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Short communication

# Demonstration of rapid multiplex PCR amplification involving 16 genetic loci☆

Peter M. Vallone *, Carolyn R. Hill, John M. Butler

*National Institute of Standards and Technology, Biochemical Science Division, 100 Bureau Drive, Mail Stop 8311, Gaithersburg, MD 20899-8311, United States*

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ABSTRACT

Current forensic DNA typing is conducted in approximately 8–10 h. Steps include DNA extraction, quantification, polymerase chain reaction (PCR) amplification of multiple short tandem repeat (STR) loci, capillary electrophoresis separation with fluorescence detection, data analysis and DNA profile interpretation. The PCR amplification portion of the workflow typically takes approximately 3 h with standard thermal cycling protocols. Here we demonstrate a rapid cycling protocol that amplifies 15 STR loci and the sex-typing marker amelogenin from the Identifiler STR typing kit in less than 36 min. This rapid protocol employs commercially available polymerases and the widely used GeneAmp 9700 thermal cycler. Complete concordance of STR allele calls (for 60 samples) between the rapid and standard thermal cycling protocols were observed although there was incomplete adenylation at several of the loci examined and some PCR artifacts were detected. Using less than 750 pg of template DNA and 28 cycles, STR peaks for all loci were above a 150 relative fluorescent unit (RFU) detection threshold with fully adequate inter-locus balance and heterozygote peak height ratios of greater than 0.84.

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## 1. Introduction

The forensic DNA typing community has settled on a core set of short tandem repeat (STR) markers that are widely used to generate DNA profiles used in database and casework applications [1,2]. Multiplex amplification using the polymerase chain reaction (PCR) copies these STR regions to detectable levels and labels the PCR products with different colored fluorescent dyes. This multiplex PCR is commonly performed with 16plex STR typing kits, such as Identifiler (Applied Biosystems, Foster City, CA) and PowerPlex 16 (Promega Corporation, Madison, WI), that simultaneously amplify 15 STRs and the amelogenin sex-typing marker [3,4].

Standard thermal cycling for these STR kits typically takes 2.5–3 h, which contributes to a significant portion of the approximately 8–10 h required to generate a DNA profile. Increasing the speed for multiplex PCR amplification has the potential to not only improve the throughput of a DNA typing laboratory (commercial, academic or governmental), but would also help speed up the overall DNA typing process and thus open new potential biometric applications, such as analysis of individuals at a point of interest like an airport or a country border.

While a great deal of effort has been invested over the past decade in developing portable, miniature and integrated DNA typing devices [5–7], very little focus appears to have been spent on rapid PCR protocols in terms of getting multiple STR loci amplified in a robust manner [8,9]. Most of the rapid PCR work to-date is for single marker targets [5,6] that do not have to worry about locus-to-locus and heterozygote intra-locus imbalance or incomplete adenylation that can impact STR data interpretation [1].

The performance and potential of rapid multiplex PCR amplification using the 16plex STR typing kit Identifiler is reported. By maximizing the speed of the widely used GeneAmp 9700 thermal cycler (Applied Biosystems) and evaluating two rapid PCR enzymes, a simple protocol for rapid amplification with a commonly used STR typing kit is demonstrated.

## 2. Materials and methods

### 2.1. DNA samples and STR typing kits

Sixty samples from a U.S. population set [10] were used for testing rapid cycling protocols. The samples were previously genotyped [11] using the Identifiler (ID) STR typing kit with the standard manufacturer-recommended thermal cycling parameters [3,4]. The primer mix from the ID kit was used without any further alteration as described in the PCR conditions.

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* Corresponding author. Tel.: +1 301 975 4872; fax: +1 301 975 8505.
*E-mail address:* petev@nist.gov (P.M. Vallone).

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### 2.2. Rapid polymerases

The two polymerases utilized in this study were PyroStart (Fermentas, Glen Burnie, MD) and SpeedSTAR (Takara Bio USA, Madison, WI). PyroStart is shipped as a 2× master mix that included the PCR buffer and dNTPs and was used as indicated in the product literature. The SpeedSTAR enzyme is shipped separately from buffer and reagent components. The SpeedSTAR enzyme (approximately 1 unit) was added to the PyroStart mastermix to increase PCR efficiency and to improve adenylation. The reported rates of nucleotide incorporation for PyroStart and SpeedSTAR based on product literature are ≈40 and ≈100 nucleotides/s, respectively.

### 2.3. Rapid thermal cycling

PCR was carried out in 10 μL reaction volumes. A typical reaction consisted of 5 μL PyroStart 2× master mix, 0.25 μL SpeedSTAR enzyme (5 units/μL), 2 μL of the ID STR primer mix, 1.25 μL water and 1.5 μL of template DNA (0.5 ng/μL).

