JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, November 2009, Vol. 54, No. 6
doi: 10.1111/j.1556-4029.2009.01200.x
Available online at: interscience.wiley.com

TECHNICAL NOTE

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Fast Multiplexed Polymerase Chain Reaction for Conventional and Microfluidic Short Tandem Repeat Analysis

ABSTRACT: The time required for short tandem repeat (STR) amplification is determined by the temperature ramp rates of the thermal cycler, the components of the reaction mix, and the properties of the reaction vessel. Multiplex amplifications in microfluidic biochip-based and conventional tube-based thermal cyclers have been demonstrated in 17.3 and 19 min, respectively. Optimized 28-cycle amplification protocols generated alleles with signal strengths above calling thresholds, heterozygous peak height ratios of greater than 0.65, and incomplete nontemplate nucleotide addition and stutter of less than 15%. Full CODIS-compatible profiles were generated using the Profiler Plus ID, COfiler and Identifiler primer sets. PCR performance over a wide range of DNA template levels from 0.006 to 4 ng was characterized by separation and detection on a microfluidic electrophoresis system, Genebench-FX™. The fast multiplex PCR approach has the potential to reduce process time and cost for STR analysis and enables development of a fully integrated microfluidic forensic DNA analysis system.

KEYWORDS: forensic science, microfluidics, polymerase chain reaction, short tandem repeat, nontemplate nucleotide addition, stutter

The polymerase chain reaction (PCR) is an enzymatic reaction that facilitates rapid exponential amplification of nucleic acid sequences in vitro. Introduced by Mullis in 1985 (1), the technique has since become an indispensable tool for genetic analysis. The ability to amplify DNA has found widespread application in areas such as basic molecular biology, clinical diagnostics, evolutionary studies, and human identification. In forensic science, PCR is typically utilized to amplify small regions of the human genome containing a class of repeated DNA known as short tandem repeats (STRs). The unit length of STRs used in human identification are 4 or 5 base pairs, and STRs generally fall within noncoding and flanking sequences but occasionally within coding regions (2). There are several hundred thousand STR loci in the human genome, occurring on average every 6–10 kb (3) and appearing to be highly polymorphic (4).

Amplification of multiple STR loci is generally followed by separation of the fluorescently labeled amplicons by electrophoresis. When a sufficient number of loci is interrogated, a genetic profile is generated providing the discriminating power needed for human identification (5,6). STR analysis was first applied in the early 1990s and has since become a major tool in the forensic armamentarium with a growing set of applications including paternity testing, human identification in mass disasters, and military and homeland security settings. In the United States, STR profiles generated from selected human samples are collected in the Combined DNA Index System (CODIS). This electronic database was established in 1997 by the FBI and standardized to a set of 13 tetrameric STR loci for data submission (7,8). Other STR databases outside the U.S. (e.g., ENFSI, Interpol, and GITAD) contain a subset of the CODIS loci, and the British National DNA Database contains 8 CODIS loci and two additional STRs (D2S1338 and D19S433).

Simultaneous amplification of all desired STR loci in a multiplex PCR reaction requires careful primer design as well as rigorous optimization of all reaction mix components and cycling parameters. Specificity is affected by conditions that impact mispriming such as annealing temperatures, and the concentrations of primers, polymerase, and magnesium chloride (9). In general, stringent annealing temperatures and low concentration of primers, polymerase, and magnesium chloride will increase specificity but at the same time reduce yield. The use of a hot start polymerase can greatly increase specificity and yield by preventing the extension of misprimed products and primer dimers (10–13). Accordingly, an optimized balance of all components and cycling conditions must be determined experimentally for successful multiplex PCR. In addition to specificity, a series of criteria must be met to satisfy forensic interpretation guidelines including signal strength, inter-loci peak height balance, heterozygous peak height ratio (PHR), incomplete nontemplate nucleotide addition (NTA), and stutter (14).

Several commercially available STR kits have been developed and allow the synthesis of the desired PCR products with high specificity (e.g., AmpFℓSTR® Profiler Plus®, COfiler®, and Identifiler® PCR Amplification Kits manufactured by Applied Biosystems [Foster City, CA]; PowerPlex® 16 System manufactured by Promega [Madison, WI]). All of these kits contain AmpliTaq-Gold™ DNA Polymerase (TaqGold) as the hot start enzyme for amplification. Many casework samples can be processed with these kits, but certain samples require alternative analyses. For STR typing of degraded DNA samples, primer sets have been designed to amplify shorter STR sequences using the same CODIS loci (15). Y-chromosome STR assays improve the chance of detecting low levels of male DNA in a high background of female DNA (16,17).

The dramatic success of STR analysis has led to significant backlogs of both casework and database samples to be tested, and

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Received 13 Feb. 2008; and in revised form 9 Dec. 2008; accepted 14 Dec. 2008.

backlog reduction is a major goal in developing new technologies and improving throughput of existing technologies. In seeking to improve STR amplification speed, sensitivity, and throughput at low cost, several avenues have been explored including development of instrumentation to allow more rapid temperature transitions, and reducing reaction volumes by making use of microfluidic biochips (18–21). With respect to instrumentation, progress has been made in building thermal cyclers with reduced thermal masses in order to improve heating and cooling rates (22). An alternative approach is continuous flow amplification in which sample is moved through reaction zones that are held at specific temperature instead of heating and cooling a static sample (23,24).

Much of the literature on increasing PCR speed and sensitivity has focused on singleplex assays. Simultaneous amplification of multiple targets as required for forensic STR typing is a more challenging task, and only a few publications have reported success in this area. For example, a 160 nL chamber coupled to an integrated heater was capable of amplification and separation of 4 STRs contained in a Y-STR assay (25). The amplification was accomplished in approximately 80 min with a detection limit of 20 copies of template DNA. Increased PCR sensitivity due to reduced PCR reaction volume has been reported for the PowerPlex® 16 System, although no attempt was made to increase reaction speed (26).

Regardless of the approach taken, when increasing speed, sensitivity, and efficiency of amplification reactions, forensic interpretation guidelines (8) must be met. Here we report for the first time successful amplification of full profiles consisting of either 9 or 15 STR loci and amelogenin in both tube reactions and 16-sample microfluidic biochip reactions in approximately 17 min. The approach allows generation of full profiles that satisfy interpretation guidelines and functions over a broad dynamic range of DNA template. The microfluidic biochip is intended to function as a major component of a fully integrated sample-in to results-out forensic DNA analysis system currently in development.

Methods and Materials

Custom Thermal Cycler and Microfluidic Biochip

Network Biosystems (Netbio) has designed and built a custom thermal cycler to perform fast cycling by allowing the PCR reaction solution temperatures to be heated and cooled rapidly, controllably, and reproducibly. This instrument accepts a 16-chamber microfluidic biochip and consists of a high output thermoelectric cooler/heater mounted to a high efficiency heat sink. Each of 16 PCR reaction solutions is placed into an individual chamber of the microfluidic biochip (see Fig. 1), which is coupled to the heat pump by applying a compressive pressure with a clamping mechanism. Each PCR chamber is 500 μm deep and *c.* 1 mm wide and holds 7 μL of PCR reaction solution.

