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Demonstration of Rapid Multiplex PCR Amplification Involving 16 Genetic Loci

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Abstract

Current forensic DNA typing is conducted in approximately eight to ten hours with steps including DNA extraction, quantitation, polymerase chain reaction (PCR) amplification of multiple short tandem repeat (STR) loci, capillary electrophoresis separation with fluorescence detection and data analysis, and DNA profile interpretation. The PCR amplification portion of the workflow typically takes approximately three hours with standard thermal cycling protocols. Here we demonstrate a rapid cycling protocol that amplifies 15 STR loci and the sex-typing marker amelogenin from either Identifiler or PowerPlex 16 STR typing kits in less than 36 minutes. This rapid protocol employs commercially available polymerases and the widely used GeneAmp 9700 thermal cycler. We observed complete concordance of STR allele calls between the rapid and standard thermal cycling protocols although there was incomplete adenylation at several of the loci examined and some PCR artifacts were detected. Using less than 1 ng of template DNA and 28 cycles, STR peaks for all loci were above a 100 relative fluorescent unit (RFU) detection threshold with fully adequate inter-locus balance and heterozygote peak height ratios of greater than 0.80.

Key Terms: rapid PCR, PCR, STR, DNA typing, multiplex PCR, PowerPlex 16, Identifiler

Introduction

The forensic DNA typing community has settled upon a core set of short tandem repeat (STR) markers that are widely used to generate DNA profiles used in database and casework applications (Butler 2005, Butler 2006). 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 (Krenke et al. 2002, Collins et al. 2004).

Standard thermal cycling for these STR kits typically takes 2.5 to 3 hours, which contributes to a significant portion of the approximately 8 to 10 hours required to generate a DNA profile (REF-Biometrics 2004 talk on STRBase?). Increasing the speed for multiplex PCR amplification has the potential to not only increase the throughput of a DNA typing laboratory (commercial, academic or governmental), but would also help speed 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 (e.g., Belgrader et al. 1998, Easley et al. 2006, Liu et al. 2007), very little focus appears to have been spent on the

rapid PCR aspect in terms of getting multiple STR loci amplified in a robust manner (Roper et al. 2005, Horsman et al. 2007). Most of the rapid PCR work to-date is for single targets (Belgrader et al. 1999, Easley et al. 2006) that do not have to worry about locus-to-locus or heterozygote intra-locus imbalance or incomplete adenylation that can impact STR data interpretation (Butler 2005).

We report here on the performance and possibilities of rapid multiplex PCR amplification using 16plex STR typing kits. By pushing the widely-used GeneAmp 9700 thermal cycler (Applied Biosystems) to its maximum speed and evaluating a number of "off-the-shelf" rapid PCR enzymes, we have developed a simple protocol for rapid amplification of two commonly used STR typing kits.

Materials and Methods

DNA Samples and STR Typing Kits

Fifteen samples from a U.S. population set (Butler et al. 2003) were used for testing rapid cycling protocols. The samples were previously genotyped (Hill et al. 2007) using PowerPlex16 (PP16) and Identifiler (ID) STR typing kits with the standard manufacturer-recommended thermal cycling parameters (Krenke et al. 2002, Collins et al. 2004). The primer mixes from these kits were used without any further alteration as described in the PCR conditions.

Rapid Polymerase

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 2x 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 rate of elongation for PyroStart and SpeedSTAR are ~40 and ~100 nucleotides/sec respectively. Other commercial enzymes AmpliTaq Gold (Applied Biosystems), Qiagen Fast Cycling PCR Kit (Qiagen, Valencia, CA), and GeneAmp Fast PCR Master Mix (Applied Biosystems) were *briefly* surveyed for rapid PCR amplification, but the PyroStart and SpeedSTAR combination performed with the most success for a large multiplex PCR (REF AAFS talk?).

Rapid Thermal Cycling

PCR was carried out in 10μL reaction volumes. A typical reaction consisted of 5μL PyroStart 2x master mix, 0.25 μL SpeedSTAR enzyme (5 units/μL), 2 μL of the commercial 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/sec. Amplification conditions consisted of 95°C for 1 minute followed by 28 cycles of 95°C 5 sec, 58°C 10 sec, 72°C 10 sec, followed by a 1 minute incubation at 72°C to aid adenylation, and then 25°C until removed from the thermal cycler.

Data Collection and Analysis

Following PCR, 1 μL of the amplified products was diluted into 9 μL of Hi-Di formamide (Applied Biosystems) and 0.3 μL of LIZ 500 or 1.0 μL of ILS 600 internal size standard (e.g., LIZ500 for Identifiler or ILS 600 for PowerPlex 16). Samples were electrokinetcally injected at 3kV for 10 sec 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.