All thermal cycling experiments were performed on a Gene Amp 9700 (Applied Biosystems) using a maximum ramp rate of 4 °C/s. Amplification conditions consisted of 95 °C for 1 min followed by 28 cycles of 95 °C 5 s, 58 °C 10 s, 72 °C 10 s, followed by a 1 min incubation at 72 °C to aid adenylation, and then 25 °C until removed from the thermal cycler.

### 2.4. Data collection and analysis

Following PCR, 1 μL of the amplified products was diluted into 8.7 μL of Hi–Di formamide (Applied Biosystems) and 0.3 μL of LIZ GS500 internal size standard (Applied Biosystems). Samples were electrokinetically injected at 3 kV for 10 s and separated on a 3130xl Genetic Analyzer (Applied Biosystems) using POP-6 polymer (Applied Biosystems) on a 36 cm capillary array (Applied Biosystems). After data collection, genotyping was performed in GeneMapper*ID* v3.2 (Applied Biosystems) using manufacturer provided bins and panels.

### 2.5. Sensitivity study

One nanogram of a pristine DNA template was serially diluted down to 50 pg (1000, 750, 500, 400, 300, 200, 100, 50 pg). The DNA template was amplified in duplicate as described above. A volume of 1 μL of the DNA template and 1.75 μL of water were used in the rapid amplification protocol. Allele calls were performed using a threshold of 50 RFU to evaluate the sensitivity limitations of the protocol.

## 3. Results and discussion

### 3.1. Rapid thermal cycling protocol and time savings

Rapid cycling parameters such as ramp rate and dwell times were altered to reduce the time required for completion of 28 cycles. A general comparison is illustrated in Table 1 contrasting the thermal cycling times for the rapid cycling protocol versus a hypothetical standard cycling protocol. A direct comparison indicates that the majority of the time is saved in the final soak step (41.2%) and in the combined time saved resulting from the reduced cycling times (≈50%). Time is also saved by using a polymerase other than AmpliTaq Gold that does not have a 10 min activation time. When compared on the same time scale, the 28 rapid cycles are completed before the end of the fourth cycle under standard thermal cycling conditions.

**Table 1**
Comparison of thermal cycling times.

| Parameter | Standard | Rapid | Difference (min) | % difference |
|---|---|---|---|---|
| Hot start (min) | 10 | 1 | 9 | 6.3 |
| Denature (s) | 60 | 5 | 25.7 | 17.9 |
| Anneal (s) | 60 | 10 | 23.3 | 16.3 |
| Elongate (s) | 60 | 10 | 23.3 | 16.3 |
| Soak (min) | 60 | 1 | 59.0 | 41.2 |
| Ramp rate (°/s) | 1 | 4 | 22.4 | 15.7 |
| Cycles | 28 | 28 | | |
| Time | 2:58:41 | 0:35:38 | 2:23:03 | |

### 3.2. Polymerases

Initial rapid multiplex PCR experiments performed poorly without the additional SpeedSTAR enzyme. This was evidenced by locus drop out, poor locus-to-locus balance and low heterozygote peak height ratios (data not shown). The improvement from the additional enzyme was drastic (full profiles, higher peak height ratios, improved adenylation). Few adjustments were made to the thermal cycling protocol indicating that the optimal fast enzyme cocktail/combination was essential for rapid multiplex PCR success. Previous attempts at rapid multiplex PCR using the relatively slower activating hot start AmpliTaq Gold [12] commonly used in the forensic community may have been a limiting factor in developing a successful rapid amplification protocol.

### 3.3. Rapid STR typing results

Complete 16 locus amplification profiles were successfully obtained in less than 36 min using primers from the ID STR typing kit. A visual inspection of the rapid amplification of the ID kit suggests that there is adequate balance between all 16 loci (Fig. 1). However, there were some consistent low intensity PCR artifacts observed in the VIC dye channel at ≈107, ≈168, ≈287 bp and incomplete adenylation was observed for several loci including D8S1179, D7S820, D3S1358, TH01, vWA, TPOX, and D5S818. D2S1338 and CSF1PO were also poorly adenylated, but the −A and +A peaks were not fully resolved (see Fig. 1). The level of incomplete adenylation was consistent for all the rapid ID PCR experiments. It should be noted that other minor non-specific PCR artifacts were observed, but under the described rapid amplification protocol these did not interfere with accurate genotyping. The D19S433 and D21S11 loci exhibited lower signal intensity on average across the samples examined for this study using 750 pg input DNA (≈850 and 1720 RFUs, respectively). This lower D19S433 and D21S11 signal was consistently observed in all 60 samples examined. The average peak height ratio (PHR) was determined from the heterozygous samples at each locus. On average, PHR ranged from 0.84 (vWA) to 0.92 (D8S1179). Our observed inter-locus performance was similar to what is to be expected from a standard protocol with greater than 0.5 ng of input DNA (PHR > 0.80) [3,4].

