HSOA Journal of Forensic, Legal & Investigative Sciences

Research Article

Validation of Low Volume, Fast PCR Amplification of STR Loci for DNA Reference Samples

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Abstract

This project was a continuation of a previous project that examined various polymerases to assess their suitability for low volume, fast PCR amplification of forensic STR loci. The primary focus of this project is to develop multiple protocols using the product selected from the previous study (KAPA2G™ Fast Multiplex PCR Kit) along with primer sets and non-fast thermal cyclers commonly used in the forensic community in order to substantially reduce PCR time without added costs or equipment. Ultimately, 3µl fast PCR protocols were validated for the PowerPlex® 16 HS System, AmpFtSTR® Identifiler® and Identifiler® Plus PCR Amplification Kit primer sets on a 384-well Veriti® thermal cycler (43-51min), as well as 5µl and 6µl Identifiler® fast reactions on a GeneAmp® PCR System 9700 thermal cycler (51min). Two-step PCR cycling utilizing a combined annealing/extension step was successfully incorporated into the fast PCR protocols for all primer sets, except PowerPlex® 16 HS. These protocols were validated for use with forensic reference-type samples, including buccal swabs or Buccal DNA Collectors™, based on optimal DNA input range (sensitivity, reproducibility, inter- and intra-locus peak balance, stutter, pull-up, incomplete adenylation (-A), and baseline assessment), stochastic, precision, stutter, automation, contamination, lot-to-lot variation and storage condition studies. Overall, a 56-73% reduction in amplification time was achieved compared to low volume, standard PCR amplification, along with STR profile pass rates of 95-98% (n=86-89) using these low volume, fast PCR protocols, all of which had an optimal DNA input range of

0.375-1.5ng. Higher percent stutter was observed compared to that of standard PCR, but non-specific amplification, incomplete non-template adenylation, and poor intra-locus peak balance were not problematic compared to other previous studies involving fast PCR.

Keywords: 2-step PCR; Fast PCR; Forensic DNA; Low volume amplification

Introduction

Reducing amplification processing time has been a major focus for forensic DNA testing over the past 10 years, especially through the advent of fast PCR polymerases, direct PCR, and rapid DNA testing [1-12]. But such a reduction in processing time has often been subject to an increase in cost due to reagents and/or equipment, and/or prone to a decrease in STR profile quality. The goal of this project was to develop fast PCR protocols for a variety of primer sets commonly used in the forensic community, without having to purchase fast thermal cyclers or incur significant reagent expenses, all the while still maintaining high quality STR profiles. Utilizing low volume reactions on non-fast thermal cyclers are key to keeping costs down [13].

The first part of this project assessed four different commercial-available products for their suitability with low volume, fast PCR, from which KAPA2G™ Fast Multiplex PCR Kit (KAPA2G; Kapa Biosystems Inc., Woburn, MA) demonstrated the greatest potential for success [13]. This paper focuses on the second half of the project, in which KAPA2G was successfully integrated into low volume (3-6µl), fast PCR protocols for three primer sets commonly used in the forensic community - PowerPlex® 16 HS System (PowerPlex 16 HS; Promega, Madison, WI) and AmpFtSTR® Identifiler® and Identifiler® Plus PCR Amplification Kit (Identifiler and Identifiler Plus, respectively; Applied Biosystems, Foster City, CA) using two non-fast thermal cyclers - 384-well Veriti® and GeneAmp® PCR System 9700 thermal cyclers (Applied Biosystems). To ensure that high STR profile quality was maintained, profiles were evaluated using criteria established by the forensic community, including but not limited to sensitivity, reproducibility, allele concordance, allele peak height, inter- and intra-locus peak balance, stutter percentages, pull-up percentages, incomplete adenylation (-A), specificity, etc.

Materials and Methods

This study began with the optimization of 5µl and 6µl fast PCR protocols for the Identifiler primer set on the 9700 thermal cycler, as well as 3µl fast PCR protocols for the Identifiler Plus and PowerPlex 16 HS primer sets on the Veriti® thermal cycler, all using KAPA2G. It should be noted that a 3µl fast PCR protocol for Identifiler with KAPA2G was previously developed by Connon et al., on the Veriti, when KAPA2G was assessed alongside three other fast PCR products [13]. Following optimization, each of these protocols (as well as the 3µl Identifiler protocol) was validated. Optimization and validation studies are discussed in more detail below.