Results and Discussion

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. Table 1 compares the thermal cycling parameters for the rapid cycling protocol versus a 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 (~50.5%). Time is also saved by using a polymerase other than TaqGold that does not have a 10 minute activation time. If placed on the same time scale, the 28 rapid cycles are completed before the end of the fourth cycle under standard thermal cycling conditions.

Rapid STR Typing Results

Complete 16 locus amplification profiles were successfully obtained in less than 36 minutes using both multiplex STR typing kits. A visual inspection of the PP16 data suggests that there is reasonable balance between all 16 loci (Figure 1). However, four PCR artifacts are present in the JOE dye channel at (~102, 162, 279, 331 bp) and incomplete adenylation exists with several loci including D7S820, D5S818, vWA and D8S1179. Likewise with the ID kit practical locus-to-locus balance is observed (Figure 2). Only one locus, D19S433 has lower signal intensity on average across the samples examined for this study using 750 ng input DNA. With the rapid ID amplifications, there were some 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, D3S1358, TH01, D2S1338, vWA, TPOX, and D5S818. Table 2 contains estimates for

loci exhibiting significant (>10 %) incomplete adenylation in both multiplexes. The level of incomplete adenylation was consistent over all the rapid ID and PP16 PCR experiments.

Signal Intensities and Peak Height Ratios

Signal intensity for TH01 and vWA in PP16 and D19S433 in ID was the lowest (<1000 RFUs). This was consistently observed in all 15 samples examined. The minimum signal present for a single sample was vWA in PP16 with 142 RFUs, still well above the calling threshold of 100 RFUs. On average, peak height ratios (PHR) ranged from 0.80 to 0.94 for either kit. The inter-locus performance was similar to what is to be expected from a standard protocol (> 0.80).

On average the PowerPlex16 results gave slightly lower average RFU values and peak height ratio than Identifiler (2697 versus 1863 RFUs). However the difference is minimal and will not affect the intended end result of genotyping. The green channel artifact (~279 bp) in D16S539 for PP16 which shows up as an incorrect 9 allele can complicate accurate genotyping. This artifact (and the others that did not fall into allele calling bins) may be overcome with further alteration of the annealing temperature which is not necessarily optimized for the PP16 PCR multiplex.

Primer Melting Temperature (T_m) and PCR Amplicon Size Dependence

The PCR primer sequences for the PP16 multiplex were previously published (Krenke et al. 2002). The average T_m for each primer pair was compared with the average signal

intensity for that specific locus. An initial evaluation indicates that there is no correlation between T_m and average signal intensity for that specific locus (correlation value of -0.2441). Using an annealing temperature of 58°C it was evident that the PCR primers were binding to their specific template targets and the elongation time was sufficient (10 sec) for a full length amplicon to be efficiently produced. (is this still too awkward?)

At the concentration of input DNA investigated (750pg) a strong correlation between amplicon size and PCR efficiency for either of the multiplexes is not evident. This can be further illustrated by the relatively low signal of D19S433 (amplicon size < 140 base pairs) in the Identifiler multiplex. This observation is reinforced by the strong signal intensities of larger amplicons such as Penta E and Penta D (>400 bp). A 'ski-slope' pattern was not evident for any of the electropherograms that would have indicated that rapid PCR efficiency was dependent on amplicon size.

Incomplete adenylation

Various loci from each of the multiplexes exhibited incomplete adenylation products. This can be an issue for profile interpretation. However, considering that the final soak time was reduced from 60 minutes down to a single minute we believe that the results are quite good. Further work in this area would be the selection and characterization of primer sequences that promote more efficient adenylation. Alternatively, additional loci (autosomal, Y chromosome, mitochondrial) could be found/selected with properties that exhibit success with rapid cycling conditions (complete adenylation, good efficiency and peak balance).

Genotyping Concordance

The genotyping results from rapid cycling experiments were fully concordant with those from standard prescribed vendor protocols. A total of 464 correct allele calls (n = 14 for ID, n = 15 for PP16) were made using either multiplex. In our limited sample cohort, allele drop out was not observed for any of the loci. Genotypes for D16S539 present in PP16 (containing the artifact '9' allele) were called by using the peak heights to correctly determine allele calls.

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. The fact that unaltered commercial multiplex primer mixes can be used for successful amplification is very promising for the developers of screening and integrated portable devices. Following complete validation studies, rapid PCR may also be useful for typing reference samples in forensic and paternity testing labs.

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 peak height ratios (data not shown). The improvement from the additional enzyme was drastic (full profiles, high peak height ratios, improved adenylation). Few adjustments were made to the thermal cycling protocol leading us to

believe that the optimal fast enzyme cocktail/combination was essential for rapid multiplex PCR success. Previous attempts at rapid multiplex PCR using the relatively slower hot start TaqGold commonly used in the forensic community may have been a limiting factor to success (REF).

