test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin. Biotechniques 1993;15:636–41.
55. Urquhart A, Kimpton CP, Downes TJ, Gill P. Variation in short tandem repeat sequences—a survey of twelve microsatellite loci for use as forensic identification markers. Int J Leg Med 1994;107:13–20.
56. Urquhart A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. Biotechniques 1995;18:116–21.
57. U.S. Department of Justice, Federal Bureau of Investigation. Quality Assurance Standards for Forensic DNA Testing Laboratories. Washington, D.C.; 1998.
58. Wallin JM, Lazaruk KD, Holt CL, Walsh PS. AmpFℓSTR multicomponent analysis. Eighth International Symposium on Human Identification, Scottsdale, AZ; 1997.
59. Wallin JM, Buoncristiani MR, Lazaruk KD, Fildes NJ, Holt CL, Walsh PS. TWGDAM validation of the AmpFℓSTR Blue PCR Amplification Kit for forensic casework analysis. J Forensic Sci 1998;43(4):854–70.
60. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 1991;10:506–18.
61. Walsh PS, Valaro J, Reynolds R. A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Res 1992;20:5061–5.
62. Walsh PS. Commentary on Kline MC, Jenkins B, Rogers S: Non-amplification of a vWA allele. J Forensic Sci 1998;43(1):250. J Forensic Sci 1998;43(5):1103–4.
63. Watson S, Kelsey Z, Webb R, Evans J, Gill P. The development of a third generation STR multiplex system (TGM). In: Olaisen B, Brinkmann B, Lincoln PJ, editors. Progress in Forensic Genetics 7: Proceedings of the 17th International ISFH Congress, Oslo 2–6 Sept 1997. Elsevier: Amsterdam, 1998;192–4.
64. Wu D, Ugozzoli L, Pal B, Qian J, Wallace R. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biol 1991; 10(3):233–8.
65. Yamamoto T, Uchihi R, Nozawa H, Huang X-L, Leong Y-K, Tanaka M, et al. Allele distribution at nine STR loci-D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317 and D7S820-in the Japanese population by multiplex PCR and capillary electrophoresis. J Forensic Sci 1999;44(1):167–70.

Additional information and reprint requests:
Jeanette M. Wallin
California Department of Justice
DNA Laboratory
626 Bancroft Way
Berkeley, CA, 94710
Phone: 510-540-2434
Fax: 510-540-2701
Email: jeanette.wallin@doj.ca.gov