For all studies, buccal swab cuttings (~¼ swab) or Buccal DNA Collector™ punches (6mm) were obtained from a total of 294 individuals and were extracted (quarter reaction with a minimum

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Citation: Connon CC, LeFebvre AK, Benjamin RC (2016) Validation of Low Volume, Fast PCR Amplification of STR Loci for Reference DNA Samples. J Forensic Leg Investig Sci 2: 008.

Received: March 10, 2016; **Accepted:** April 06, 2016; **Published:** April 21, 2016

	Identifiler (3µl)	Identifiler (5µl)	Identifiler (6µl)	IdentifilerPlus (3µl)	PowerPlex 16 HS (3µl)
Fast PCR	1.5µl KAPA2G™ Fast Multiplex Mix	2.5µl KAPA2G™ Fast Multiplex Mix	3.0µl KAPA2G™ Fast Multiplex Mix	1.5µl KAPA2G™ Fast Multiplex Mix	1.5µl KAPA2G™ Fast Multiplex Mix
	0.6µl Primers	1.0µl Primers	1.2µl Primers	0.6µl Primers	0.3µl Primers
	0.9µl DNA Template	1.5µl DNA Template	1.8µl DNA Template	0.9µl DNA Template	0.9µl DNA Template
					0.3µl Water
Standard PCR	1.145µl PCR Reaction Mix	1.909µl PCR Reaction Mix	2.290µl PCR Reaction Mix	1.2µl Identifiler® Plus Master Mix	0.6µl PowerPlex® HS 5X Master Mix
	0.055µl AmpliTaq Gold®	0.091µl AmpliTaq Gold®	0.110µl AmpliTaq Gold®	0.6µl Primers	0.3µl Primers
	0.6µl Primers	1.0µl Primers	1.2µl Primers	1.2µl DNA Template	1.2µl DNA Template
	1.2µl DNA Template	2.0µl DNA Template	2.4µl DNA Template		0.9µl Water

Table 1: Fast PCR Reaction Compositions Compared to Standard PCR.

Reaction composition was based on established 3-6µl amplifications (using standard PCR) at Cellmark Forensics, as well as manufacturer recommendations. Final concentrations of primer sets were unchanged between fast and standard amplification for the same primer set.

of a one hour incubation at 56°C) using the ChargeSwitch® Forensic DNA Purification Kit (ChargeSwitch; Applied Biosystems) [14] on a BioSprint 96 (QIAGEN) or KingFisher® 96 (Thermo Scientific, Vantaa, Finland). Samples were then quantified using the Quant-iT™ PicoGreen® dsDNA Quantitation Kit coupled with the Quant-iT™ PicoGreen® dsDNA Quantitation Reagent (PicoGreen; Applied Biosystems) and a FLUOstar microplate reader (BMG LABTECH, Ortenberg, Germany). As discussed previously by Connon et al., [13], this quantification method is acceptable per FBI QAS guidelines for DNA reference samples [15,16] and was successfully utilized by the databasing unit of Cellmark Forensics from 2008-2015. Following quantification, a pre-amplification dilution was performed in order to normalize samples for amplification. Amplification is discussed in more detail below (Table 1 for reaction composition). Following amplification, amplification product was diluted with water (4µl for 3µl and 6µl reactions, and 5µl for 5µl reactions). One microliter of diluted amplification product was combined with 10µl of a formamide/size standard mixture (10µl formamide and either 0.2µl GeneScan™ 500 LIZ™ [LIZ; Applied Biosystems] for Identifiler/Identifiler Plus or 0.5µl ILS 600 [Promega] for PowerPlex 16 HS) for each sample or allelic ladder. Prepared amplification product was detected using a 3130xl Genetic Analyzer (3130xl; Applied Biosystems) equipped with POP-4® (POP-4; Applied Biosystems) and a 36cm array using a 3kV, 7sec injection. All profiles were analyzed with GeneMapper™ ID v3.2 software using a 75rfu threshold. Specific analysis is discussed below. Any tests for statistical significance (t-test, one-way ANOVA or Tukey HSD [honest significant difference] [17,18]) were performed using a 5% significance level.

Multiple thermal cyclers were used during the duration of this project. Unless otherwise noted, the same thermal cycler was used during development and optimization of each low volume, fast PCR protocol, but the same thermal cycler was not used between protocols. Unless otherwise noted, all PCR product was detected on a single 3130xl.