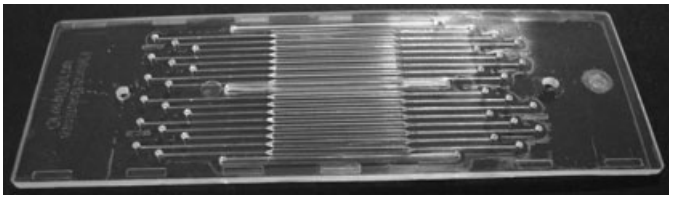

FIG. 1—*Photograph of the 16-lane microfluidic biochip.*

Instrumentation and Temperature Profiles

All amplification reactions in tube were performed with an Eppendorf Mastercyler ep gradient S (Eppendorf North America, Westbury, NY). Block temperature profiles of the above instrument were obtained using a 127 μm diameter type K thermocouple sensor which was attached directly to the block. For reaction solution profiles a 127 μm diameter type K thermocouple sensor was placed in the 20 μL reaction solution, within a thin-walled PCR tube. Data acquisition was performed with an Omega HH506RA Multilogger thermometer set to acquire data at a rate of 100 Hz. Amplification in biochip was accomplished using the Netbio thermal cycler with 16-sample plastic biochips as the reaction vessels. The solution temperature within the microfluidic biochip was monitored by inserting a thermocouple into one of the channels of the biochip.

PCR—Reaction Mix Components and Cycling Conditions

Multiplex PCR reactions were performed with the AmpFℓSTR® Profiler Plus® ID PCR Amplification Kit (Profiler Plus ID kit) (Applied Biosystems, Foster City, CA) using 9947A genomic DNA (Promega, Madison, WI) as a template. Polymerases used for amplification were either AmpliTaq Gold® DNA Polymerase (TaqGold) supplied with the Profiler Plus ID kit or other polymerases (SpeedSTAR™ HS DNA Polymerase [SpeedSTAR; Takara BIO USA Inc., Madison, WI], KOD Hot Start DNA Polymerase [KOD; EMD Biosciences Inc., Gibbstown, NJ], PyroStart™ Fast PCR Master Mix [PyroStart; Fermentas Inc., Glen Burnie, MD]). Multiplex PCRs with other polymerases were carried out using the labeled multiplex primer set from the Profiler Plus ID kit in combination with the polymerase specific buffers and dNTPs. All tube PCRs were carried out in 0.2 mL thin-walled PCR tubes (Eppendorf North America) using the Eppendorf Mastercyler ep gradient S. All chip reactions were amplified in the Netbio thermal cycler using 16-sample biochips.

The following PCR reaction mixes were prepared and cycled:

Standard TaqGold reactions: Standard TaqGold multiplex reactions consisted of 9.55 μL Profiler Plus ID reaction mix, 1 ng 9947A genomic DNA, 5 μL Profiler Plus ID Primer set, and 2.25 U TaqGold in a 25 μL reaction volume. Cycling conditions (block temperatures and times) were chosen following the manufacturer's recommendations and set to an initial 95°C for 11 min (hot start) followed by 28 cycles of 1 min at 94°C (denaturing), 1 min at 59°C (annealing), 1 min at 72°C (extension), and a final extension of 45 min at 60°C. *Optimized TaqGold reactions*: TaqGold reactions optimized for fast cycling were carried out in a 10 μL reaction volume containing 3.82 μL Profiler Plus ID reaction mix, 1 ng 9947A genomic DNA, 2 μL Profiler Plus ID Primer set, and 0.9 U TaqGold. Reactions were cycled at 95°C for 11 min; 28 cycles 10 sec, 98°C; 45 sec, 59°C; 30 sec, 72°C; and a final extension of 15 min at 72°C. *SpeedSTAR tube reactions*: SpeedSTAR PCR mix components for tube PCR were: 2 μL Profiler Plus ID primer set, 9947A genomic DNA, 1× Fast Buffer I (Takara BIO USA Inc.), 200 μM dNTPs, and 0.315 U SpeedSTAR in a 10 μL reaction volume. Cycling conditions were systematically optimized for fast performance and set to: 1 min at 95°C (enzyme activation) followed by 28 cycles of 4 sec at 98°C, 15 sec at 59°C, 5 sec at 72°C and a 1 min at 72°C, final extension. *SpeedSTAR biochip reactions*: For biochip PCR the 7 μL reaction mix contained 1.4 μL Profiler Plus ID primer set, 9947A genomic DNA, 1× Fast Buffer I buffer, 200 μM dNTPs, and 0.42 U SpeedSTAR. Cycling parameters were set to 70 sec at 95°C; 28 cycles of 4 sec, 98°C;

15 sec, 59°C; 7 sec, 72°C; and a final extension of 1:30 min at 70°C. *KOD reactions*: Amplification with KOD was performed with 2 µL Profiler Plus ID primer set, 1× KOD buffer (EMD Biosciences Inc.), 200 µM dNTPs, 1 ng 9947A genomic DNA, 1.5 mM $MgSO_4$, 0.2 U KOD in a 10 µL reaction volume. Cycling conditions were: 2 min, 95°C followed by 28 cycles of 4 sec, 98°C; 30 sec, 59°C; 10 sec, 72°C; with a final extension of 1 min, 72°C. *PyroStart reactions*: Reaction mixtures with PyroStart in a 1× final concentration also contained 2 µL Profiler Plus ID primer set and 1 ng 9947A genomic DNA in a 10 µL reaction and were cycled at: 1 min, 95°C and 28 cycles of 4 sec, 98°C; 20 sec, 59°C; 30 sec, 72°C; followed by a final extension of 1 min at 72°C.

Multiplex PCR with Other STR Typing Kits

The suitability of SpeedSTAR to generate full STR profiles with other STR typing kits (AmpFℓSTR® Identifiler® [Identifiler], AmpFℓSTR® COfiler® PCR Amplification Kit [COfiler]; Applied Biosystems) was tested in tube and biochip with the reaction conditions as described above for SpeedSTAR with the Profiler Plus ID kit. In these reactions, the Profiler Plus ID primer sets were replaced with the primer set from each of the kits.

Reproducibility

Reproducibility studies in tube and biochip were performed with TaqGold and SpeedSTAR using 1 ng 9947A genomic DNA as a template. For tube reproducibility five individual reactions were prepared. Biochip reproducibility was determined in three biochip PCR runs with eight reactions each.

Sensitivity

Sensitivity studies for SpeedSTAR amplification in tube and biochip were performed using the following amounts of 9947A template DNA: In tube: 4, 2, 1.5, 1, 0.5, 0.25, 0.125, 0.1, 0.05, 0.03, 0.02, 0.01, 0.006 ng; in biochip: 4, 2, 1.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.02, 0.015, 0.01, 0.006 ng. The reactions at each template level were performed in duplicate.

STR Separation and Detection Instrumentation

Amplified products were separated and detected using NetBio's Genebench-FX™ Series 100 (27). This instrument was developed and optimized specifically for STR analysis and has been ruggedized for laboratory and field-forward utilization. To 2.7 µL of each amplified product 10.2 µL Hi-Di formamide and 0.1 µL of Genescan 500 LIZ internal lane standard (both Applied Biosystems) were added. After denaturation at 95°C for 3 min and snap cooling on ice, samples were loaded into the separation biochip and electrophoretically moved into the separation channels by applying a 350 V/cm electric field for 90 sec. This was followed by the application of a 150 V/cm electric field along the separation channel to separate the DNA fragments. All separations were carried out at 50°C.