### 3.4. PCR amplicon size

By using an annealing temperature of 58 °C in our rapid cycling protocol it was evident that the PCR primers were binding to their specific template targets and the elongation time was sufficient (10 s) for efficient production of a full length amplicon of greater than 300 bp. The larger molecular weight loci, CSF1PO, D2S1338 and D18S51, all produced strong signal intensities.

At the concentration of input DNA investigated (750 pg), a strong correlation between amplicon size and PCR efficiency is not

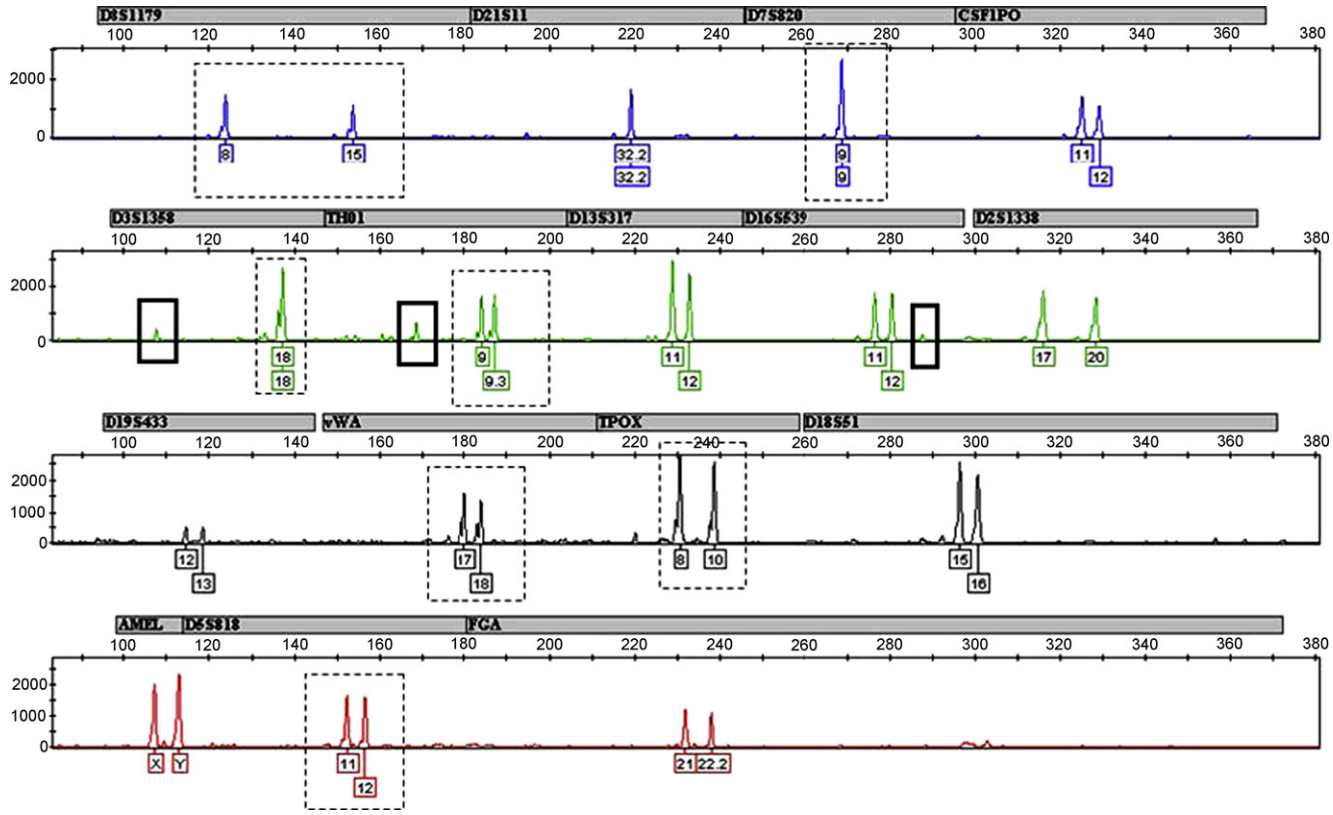


**Fig. 1.** An Identifiler result utilizing the rapid PCR protocol demonstrating that all 16 loci are amplified. Several amplification artifacts that were consistently observed are outlined with solid boxes. Dotted boxes indicate loci with incomplete adenylation.

evident. This can be further illustrated by the relatively low signal of D19S433 (amplicon size < 140 bp). A 'ski-slope' pattern with a lower signal for larger amplicons that one might have predicted was not evident for any of the electropherograms suggesting that rapid PCR efficiency is independent of amplicon size. This is in contrast to previous rapid PCR results obtained with a 3 locus multiplex system [12]. Belgrader et al. observed drop out for the largest locus FGA (≈240 bp) when using 4 units of AmpliTaq Gold, 10 ng of template DNA and 30 cycles while attempting to reduce cycling time to less than 1 h [12].