Optimization of low volume, fast PCR protocols using KAPA2G

The same general criteria with regard to profile quality that were used by Connon et al., during the assessment of various fast PCR products were also desired for these protocols [13]. These included: the generation of full STR profiles free of oversaturation, -A (incomplete non-template adenylation), and non-specific amplification (NSA) products; average peak heights of 750-1500rfu;

average peak height ratios (PHR) of >85%; minimal occurrences of PHR <50% (but preferably no occurrences); and inter-locus balance of ~0.35 or less (as measured via the coefficient of variation of locus peak height to the profile's total sum of peak height ratios). Additionally, percent stutter and percent pull-up had to be below 20% of the true allele; actual number of occurrences of stutter, pull-up and elevated baseline were qualitatively assessed during fast PCR protocol development/optimization, but were quantitatively assessed during protocol comparison. Optimization of each product is discussed below.

5µl and 6µl Identifiler fast PCR protocols: For all studies, one or two samples were amplified using ~0.25ng, 0.50ng, 0.75ng and 1.0ng DNA, each in triplicate (unless otherwise noted), with appropriate controls. Amplification reaction composition consisted of 2.5µl KAPA2G™ Fast Multiplex Mix, 1.0µl Identifiler primer set and 1.5µl DNA template for 5µl reactions (Table 1). Amplification was carried out in a 96-well plate in a 9700, and thermal cycling parameters initially began with the same parameters used for the 3µl fast PCR reaction developed for KAPA2G as presented by Connon et al., (except with a 5min final extension) [13]. Improvements to amplification efficiency were evaluated by using the “Max” versus “9600 Emulation” modes on the thermal cycler, assessing different numbers of amplification cycles (27 and 28, compared to 26 used for the 3µl reaction), assessing longer annealing/extension times (45sec and 50sec, compared to 40sec) and assessing a longer final extension (10min compared to 5min; n=24). See Table 2 for specific amplification parameters tested.

Development of the 6µl Identifiler fast PCR protocol was based upon the 5µl protocol and required changes to reaction composition only, which consisted of 3.0µl KAPA2G™ Fast Multiplex Mix, 1.2µl Identifiler primer set and 1.8µl DNA template.

Lastly, 24 buccal samples (all from different donors) were processed using the 5µl and 6µl Identifiler fast amplification protocols, and fast profiles were compared to those obtained using the Identifiler standard amplification protocols used at Cellmark Forensics (Table 3), including determination of first pass success rate. First pass success rate was defined as the percentage of passing profiles obtained during the first round of testing (i.e., without re-extraction, re-amplification, re-injection, etc.). Passing profiles must have all alleles detected at or above threshold, all PHR ≥50%, no called stutter peaks >20%, no called pull-up peaks >20% and no -A; see Table 4 for a complete list of guidelines.

PCR Step	Amplification Mode	# Cycles	Annealing/ Extension	# Cycles	Final Extension	Final
Thermal Cycler Mode	9600 Emulation, Max	Max	Max	Max	Max	Max
Polymerase Activation	95°C 1min	95°C 1min	95°C 1min	95°C 1min	95°C 1min	95°C 1min
# of Cycles	26	26, 28	26	26, 27	27	26, 27
Denaturation	95°C 5sec	95°C 5sec	95°C 5sec	95°C 5sec	95°C 5sec	95°C 5sec
Annealing/Extension	61°C 40sec	61°C 40sec	61°C 40, 45, 50sec	61°C 40sec	61°C 40sec	61°C 40sec
Final Extension	72°C 5min	72°C 5min	72°C 5min	72°C 5min	72°C 5, 10min	72°C 10min
Hold	25°C	25°C	25°C	25°C	25°C	25°C
Total Time	44, 55min	44, 46min	44, 46, 48min	44, 46min	46, 51min	51min

Table 2: Thermal Cycling Parameters for 5µl Identifiler Fast PCR Development.

The PCR step under evaluation is listed at the top of each column. Each of the thermal cycling parameters was assessed using a 96-well 9700 thermal cycler (gold or silver block). The final thermal cycling parameters that were developed for the 5µl amplification was also used for 6µl Identifiler fast PCR.