Data Analysis

Data were analyzed with the GeneMarker® HID STR Human Identification Software, Version 1.51 (SoftGenetics LLC, State College, PA). Signal strengths were normalized to the internal lane standard and the percentages of stutter, incomplete nontemplate addition (NTA) as well as peak height ratios (PHRs) were determined. The level of incomplete NTA is calculated by dividing the signal strength of the template fragment (−A) by the signal strength of the adenylated fragment (+A) and reported as percentage. The PHR is calculated by dividing the peak height of the lower signal strength allele by the higher signal strength allele within the locus.

Results

Temperature Profiles of Thermal Cycling Instruments and Reaction Solutions in Conventional PCR Tubes and Microfluidic Biochips

Amplification reactions were performed in thin-walled PCR tubes using a commercial thermal cycler and in microfluidic biochips using the NetBio thermal cycler. For tube reactions, the Eppendorf Mastercycler ep gradient S thermal cycler, one of the fastest block-base thermal cyclers, was utilized. Figure 2*a* shows the temperature of the block and the reaction solution within a tube for one of the 28 thermal cycles using a conventional STR cycling protocol. The Mastercycler's heating and cooling system is based on a heat pump with an integrated block for tube insertion. The time and temperature setpoints are 1 min at 98°C for denaturation, 1 min at 59°C for annealing, and 1 min at 72°C for extension. A comparison of the temperature profiles for the heat block and the reaction solution shows a lag in the response of the solution temperature relative to the block temperature. The measured heating and cooling rates of the block are 5.6°C/sec and 4.9°C/sec and of the solution are 4.8°C/sec and 3.3°C/sec. The block makes the temperature transition from extension (72°C) to denaturation (98°C)

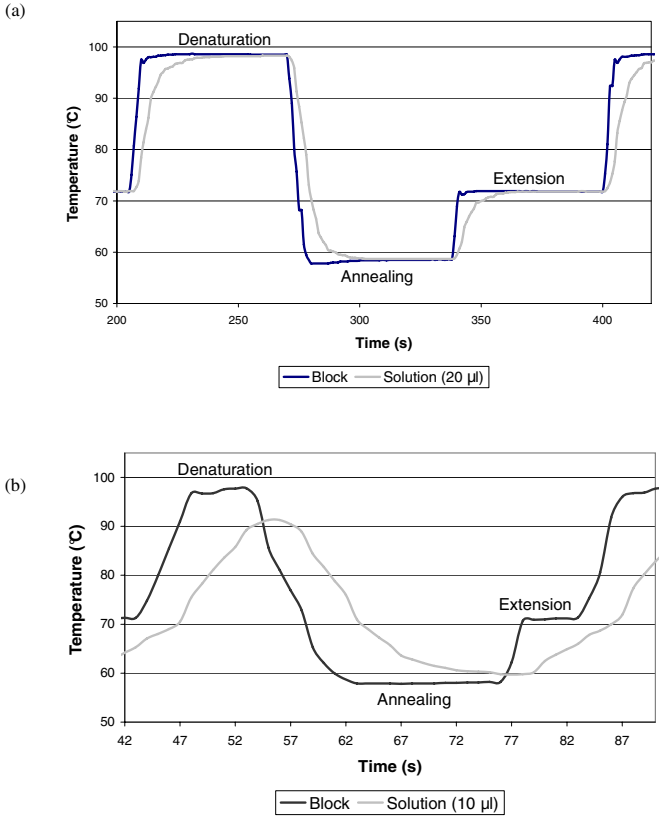

FIG. 2—*Temperature profiles of block and reaction solution for one thermal cycle of the (a): standard STR cycling protocol (total cycling time: 145.1 min) and (b): fast cycling protocol (total cycling time: 19.6 min).*

in 14 sec, but the solution does not achieve the setpoint temperature for 39 sec. Transitions between the denaturation and annealing steps (59°C) take 10 and 27 sec and between the annealing and extension steps 7 and 24 sec for the block and solution, respectively.

The temperature profiles of the Eppendorf Mastercyler block and the reaction solution for one of the 28 thermal cycles under fast cycling conditions are shown in Fig. 2*b*. The time and temperature setpoints are 5 sec at 98°C for denaturation, 15 sec at 59°C for annealing, and 5 sec at 72°C for extension. The delayed and dampened response of the solution prevents it from achieving the desired setpoint temperatures.

The temperature profiles of the heat pump and the reaction solution for one of the 28 thermal cycles for the NetBio thermal cycler using fast cycling conditions were also determined (Fig. 3). The time and temperature setpoints are 4 sec at 95°C for denaturation, 15 sec at 59°C for annealing, and 7 sec at 72°C for extension. The measured heating and cooling rates of the heat pump are 21.5°C/sec and 21.7°C/sec, and the measured heating and cooling rates of the reaction solution are 14.8°C/sec and 15.4°C/sec. Accordingly, the NetBio thermal cycler is capable of heating and cooling the reaction solution at a rate that is three to five times faster than the commercial block-based cycler. The transition times between extension, denaturation, and annealing steps for the heat pump are 1.7, 2.1, and 0.7 sec and for the solution 2.7, 4.5, and 2.2 sec. The NetBio cycler allows the reaction solution to reach the required temperatures approximately sevenfold faster than the block-based cycler. The NetBio thermal cycler has achieved its design goals of rapidly heating and cooling the reaction solutions, resulting in defined and controlled incubation temperatures and times under fast cycling conditions.

Evaluation of PCR Enzymes and Initial Optimization in Tubes

A large number of polymerases were evaluated for potential use for fast, multiplexed STR analysis, and candidates were selected based in part on hot-start activation time and extension rate. Four polymerases with reported extension rates ranging from 15 to 200 nucleotides/sec were selected for experimental evaluation and compared with recommended conditions for TaqGold. PCR conditions in tubes were initially optimized for the four enzymes with the goal of achieving full STR profiles in the least amount of time. For all reactions, 1 ng of human genomic DNA was amplified using primer pairs from the Profiler Plus ID kit and vendor recommended buffers and enzyme concentrations, and resulting profiles were separated, detected, sized, and quantified using GeneBench-FX™ Series 100. Optimization of cycling was achieved by systematic variation of the time and temperature of the denaturation, annealing, and extension steps. Following optimization, the total time for PCR amplification with signal strengths suitable for STR interpretation ranged from 19.1 min for SpeedSTAR to 71.7 min for TaqGold. These optimized conditions allow amplification to be performed 2–10 fold more rapidly than recommended conditions for TaqGold. Signal strengths for SpeedSTAR, Pyrostart, KOD, and optimized TaqGold reactions with the Profiler Plus primer set and 9947A DNA are all either approximately the same or higher than those generated using standard TaqGold PCR conditions. SpeedSTAR, PyroStart and optimized TaqGold reactions exhibit levels of incomplete NTA that are up to three times higher than those of standard TaqGold reactions. For most alleles, incomplete NTA levels fall below the 15% interpretation threshold (data not shown). Further optimization to reduce the higher levels of incomplete NTA below 15% was performed (see below). The KOD polymerase possesses 3′–5′ exonuclease activity and does not generate fragments with A-overhangs; accordingly, all alleles were 1 nucleotide shorter than their allelic ladder counterpart.