### 3.5. Incomplete adenylation

Various loci exhibited products with incomplete adenylation. This can be an issue for profile interpretation. However, the results are promising considering that the final soak time was reduced from 60 min down to a single minute. Further work in this area could include the selection and characterization of primer sequences that promote more efficient adenylation under rapid PCR conditions similar to what was done previously with conventional thermal cycling [13]. Alternatively, additional loci (autosomal, Y chromosome, mitochondrial) could be specifically selected with properties that exhibit success with rapid cycling conditions such as complete adenylation, good amplification efficiency and peak balance.

### 3.6. Genotyping concordance

The genotyping results from rapid cycling experiments were fully concordant with those from the standard vendor protocol. A total of 960 correct genotype calls were made using the 16 locus multiplex (60 samples × 16 loci = 960). In our limited sample cohort, allele drop out was not observed for any of the loci.

### 3.7. Sensitivity study

An evaluation of the minimal amount of DNA sample that can reliably be amplified with the rapid amplification protocol was performed. A highly characterized sample was amplified in duplicate for 28 cycles. The DNA template concentrations tested were; 1000, 750, 500, 400, 300, 200, 100 and 50 pg. Signal intensity for D21S11 and D19S433 was consistently lower for all the concentration tested. Allele drop out was observed between 200 and 300 pg of DNA template whereas conventional thermal cycling with 28 cycles could routinely produce results with DNA template in the 100–200 pg concentration range (data not shown). This lower efficiency may be a result of the extremely short hold times (5 and 10 s) employed in the rapid amplification protocol.

## 4. Conclusions

The initial work presented here indicates the great potential of reduced thermal cycling times for large STR multiplexes using enzymes other than the standard AmpliTaq Gold DNA polymerase provided with the commercial STR kits. It should be stated that the ID primer mix performed surprisingly well under PCR conditions quite different than prescribed by the manufacturer. Results obtained from a commercial multiplex provide insight on the needs to be addressed for further rapid PCR protocol optimization. The fact that an unaltered commercial multiplex primer mix can be used for successful amplification of 16 STR loci is very promising for the developers of screening and integrated portable devices. Portable or integrated devices often use smaller PCR volumes and a

custom thermal cycling platform. Results presented here provide the potential for optimizing a larger STR PCR multiplex on a microfluidic platform. Following complete validation studies, rapid PCR may also be useful for typing reference samples in forensic and paternity testing labs.

The goal of this work is to provide a starting point for investigating and further optimizing rapid PCR protocols. This initial work shows that a large STR multiplex can be run in less than 36 min using commercially available primer sets and enzymes and a thermal cycler that is used in most forensic laboratories. Even if some artifacts arise during rapid PCR protocols, such as incomplete adenylation or a few non-specific products, the STR profile information can still be valuable for general screening and informational purposes. In simple screening situations there should be sufficient quantities of single-source high-quality DNA available.

Until recently, success with amplifying large (>4 loci) multiplexes in less than 1 h has been limited [9,12]. The STR performance parameters of locus-to-locus balance, locus or allelic drop out, incomplete adenylation, low peak height ratio and general robustness must be evaluated when developing a rapid PCR protocol. Some of these problems were addressed with additional enzyme or altering cycling times. But others are specific to a locus, primer pair sequence or primer pair concentration present in a commercial primer mix. From the results presented with a commercial kit there is now a basis for understanding the limitations of using a 'fixed' (in terms of primer sequence and concentration) primer mix. It also allows us to direct future focus on specific aspects of primer design to resolve these issues when developing new rapid multiplex PCR assays.

The PyroStart/SpeedSTAR polymerase combination will add slightly to the enzyme cost ($1.15) of a rapid cycling protocol per reaction compared to 2 units of AmpliTaq Gold ($0.86). However, the additional cost of a rapid cycling protocol should be balanced against the time savings and potential for increased throughput.

There are a number of additional experiments to perform including evaluating more rapid thermal cycling instrumentation capable of faster temperature ramp rates, investigating the impact of further variations in PCR volumes, further optimization of annealing temperatures, and determining levels of sensitivity. We also plan to examine additional non-kit STR loci [14] where primer concentrations can be modified to adjust locus-to-locus balance and primers ends can be modified to aid adenylation [13]. While our initial results are promising, there is still much to do to further the understanding of performance characteristics with rapid multiplex PCR.

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