	PCR Step	Identifiler (3µl) ^a	Identifiler (5µl) ^b	Identifiler (6µl) ^b	Identifiler Plus (3µl) ^a	PowerPlex 16 HS (3µl) ^a
Fast PCR	Polymerase Activation	95°C 1min	95°C 1min	95°C 1min	95°C 1min	96°C 1min
	# of Cycles:	26	27	27	26	10/18
	Denaturation	95°C 5sec	95°C 5sec	95°C 5sec	95°C 10sec	94/90°C 15sec
	Annealing	61°C 40sec	61°C 40sec	61°C 40sec	63°C 50sec	60°C 15sec
	Extension					70°C 30sec
	Final Extension	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min
	Hold	25°C	25°C	25°C	25°C	25°C
	Total Time	43min	51min	51min	49min	51min
Standard PCR	Polymerase Activation	95°C 11min	95°C 11min	95°C 11min	95°C 11min	96°C 2min
	# of Cycles:	26	27	27	26	10/18
	Denaturation	94°C 1min	94°C 1min	94°C 1min	94°C 20sec	94/90°C 30sec
	Annealing	59°C 1min	59°C 1min	59°C 1min	59°C 3min	60°C 30sec
	Extension	72°C 1min	72°C 1min	72°C 1min		70°C 45sec
	Final Extension	60°C 60min	60°C 60min	60°C 60min	60°C 10min	60°C 30min
	Hold	4°C	4°C	4°C	4°C	4°C
	Total Time	2hr 42min	3hr 0min	3hr 0min	2hr 0min	1hr 55min
Time Saved		1hr 59min	2hr 9min	2hr 9min	1hr 11min	1hr 4min
		-73%	-72%	-72%	-59%	-56%
KAPA2G Cost		\$0.06	\$0.10	\$0.12	\$0.06	\$0.06

Table 3: Fast PCR Thermal Cycling Parameters Compared to Standard PCR.

Thermal cycling parameters for each of the five fast PCR protocols are compared to the standard amplification parameters (established at Cellmark Forensics). Cost of KAPA2G is offset by a savings associated with reduced reaction volumes compared to standard 25µl reactions.

^aPCR performed on a 384-well Veriti thermal cycler; ^bPCR performed on a 9700 thermal cycler

3µl Identifiler Plus fast PCR protocol: For all studies, two samples were amplified using ~0.25ng, 0.50ng and 0.75ng DNA, each in triplicate (unless otherwise noted), along with controls. Amplification reaction composition consisted of 1.5µl KAPA2G™ Fast Multiplex Mix, 0.6µl Identifiler Plus primer set and 0.9µl DNA template (Table 1). Amplification was carried out on a 384-well plate in a Veriti thermal cycler. The initial thermal cycling parameters that were utilized were based upon the protocol that was developed for Identifiler fast PCR using KAPA2G. Improvements to amplification efficiency began with assessing final extension times (1min, 5min and 10min) to prevent the formation of -A peaks and different annealing/extension temperatures (59°C, 61°C and 63°C) to prevent non-specific amplification, followed by efforts to minimize amplification time via reducing activation length (3min, 2min and 1min), denaturation (15sec, 10sec and 5sec) and annealing/extension (60sec and 50sec). Eighty-eight buccal samples (all from different

donors) were processed using two potential 3µl Identifiler Plus fast amplification protocols in order to identify which performed better, and these fast profiles were compared to those obtained using the 3µl Identifiler Plus standard amplification protocol used at Cellmark Forensics (Table 3), including determination of first pass success rate. See Table 5 for specific amplification parameters tested.

3µl PowerPlex 16 HS fast PCR protocol: For all studies, two samples were amplified using ~0.25ng, 0.50ng and 0.75ng DNA, each in triplicate (unless otherwise noted), with appropriate controls. Amplification reaction composition consisted of 1.5µl KAPA2G™ Fast Multiplex Mix, 0.3µl PowerPlex 16 HS primer set and 1.2µl DNA template/water (Table 1). Amplification was carried out in a 384-well plate in a Veriti thermal cycler. The initial thermal cycling parameters that were utilized were based upon the 3µl protocol that was used for PowerPlex 16 HS at Cellmark Forensics and information learned from development of the other fast PCR