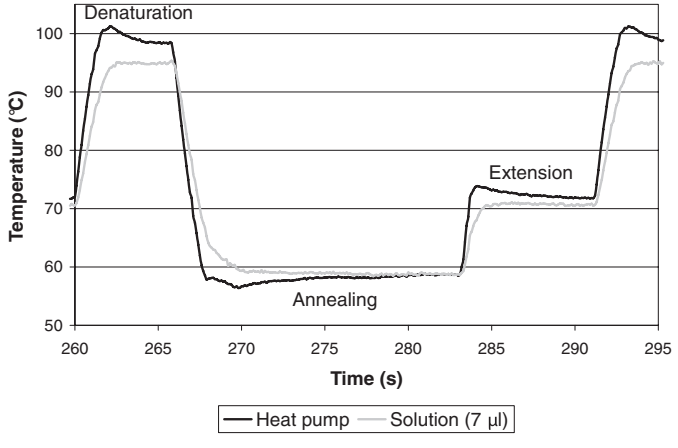

FIG. 3—*Temperature profiles of the heat pump and the reaction solution for one thermal cycle for the NetBio thermal cycler using fast cycling conditions (total cycling time: 17.3 min).*

The range of stutter produced for optimized TaqGold, SpeedSTAR, and PyroStart reactions is slightly higher than the range of stutter produced with standard TaqGold reactions; the range of stutter generated with KOD is higher than the range of stutter for standard TaqGold (data not shown). Based on the initial optimization results presented above, the SpeedSTAR polymerase was selected for further optimization in biochips with the goal of minimizing the total cycling time and achieving full STR profiles that satisfy signal strength, PHR, incomplete NTA and stutter interpretation requirements.

Fast PCR Protocol Optimization and Allele Characterization Using the SpeedSTAR Polymerase in Tubes and Biochips

The SpeedSTAR polymerase is a robust, sensitive, and reliable PCR enzyme with a 4.5-fold higher fidelity than Taq polymerase (28). The fidelity is a measurement of the extent to amplify a DNA template without introducing sequence errors.

The optimized time and temperature setpoints for amplification using SpeedSTAR in the microfluidic 16-sample biochip on the NetBio thermal cycler are 70 sec 95°C for hot-start activation followed by 28 cycles of 4 sec at 95°C for denaturation, 15 sec at 59°C for annealing, and 7 sec at 72°C for extension. A final extension of 90 sec at 72°C is performed for a total protocol time of 17.3 min. Tube reactions in the Eppendorf Mastercycler were performed in 19.1 min comprising of block times and temperatures set to an initial activation time of 1 min at 95°C, 28 cycles of 4 sec at 98°C, 15 sec at 59°C, and 5 sec at 72°C, followed by a final extension of 1 min at 72°C.

Figure 4 shows STR profiles generated with the optimized cycling conditions in (a) 7 µL biochip and (b) 10 µL tube reactions using 0.5 ng of DNA and Table 1 presents signal strengths for all Profiler Plus ID alleles from the SpeedSTAR biochip and tube reactions as well as TaqGold in tubes using the standard protocol. Signal strengths of the 0.5 ng SpeedSTAR biochip reactions are on average approximately two times higher than those of the 1 ng standard TaqGold reactions, while the signal strengths of the 0.5 ng SpeedSTAR tube reactions are on average approximately the same as those of the TaqGold reactions. The same fast biochip and tube conditions described above are also capable of generating full

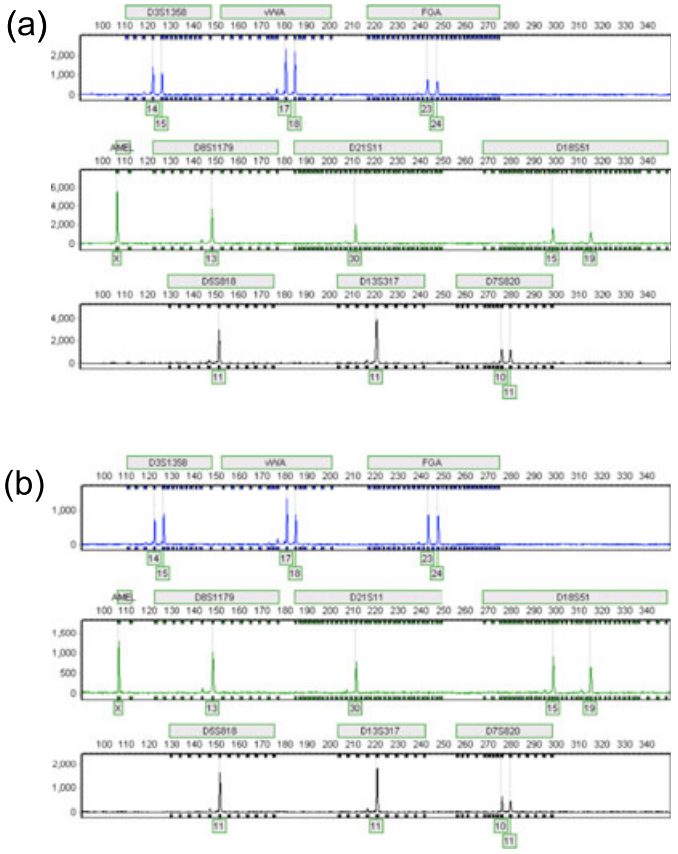

FIG. 4—*STR profile generated with optimized cycling conditions in (a): SpeedSTAR biochip reaction and (b): SpeedSTAR tube reactions using 0.5 ng template DNA.*

profiles with the COfiler and Identifiler primer sets (profiles not shown).

To characterize the products of fast PCR reactions, quantification of PHR, incomplete NTA, and stutter was performed (data summarized in Table 1). Biochip and tube reactions using the SpeedSTAR polymerase show more inter-locus peak height imbalance compared to that for the TaqGold reactions. The PHR for alleles generated in biochip reactions is between 0.70 and 0.95 and is approximately the same in tubes; all fall within acceptable interpretation guidelines. Reactions using SpeedSTAR have PHRs that are *c.* 10% lower than those determined for standard TaqGold reactions. Similarly, the level of incomplete NTA for most alleles in both biochip and tube reactions using SpeedSTAR are approximately the same (2.0–10.5%); these levels are about two times higher than reported incomplete NTA levels for TaqGold control reactions. The exception is incomplete NTA for the D3S1358 alleles, which is 4.8 to 7 times higher with SpeedSTAR than with TaqGold; even in this case, the level of incomplete NTA is below 12% for the SpeedSTAR enzyme. Finally, the level of stutter in both biochip and tube based reactions using SpeedSTAR is between *c.* 6.0% and 14.1%. A maximum level of stutter of 17.1% (average + 3 standard deviations [SD]) is observed for loci D18S51, which is on average approximately 1.6-fold higher than that for standard TaqGold tube reactions.