The goal of this work is to provide a starting point for investigating and optimizing rapid PCR protocols. We have shown that a large STR multiplex can be run in less than 36 minutes using commercially available primer sets and enzymes and a thermal cycler that is present in many 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/information 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 hour has been limited (REFs, Belgrader, Horsman,). The issues 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. From the results presented here with commercial kits we have a basis for understanding the limitations of using a 'fixed' (in terms of primer sequence and concentration) primer mix. It also allows us to focus on specific aspects of primer design to resolve these issues when developing new rapid multiplex assays.

There are a number of additional experiments to perform including evaluating more rapid thermal cycling instruments capable of faster temperature ramp rates, investigating the impact of further reduction in PCR volumes, optimizing annealing temperatures, and determining levels of sensitivity. We also plan to examine additional non-kit STR loci (Hill et al. 2008) where primer concentrations can be modified to adjust locus-to-locus balance and primers ends can be modified to aid adenylation (Brownstein et al. 1996). There is still much to do to further the understanding of performance characteristics with rapid multiplex PCR.

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Table 1 Comparison of Thermal Cycling Times

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

Table 2 Estimates of incomplete adenylation (-A)

Identifiler

Locus	% -A
vWA	42.4
D3S1358	39.9
TPOX	27.1
D8S1179	23.5
TH01	22.0
D7S820	15.3
D5S818	13.8

PP16

Locus	% -A
vWA	>90%
D5S818	30.9
D7S820	25.5
D8S1179	17.2

Figure 1. A PowerPlex 16 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 (see Table 2).

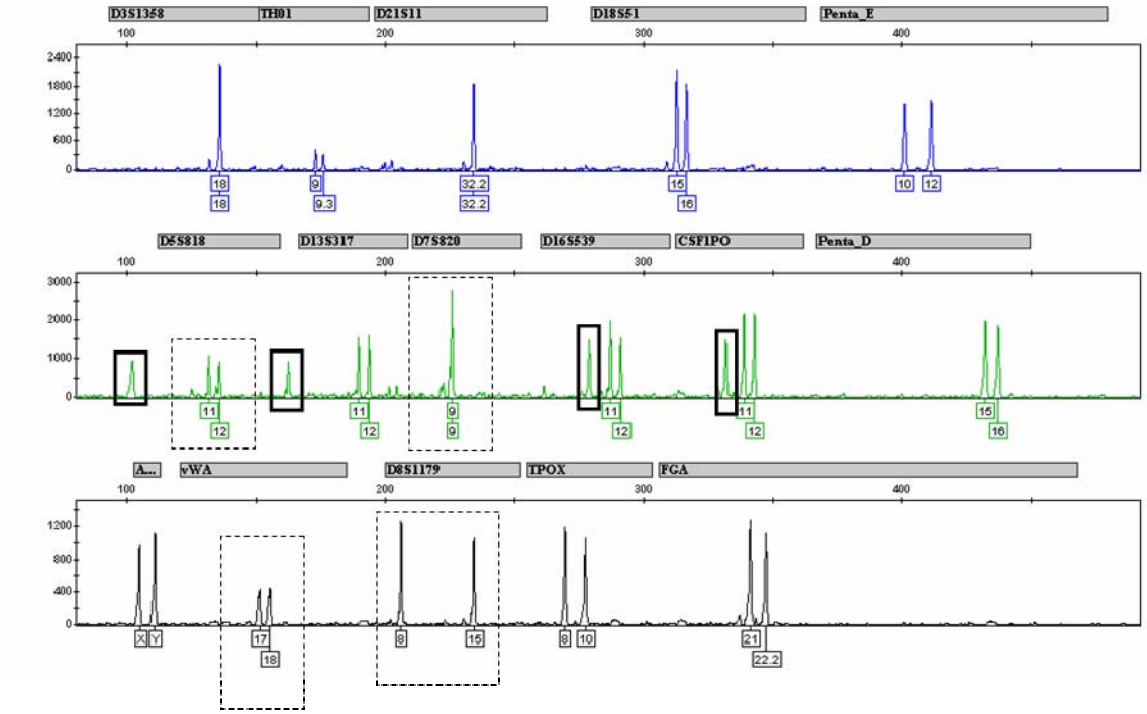

Figure 2. An Identifiler result utilizing the rapid PCR protocol demonstrating that all 16 loci are amplified. Several amplification artifacts that were consistently observed are outlined with solid boxes. Dotted boxes indicate loci with incomplete adenylation (see Table 2).