Criteria	Pass	Fail
% Alleles Detected ^a	100%, no signs of mixture	<100%
PHR at all heterozygous loci	≥50%	<50% at any locus
Pull-up	≤20%	>20%
Stutter (n-4, n+4)	≤20%	>20%
Stutter (n-8)	≤2 occurrences, ≤5%	>2 occurrences (≤5%) or any occurrence >5%
Trialleles and microvariants ^b	None	Any occurrence
-A	None	Any occurrence
+A	None	Any occurrence
Elevated Baseline	Occurrences at ≤3 loci	Occurrences at >3 loci
Non-specific Amplification	None	Any occurrence
Oversaturation	≤2 oversaturated peaks	>2 oversaturated peaks
Migration	All allele calls correct	Any occurrence of poor migration ^c
Injection Failure	None	Any occurrence
Loss of Resolution ^d	None	Any occurrence
Spikes	≤1 occurrence ^e	>1 occurrence ^f

Table 4: First Pass Analysis Guidelines.

“Occurrence” relates to a detected peak that is called using GeneMapper® ID.

^aAt a 75rfu threshold; ^bMust be reprocessed to confirm; however, given that all samples that were tested either originated from a known source or were also processed using a current procedure for comparison purposes, these would only fail if non-concordant with the known profile or profile obtained from the current process; ^cThat results in OL or miscalled allele(s); ^dPoor ILS or unresolved peaks; ^eOkay if in multiple dye channels but occur at the same base size; ^fAt different base sizes

PCR Step	Final Extension	Primer Annealing	Initial Activation	Denaturation	Annealing/ Extension	Final
Polymerase Activation	95°C 3min	95°C 3min	95°C 1, 2, 3min	95°C 1min	95°C 1min	95°C 1min
26 of Cycles:						
Denaturation	95°C 15sec	95°C 15sec	95°C 15sec	95°C 5, 10, 15sec	95°C 10sec	95°C 10sec
Annealing/Extension	59°C 1min	59, 61, 63°C 1min	63°C 1min	63°C 1min	63°C 50sec, 1min	63°C 50sec
Final Extension	72°C 1, 5, 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min
Hold	25°C	25°C	25°C	25°C	25°C	25°C
Total Time	50, 55, 59min	59, 58, 57min	55, 56, 57min	51, 53, 55min	49, 53min	49min

Table 5: Thermal Cycling Parameters for 3µl Identifier Plus Fast PCR Development.

The PCR step under evaluation is listed at the top of each column. Each of the thermal cycling parameters was assessed using a 384-well Veriti thermal cycler.

protocols using KAPA2G. Improvements to amplification efficiency began with assessing final extension times (1min, 5min and 10min) to prevent the formation of -A peaks, 2-step PCR cycling with different annealing/extension temperatures (58°C, 60°C and 62°C) to prevent non-specific amplification and increasing ramp rates to 100% for annealing and extension steps. These were followed by efforts to minimize amplification time via reducing annealing time (30sec and 15sec), extension (30sec, 20sec and 15sec), denaturation (15sec, 10sec and 5sec), initial activation (2min and 1min) and final hold (4°C and 25°C). Eighty-nine buccal samples (all from different donors) were processed using this 3µl PowerPlex 16 HS fast amplification protocol, and fast profiles were compared to those obtained using the 3µl PowerPlex 16 HS standard amplification protocol used at Cellmark Forensics (Table 3), including determination of first pass success rate. See Table 6 for specific amplification parameters tested.

Validation of low volume, fast PCR protocols using KAPA2G

A validation study of KAPA2G consisted of an assessment its performance utilizing the five fast PCR protocols that were developed for the two non-fast thermal cyclers (Tables 2 and 3 for reaction

composition and thermal cycling parameters), lot-to-lot variation and storage conditions. PCR performance was evaluated in a manner similar to that which had been employed during the initial assessment of KAPA2G [13] and was based on optimal DNA input ranges (determined by sensitivity, reproducibility, inter-locus balance, intra-locus balance, stutter, pull-up, -A, non-specific amplification and baseline noise), stochastic threshold, precision of allele sizing, stutter, automation suitability, and contamination assessment. Each of these analyses is discussed in more detail below. Studies comparing each fast PCR method to its respective standard amplification were performed during the development process for each method (see above), and therefore were not repeated during the validation.