DNA Template Levels and Allele Characteristics in Fast PCR Reactions

The effects of template DNA on signal strength for fast PCR reactions using the SpeedSTAR polymerase in (a) biochip and (b) tube reactions are presented in Fig. 5. The alleles selected for analysis are amelogenin, the allele with the highest signal in the STR profile and FGA 23 and 24 and D7S820 10 and 11, the alleles with the lowest signal in the profile. Signal strengths for all alleles increase as the DNA template level increases from 0.006 ng to 4 ng in both SpeedSTAR biochip and tube reactions. DNA template concentration was determined by both UV spectrometry and gel analysis using human genomic DNA (Roche, Branchburg, NJ) as a reference. The quantified DNA solution was serially diluted to achieve the DNA template levels from 0.006 ng to 4 ng. At a template level of 0.006 ng, signal strengths for the amelogenin peak of 111 RFU for biochip and 58 RFU for tube reactions are observed. Background levels and SD for blue, green, yellow and orange traces are 5 ± 3.6, 10 ± 6.4, 6 ± 4.8, and 7 ± 4 RFU, respectively**.** The signal strength of the amelogenin peak at a template level of 0.006 ng is separated from the background level by 15 and 7.5 SD of noise for biochip and tube reactions, respectively. The STR profiles show that 11 and 7 of 15 alleles for biochip and tube reactions, respectively exhibit a signal to noise level of greater than 3. At a template level of 4 ng, signal strengths of 12680 RFU are seen for biochip and 5570 RFU for tube reactions. All alleles observed in both reaction types show good peak morphology.

For fast biochip reactions (Fig. 6*a*), the PHR is between 0.6 and 1.0 for template levels ranging from 0.05 to 4.0 ng. For template levels below 0.05 ng, the PHR decreases until 0.025 ng, when instances of allelic dropouts occur resulting in PHRs of zero. Similar results are observed for fast tube reactions, although they generally exhibit somewhat lower PHRs than biochip reactions (Fig. 6*b*). For biochip reactions, the level of incomplete NTA is 15% or less for template levels of 2.0 ng and below (Fig. 7*a*). For tube reactions, the incomplete NTA levels surpass 15% at template levels of 1 ng and increase dramatically by 4 ng (Fig. 7*b*). The two major differences between biochip and tube reactions are the temperature profiles of the reaction solutions and the relative concentration of template and polymerase. The level of incomplete NTA decreases as more polymerase is available. For biochip reactions experimental data show that over a DNA template range from 0.5 to 4 ng the level of incomplete NTA decreases by *c.* 50% as the amount of SpeedSTAR polymerase increases from 0.3 U to 1.2 U (data not shown). The level of stutter for fast biochip and tube reactions is relatively constant and generally less than 15% for all alleles over a template level range of 0.25–4 ng (Fig. 8).

Repeatability and Reproducibility Studies

The repeatability and reproducibility of the fast biochip (Table 2*a*) and tube (Table 2*b*) reactions using the SpeedSTAR polymerase was evaluated by performing 24 identical PCR reactions in three PCR biochips and by performing five identical tube reactions. For biochip reactions, the coefficient of variation (CV) for signal strength ranges from 17% to 24% and for tube reactions from 15% to 34%. The CV for standard TaqGold reactions is between 6% and 21%.

The CV for PHRs is up to 14% for biochips and 21% for tube reactions, compared to the 5% to 10% range observed for standard TaqGold reactions. CVs for incomplete NTA in biochip reactions vary between 6% and 28%, and for tube reactions between 2% and 13%. Again, these variations are similar to the 4–28% range observed for standard TaqGold reactions. The CVs for stutter in biochips are 4–9%, in tubes 2–6%, and are also similar to the range of 4–13% observed for standard TaqGold reactions.

TABLE 1—*Comparison of signal strength, PHR, incomplete NTA and stutter for SpeedSTAR biochip and tube reactions and TaqGold standard reactions.*

Locus	Allele	Signal Strength (RFU)	SD	PHR	SD	Incomplete NTA (%)	SD	Stutter (%)	SD
(a) *SpeedStar biochip reactions*									
D3S1358	14	1404	302	0.79	0.05	9.08	0.71	10.35	1.02
D3S1358	15	1111	244			8.05	1.40		
vWA	17	2339	401	0.95	0.08	6.14	1.10	12.87	1.21
vWA	18	2217	288			6.83	0.97		
FGA	23	748	312	0.87	0.08	4.21	0.93	9.72	1.33
FGA	24	649	278			3.34	1.40		
Amelogenin	X	5645	941			4.04	0.29		
D8S1179	13	3784	408			5.62	0.78	10.70	0.36
D21S11	30	2009	387			3.15	0.61	13.05	1.19
D18S51	15	1656	200	0.70	0.09	6.90	0.89	11.44	0.79
D18S51	19	1158	298			9.53	1.51	14.12	1.01
D5S818	11	2904	340			7.56	1.16	9.65	0.48
D13S317	11	3906	769			3.32	0.33	6.78	0.71
D7S820	10	1233	223	0.93	0.14	7.23	1.41	7.37	0.69
D7S820	11	1150	195			10.16	0.29		
(b) *SpeedStar tube reactions*									
D3S1358	14	727	114	0.81	0.10	9.74	0.92	8.14	0.60
D3S1358	15	895	103			10.50	1.05		
vWA	17	1347	178	0.65	0.16	6.62	0.68	11.90	0.64
vWA	18	876	184			6.78	0.81		
FGA	23	877	179	0.96	0.15	2.54	0.41	8.32	0.83
FGA	24	840	186			2.30	0.38		
Amelogenin	X	1325	204			2.02	0.47		
D8S1179	13	1012	149			5.47	0.43	11.56	0.47
D21S11	30	788	164			3.45	0.52	13.29	0.71
D18S51	15	924	134	0.72	0.18	5.48	0.46	9.09	1.19
D18S51	19	664	128			8.92	0.91	13.90	0.78
D5S818	11	1608	186			3.97	0.64	8.73	0.26
D13S317	11	1867	243			3.21	0.35	7.74	0.29
D7S820	10	647	203	0.73	0.17	8.01	0.71	5.96	1.10
D7S820	11	470	136			10.00	0.93		
(c) *Standard TaqGold reactions*									
D3S1358	14	832	167	0.92	0.04	1.88	0.52	5.31	0.23
D3S1358	15	803	142			1.54	0.34		
vWA	17	766	84	0.90	0.08	4.22	0.44	7.38	0.48
vWA	18	737	88			4.23	0.19		
FGA	23	735	130	0.93	0.05	2.14	0.45	7.19	0.43
FGA	24	727	123			1.98	0.44		
Amelogenin	X	1656	344			3.35	0.49		
D8S1179	13	1406	105			4.52	0.58	6.51	0.62
D21S11	30	1280	81			3.00	0.66	7.33	0.60
D18S51	15	879	138	0.88	0.05	4.68	0.74	7.89	0.80
D18S51	19	814	126			5.95	0.68	11.16	0.52
D5S818	11	1599	224			3.71	0.17	4.94	0.44
D13S317	11	1486	196			2.33	0.28	4.10	0.47
D7S820	10	635	114	0.89	0.08	4.69	0.99	4.57	0.62
D7S820	11	561	92			7.67	0.72		

PHR, peak height ratio; NTA, nontemplate nucleotide addition.

Discussion

Fast Thermal Cycling Instrumentation Requirements and the Microfluidic Biochip

To achieve fast multiplexed PCR amplification suitable for forensic interpretation, development of thermal cycling instrumentation and reaction vessels, and selection and optimization of amplification enzyme and reaction conditions were performed in parallel. Most commercially available thermal cycling instruments are limited in that they receive temperature feedback directly from the block and control the block temperature as opposed to the PCR solution temperature. As a consequence, the temperature profile of the solution, which is critical to the success of the PCR, is likely to be grossly different from the desired profile. For conventional PCR amplification protocols, the difference between the time the block and the solution reach the desired temperature is not critical; the long incubation times utilized at each step allow the solution temperatures to reach the block temperatures. Under fast cycling condition in commercial thermal cyclers, however, the solution either does not achieve the desired temperature or does not experience the desired temperature for sufficient time.

To achieve fast thermal cycling, a custom thermal cycler with the capability of heating and cooling the PCR solution rapidly, controllably, and reproducibly was designed and built. The ability to rapidly heat and cool the reaction solution temperatures allows ramping and settling times to be minimized and incubation time at the desired temperature to dominate the total step time. These properties are critical for minimizing multiplex cycling times without compromising STR profiles. High rates of heating and cooling are achieved by utilizing a high heating and cooling capacity heat pump and maximizing the thermal transfer between the heatpump and the PCR solution. Heat transfer between heatpump and solution

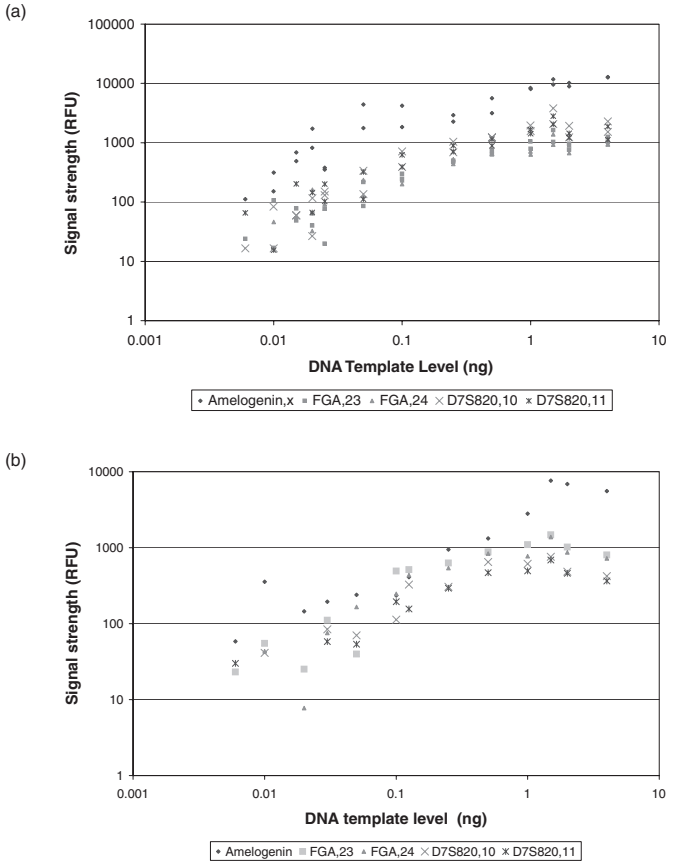

FIG. 5—*Effect of DNA template level on signal strength in (a): SpeedSTAR biochip reactions and (b): SpeedSTAR tube reactions.*

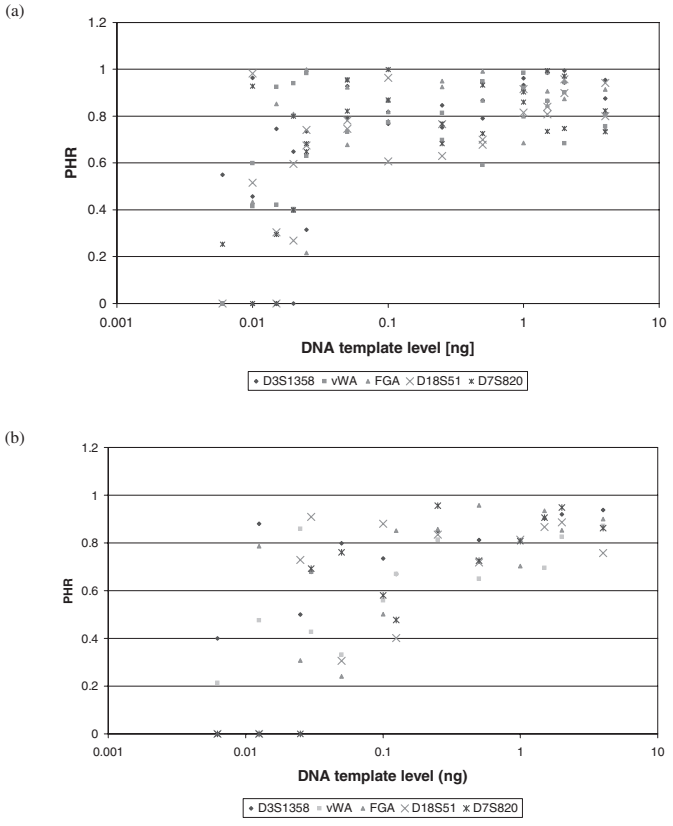

FIG. 6—*Effect of DNA template level on PHR in (a): SpeedSTAR biochip reactions and (b): SpeedSTAR tube reactions.*

is enhanced compared to block thermal cyclers by placing the reaction vessel directly on the heatpump.

The microfluidic biochip design leverages the benefits of microfluidics including having a high surface to volume ratio and reduced diffusion times to maximize heat transfer, and uniform heating and cooling. The use of microfluidic technology also provides benefits with respect to a fully integrated forensic DNA analysis instrument. Schematically, one end of the biochip will receive PCR reaction solutions following DNA extraction and quantification and the other end will pass amplified DNA to Genebench-FX™ for fragment separation and generation of STR profiles. The use of plastic biochips is critical in this regard as the relatively low cost of plastic manufacture allows the biochips to be disposable, eliminating the labor required to reuse the biochip and reducing the possibility of contamination. A single-use disposable would be particularly advantageous for low copy number analyses to avoid any risk of contamination other than initial sample collection.

Polymerase Selection and Fast PCR Protocol Optimization

It is emphasized that many commercially available polymerases can be adapted for use in fast PCR applications using the approaches described here; no single enzyme possesses all the ideal properties that may be of interest to the forensic scientist. The criteria for optimization of the protocols include the generation of full profiles, signal strength, dynamic range, inter-locus signal strength balance, PHR, incomplete NTA, stutter, and total cycle time. The annealing temperature and time influence the specificity and efficiency of primer binding to the template DNA and are particularly important for multiplexed PCR reactions. A complete set of primer pairs must bind correctly during the annealing step in order to achieve full STR profiles with acceptable PHRs and inter-locus signal strength balance. For a given enzyme, we suggest evaluation of annealing temperatures from 57°C to 62°C and times from 5 to 30 sec. Extension temperature and time primarily impact the allele product yield and are an inherent property of the enzyme under study. It should be noted that the extension rates reported by the manufacturer are often provided for singleplex reactions; extension rates for multiplex reactions can be much slower. Final extension times can be reduced significantly until incomplete NTA begins to increase. With the optimized protocols using the SpeedSTAR enzyme, total cycling times for biochip and tube are 17.3 and 19.1 min, respectively. We believe that these are the fastest cycling times generating full STR profiles reported to date.