Determination of the optimal range of input DNA: Two known, highly heterozygous male samples were manually serially diluted to create ten dilutions such that the total amount of input DNA (ng) in each amplification ranged from approximately 5.86×10^{-3} ng to 3.00ng; each dilution was amplified in triplicate. As described previously by Connon et al., optimal DNA input ranges were determined for each of the five fast PCR protocols based upon sensitivity, reproducibility, inter- and intra locus peak balance and presence of artifacts called by the analysis software [13].

	3-Step PCR	2-Step PCR	3-Step PCR					
PCR Step	Final Extension	Primer Annealing	Ramp Rates	Annealing	Extension	Denaturation	Initial Activation	Final Hold
Polymerase Activation	96°C 2min	96°C 2min	96°C 2min	96°C 2min	96°C 2min	96°C 2min	96°C 1, 2min	96°C 1min
10 of Cycles:								
Denaturation	94°C 15sec	94°C 15sec	94°C 15sec	94°C 15sec	94°C 15sec	94°C 5, 10, 15sec	94°C 15sec	94°C 15sec
Annealing	60°C 30sec ^a	58, 60, 62°C 1min ^a	60°C 30sec ^{a,b}	60°C 15, 30sec	60°C 15sec	60°C 15sec	60°C 15sec	60°C 15sec
Extension	70°C 30sec ^c		70°C 30sec ^{b,c}	70°C 30sec	70°C 15, 20, 30sec	70°C 30sec	70°C 30sec	70°C 30sec
18 of Cycles:								
Denaturation	90°C 15sec	90°C 15sec	90°C 15sec	90°C 15sec	90°C 15sec	90°C 5, 10, 15sec	90°C 15sec	90°C 15sec
Annealing	60°C 30sec ^a	58, 60, 62°C 1min ^a	60°C 30sec ^{a,b}	60°C 15, 30sec	60°C 15sec	60°C 15sec	60°C 15sec	60°C 15sec
Extension	70°C 30sec ^c		70°C 30sec ^{b,c}	70°C 30sec	70°C 15, 20, 30sec	70°C 30sec	70°C 30sec	70°C 30sec
Final Extension	72°C 1, 5, 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min
Hold	4°C	4°C	4°C	4°C	4°C	4°C	4°C	4, 25°C
Total Time	1hr 12min,	1hr 12min, 1hr 10min, 1hr 8min	1hr 0min,	53min,	45, 48, 53min	48, 50, 53min	52, 53min	52, 51min
	1hr 16min,		1hr 21min	1hr 0min				
	1hr 21min							

Table 6: Thermal Cycling Parameters for 3µl PowerPlex 16 HS Fast PCR Development.

The PCR step under evaluation is listed at the top of each column. Each of the thermal cycling parameters was assessed using a 384-well Veriti thermal cycler. All ramp rates are 100%, unless otherwise noted.

^a29% ramp rate, ^balso tested at 100% ramp rate, ^c23% ramp rate.

Stochastic threshold, precision, stutter, automation and contamination: Stochastic, precision and stutter ($n \pm 4$ and $n-8$) studies were also performed as described previously by Connon et al., [13], with the following two modifications to the precision study. First, nine amplifications of 9947A positive control DNA were assessed for each of the primer sets using the 3µl fast PCR protocols (additional studies for 5µl and 6µl Identifiler fast PCR were deemed unnecessarily redundant). Second, fast results were not compared to standard PCR, but were instead held to the allele sizing standard deviation (<0.15) requirement per Applied Biosystems for standard capillary electrophoresis detection and GeneMapper® ID analysis. Next, for each primer set, a large number ($n=86-89$) of samples previously amplified using standard PCR were amplified using fast PCR to demonstrate suitability for high-throughput automation; STR profiles were assessed for concordance, profile completeness, peak height, intra- and inter-locus peak balance and first pass success rate. Contamination studies examined all negative amplification controls (water) for signs of contamination (25rfu threshold). All other samples were expected to be single-source; therefore they were assessed for signs of contamination via the presence of a profile consistent with a mixture.

Lot-to-lot variation: Lot-to-lot variation of KAPA2G™ Fast Multiplex PCR Kit was assessed using five known samples and controls via amplification with 3µl Identifiler fast PCR reactions (~0.75ng template DNA) using three different lots of KAPA2G™ Fast Multiplex Mix. All three lots were stored frozen until use and were all used prior to their expiration dates. The preparation dates of the lots were not known, but Lot 1 had the earliest expiration date, followed by Lot 2 and then Lot 3. Following GeneMapper® ID analysis, STR profiles were assessed for profile completeness, peak height, intra- and inter-locus peak balance.