The fast PCR conditions presented here allow the generation of full STR profiles with high efficiency. The microfluidic biochip reactions using 0.5 ng template DNA generate signal strengths that are approximately two times higher than those for standard TaqGold reactions using 1 ng template. This result suggests that the SpeedSTAR enzyme in the biochip and the TaqGold enzyme in the conventional reaction act with similar efficiencies; the DNA concentration is approximately 1.8-fold higher in the biochip as compared to the tube, which corresponds to the twofold greater signal strength. In contrast, the fast tube-based reactions are less efficient than the control TaqGold reactions; the approximately 40% reduction in product yield is likely the consequence of the poor cycling profile that results when commercial thermal cyclers are used for fast thermal cycling. Even in this circumstance, signal strengths are well over the levels required for interpretation and can be raised significantly by increasing the extension time by a

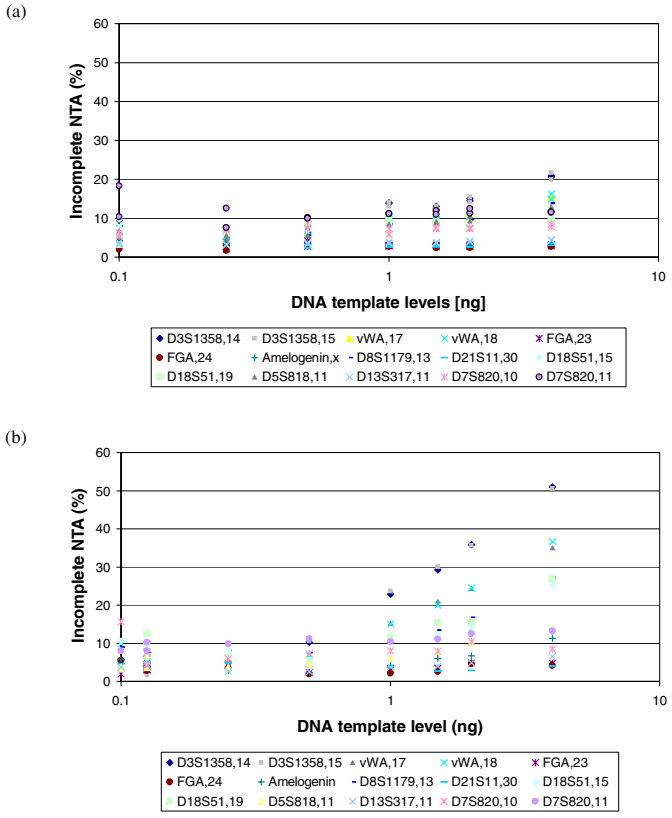

FIG. 7—*Effect of DNA template level on incomplete NTA in (a): SpeedSTAR biochip reactions and (b): SpeedSTAR tube reactions.*

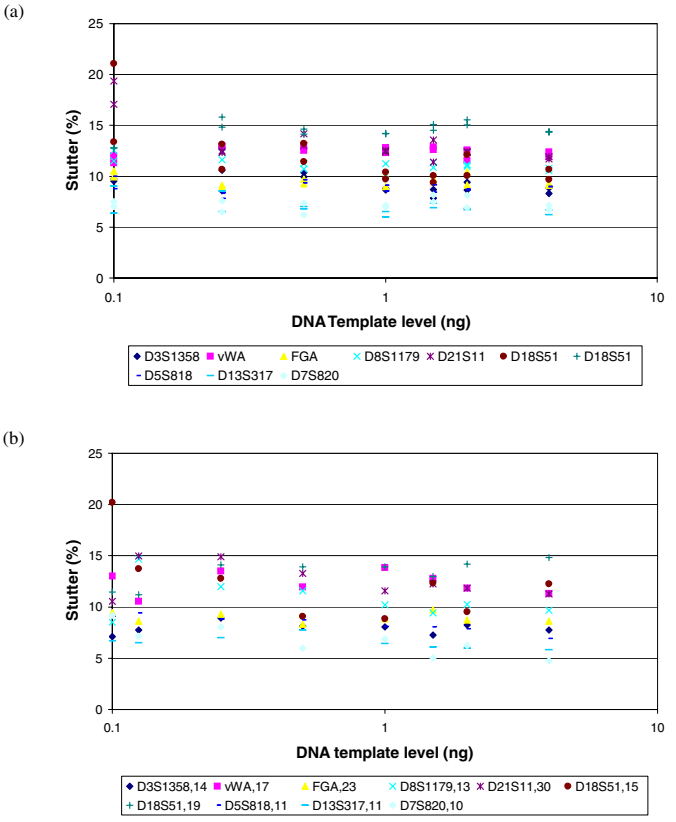

FIG. 8—*Effect of DNA template level on stutter in (a): SpeedSTAR biochip reactions and (b): SpeedSTAR tube reactions.*

few seconds per cycle (data not shown). Repeatability and reproducibility of the signal strength for fast PCR reactions in biochips and tubes are similar to those in conventional reactions.

The inter-locus allele signal strength for the fast biochip and tube reactions shows a higher level of imbalance as compared to the TaqGold reaction. The inter-loci signal strength balance is influenced by numerous factors including primer concentration, annealing temperature and time, and molecular weight of the loci. The STR amplification kit used for these experiments has a set of primer concentrations that are optimized for the TaqGold enzyme and the recommended cycling protocols. The signal strengths of the loci can be modified by adjusting the primer concentrations utilized in the amplification reactions (29).

The relationship between signal strength and template level for fast biochip and tube reactions is as expected with signal strength generally increasing with template. Good peak morphology is observed for all alleles at high template levels of 4 ng (which generate alleles with signal strengths of greater than 12,000 RFU). At template levels of 0.03 ng and below some allele drop-outs occurs. This effect is observed when amplification reactions are carried out with limited number of template DNA strands in the solution leading to stochastic amplification (30). The presence of 11 and 7 alleles for biochip and tube reactions respectively with a detectable signal, that is greater than 3 SD above the baseline level, at a template level of 0.006 ng or near single copy demonstrates the high sensitivity of the fast biochip and tube reaction coupled with Genebench-FX™ Series 100 separation and detection and the potential utility of this system for low copy number analysis. The absolute quantification of 0.006 ng DNA template is subject to uncertainties in DNA quantification based on UV spectrometry and gel analysis and serial dilution to this template level. Nevertheless, it is clear that successful amplification of near single copy template is routinely observed. Taken together, this data also suggests that the fast PCR approach and the thermal cycler and Genebench instrumentation have a wide template dynamic range.

Fast Multiplexed PCR and Forensic Interpretation Guidelines

The speed of an STR amplification reaction is only relevant if the reaction itself generates actionable data that meets forensic interpretation guidelines. The FBI has general guidelines that are used for STR interpretation (14), and individual laboratories set thresholds that must be met before a profile can be considered acceptable based on their validation work (31,32). The conditions presented here represent an initial series of experiments designed to generate fast STR profiles that will meet these guidelines; further optimization of conditions in biochips will be conducted. Nevertheless, the data obtained to date is promising. PHRs for 0.5 ng template in biochip and tube reactions meet with the interpretation guidelines that state a level of 0.6 or greater is required and are consistent with previously reported results (32,33). For higher DNA template amounts, PHRs remain relatively constant but are lower than those for the 1 ng TaqGold reactions. PHRs can be improved by further optimization of the reaction conditions, specifically, the denature temperature and concentration of polymerase in the reaction (30). For low copy numbers, the PHR is dominated by amplification due to stochastic fluctuations.

The level of incomplete NTA is based on the ability of the polymerase to fully adenylate all STR amplicons. For conventional amplification, this is accomplished by attaching a "pigtail"

TABLE 2—*(a) Reproducibility of SpeedSTAR biochip reactions. (b) Reproducibility of SpeedSTAR tube reactions.*

	Signal Strength (RFU)				PHR				Incomplete NTA (%)				Stutter (%)			
Allele	Average	SD	Max	Min	Average	SD	Max	Min	Average	SD	Max	Min	Average	SD	Max	Min
(a)																
D3S1358,14	1235	271	1964	782	0.85	0.11	1.00	0.63	9.11	1.77	11.86	6.64	8.78	0.63	9.84	7.67
D3S1358,15	1067	252	1794	689					8.97	1.80	12.08	6.53				
vWA,17	2765	551	3818	1732	0.81	0.12	0.98	0.56	6.19	1.27	8.25	4.76	12.95	0.66	14.35	11.78
vWA,18	2274	398	3198	1379					6.22	1.34	8.29	4.71				
FGA,23	885	154	1277	621	0.84	0.11	0.99	0.61	2.51	0.58	3.51	1.03	10.10	0.95	12.18	8.63
FGA,24	795	176	1109	376					2.68	0.48	3.52	1.71				
Amelogenin	5085	1139	7276	3272					3.70	0.40	4.54	3.01				
D8S1179,13	3787	779	5700	2420					7.35	0.74	8.82	6.17	10.96	0.53	12.08	10.01
D21S11,30	1914	424	2726	1005					2.91	0.61	4.14	2.04	12.74	0.85	14.44	10.73
D18S51,15	2249	430	3056	1475	0.83	0.10	0.99	0.59	5.80	2.08	7.99	0.00	10.61	0.64	11.54	9.04
D18S51,19	1844	358	2550	1271					5.90	2.23	8.19	0.00	14.52	0.85	16.64	12.96
D5S818,11	2735	501	3847	1630					9.04	1.88	12.07	6.34	8.90	0.33	9.89	8.35
D13S317,11	4098	701	5280	2724					3.02	0.85	6.39	2.25	6.87	0.42	7.95	6.09
D7S820,10	1877	385	3263	1398	0.84	0.09	1.00	0.69	6.68	0.60	7.67	5.67	7.26	0.47	7.99	6.42
D7S820,11	1594	310	2336	1124					10.60	0.68	11.81	9.19				
CV Min: 17%			CV Min: 11%				CV Min: 6%				CV Min: 4%					
CV Max: 24%			CV Max: 14%				CV Max: 28%				CV Max: 9%					
(b)																
D3S1358,14	1719	465	2322	1250	0.86	0.08	0.94	0.74	19.51	0.44	20.04	18.84	8.34	0.49	8.88	7.65
D3S1358,15	1455	285	1719	1111					19.68	0.78	20.71	18.56				
vWA,17	1934	299	2150	1483	0.83	0.11	0.98	0.71	12.12	0.22	12.32	11.75	12.72	0.50	13.37	12.18
vWA,18	1722	451	2439	1258					12.04	0.27	12.29	11.65				
FGA,23	1625	289	2020	1276	0.91	0.08	0.99	0.80	3.57	0.21	3.84	3.28	9.57	0.53	10.22	8.90
FGA,24	1561	347	2004	1185					3.07	0.21	3.30	2.75				
Amelogenin	3670	845	4967	2799					5.02	0.27	5.41	4.68				
D8S1179,13	2447	659	3268	1820					8.20	0.40	8.61	7.58	10.47	0.45	10.86	9.72
D21S11,30	1629	436	2147	1222					4.85	0.33	5.26	4.46	11.78	0.28	12.14	11.54
D18S51,15	1603	545	2262	1067	0.79	0.17	0.95	0.61	8.30	0.34	8.67	7.91	9.67	0.31	10.09	9.30
D18S51,19	1296	435	2043	963					8.79	0.99	10.49	7.91	13.56	0.88	14.69	12.28
D5S818,11	3412	895	4629	2507					6.27	0.17	6.47	6.02	8.38	0.31	8.86	8.03
D13S317,11	3093	749	3987	2204					3.94	0.14	4.16	3.81	6.49	0.25	6.86	6.25
D7S820,10	1009	229	1298	781	0.93	0.03	0.98	0.90	7.86	0.65	8.55	6.86	6.52	0.34	7.02	6.15
D7S820,11	946	209	1228	705					12.13	1.53	14.38	10.56				
CV Min: 15%			CV Min: 3%				CV Min: 2%				CV Min: 2%					
CV Max: 34%			CV Max: 21%				CV Max: 13%				CV Max: 6%					

PHR, peak height ratio; NTA, nontemplate nucleotide addition.

to the primer and increasing the final extension time. The level of incomplete NTA for 0.5 ng biochip and tube reactions are within interpretation guidelines. Pigtail primer sequences have been characterized empirically (34) for Taq and can be similarly optimized for SpeedSTAR. Levels of incomplete NTA increase with increasing DNA template (a consequence of the increasing ratio of DNA to polymerase) and can be reduced by increasing the amount of polymerase, extension time per cycle, and final extension time. The later two approaches are not well suited to fast multiplexed amplification, as they increase the reaction time. Increasing polymerase concentration is effective and compatible with fast PCR. Stutter is a result of DNA strand slippage during extension (35) and affects the ability to resolve samples consisting of mixtures. The level of stutter for the alleles of the 9947A DNA generated in 0.5 ng biochip and tube reactions fall within the interpretational guidelines and are also consistent with previous reports (32,33). Previous reports (33) also show that the level of stutter for alleles within a locus vary with fragment size and confirmation of this initial finding over all alleles within each locus remains to be performed. As expected, the level of stutter for each of the alleles appears to be independent of the DNA template level. The results observed and initial conclusions drawn from these fast multiplex PCR experiments were performed with 9947A DNA and are being expanded to include a variety of forensic samples to further evaluate fast multiplex PCR performance.

Conclusions

The protocols and instrumentation presented here enable significant reductions in the time required to generate full profiles that satisfy interpretation guidelines for STR analysis. Fast amplification times as short as 17 min have been achieved, well under the conventional TaqGold reaction time of 145.1 min. Critically, profile quality has not been sacrificed for speed. It is reasonable to expect that intensive optimization will ultimately lead to amplification times of only a few minutes. The described optimization approach can be applied to the optimization of STR amplification in tubes using essentially any enzyme or instrumentation; the time savings in tube reactions should contribute to reducing the time and cost required to generate STR profiles. The fast PCR method is effective over a wide dynamic range and is compatible with commercially available kits. Fast multiplex amplification will also have application to other fields, including clinical diagnostics.

A major goal in embarking on these studies was to develop a fast cycling instrument and accompanying biochip that would be major components of a fully integrated STR analysis instrument. The fully integrated instrument will extract, purify, and quantify DNA from forensic samples, perform fast PCR, and separate and detect STR fragments. The two major advantages of the microfluidic approach to integration are the reaction efficiencies and the ability to perform disparate reactions sequentially on a single biochip. Although significant optimization of the fast PCR module will

be required, it is well suited for incorporation into the fully integrated forensic DNA analysis system.

Acknowledgments

The authors would like to thank Dr. John Butler of the National Institute of Standards and Technology, and Dr. Martin R. Buoncristiani of the California Department of Justice for critical reading of the manuscript, Dr. John Wright for instrument development, and Mark McSheehy and Jacques Altenord for Genebench-FX™ Series 100 operation.

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