Storage conditions: Kapa Biosystems indicates that KAPA2G™ Fast Multiplex Mix may be stored at 4°C for up to one month and is stable for

up to fifty freeze/thaw cycles. To assess reagent performance as a result of number of thaws and storage at 4°C, a total of 12 aliquots was thawed 1-3 times and used either immediately or following storage at 4°C for one week, two weeks or one month to amplify five known samples and controls with 3µl Identifiler fast PCR reactions (~0.75ng template DNA). Following GeneMapper®ID analysis, STR profiles were assessed for profile completeness, peak heights and intra- and inter-locus peak balance.

Results and Discussion

Optimization of low volume, fast PCR protocols using KAPA2G

5µl and 6µl Identifiler fast PCR protocols: Per manufacturer recommendations, standard Identifiler amplification on 9700 thermal cycler utilizes the “9600 Emulation” mode, but the 9700 also offers a “Max” mode with faster ramp rates. A comparison of fast PCR performance using the 9600 emulation and max modes demonstrated slightly higher quality STR profiles using the maximum ramp rate mode, but improvements were still needed with respect to lower than desired peak heights (which caused dropout) and inter-locus peak imbalance. Thus, the number of amplification cycles was increased from 26 (used by Cellmark for 3µl standard amplifications) to 28 (used by Cellmark for 5µl standard amplifications), but this introduced -A peaks. Efforts continued with the 26 cycle amplification, coupled with longer annealing/extension times of 45sec and 50sec (compared to 40sec), but did not result in a significant improvement in allele dropout. Therefore, a 27 cycle amplification (used by Cellmark for 6µl standard amplifications) was evaluated next with a 40sec annealing/extension step, which resulted in nearly doubling average peak height from 503rfu to 976rfu; furthermore, full profiles were obtained from all samples using 27 cycles, but -A was also

present. A 10min final extension successfully eliminated -A (n=24) and resulted in a 51min amplification protocol.

The 24 samples that were amplified using the final 5 μ l and 6 μ l Identifiler fast PCR protocols exhibited 100% allele concordance to profiles obtained using standard PCR, had a 100% pass rate compared to 83% using standard PCR (all failures due to PHR<50%). It should be noted that standard PCR pass rates from these 24 samples were lower than normal (typically >90%) because samples were specifically selected that had PHR<50% using standard PCR to better assess fast PCR's intra-locus balance. Peak height (averages of 1153rfu and 1205rfu, respectively), intra-locus (average PHR of 87.4% and 88.8%, respectively) and inter-locus peak balance (average CV of LPH:TPH of 0.323 and 0.344) were all acceptable from 5 μ l and 6 μ l fast amplifications.

3 μ l Identifiler Plus fast PCR protocol: Optimization of the 3 μ l Identifiler Plus PCR protocol began with assessing different final extension lengths to prevent the formation of -A. Similar to the other fast PCR protocols, -A was present using the 1min and 5min final extensions, but was eliminated using 10min. However, low-level NSA was observed using the 59°C annealing/extension temperature, primarily at TH01 (~169b, ~185b and occasionally at ~187b), D16S539 (~287b), vWA (~153b) and TPOX (~220b). Therefore, 61°C and 63°C were evaluated to improve primer specificity; NSA continued at a lesser extent with the use of 61°C, but was eliminated using 63°C. Since full profiles were obtained from all samples, average peak height (1015rfu) and inter-locus peak balance (average CV of LPH:TPH of 0.292) were acceptable and all PHR were >50% using 63°C, that temperature was selected, and development shifted to reducing amplification time.

Shorter initial activation times of 1min and 2min were assessed in comparison to 3min, both of which exhibited acceptable profiles, though average peak height was reduced to 874rfu and 849rfu, respectively, while average CV of LPH:TPH improved somewhat (0.278 and 0.275, respectively) for both activation times. Therefore, the 1min activation was selected for further testing. Shorter denaturation times (5sec and 10sec, compared to 15sec) were assessed next. Both data sets yielded full profiles, but the 5sec data set exhibited a decrease in average peak height (711rfu), while the 10sec data set exhibited an increase (1269rfu), compared to 15sec. Though the explanation for the peak height increase using a 10sec denaturation (compared to those obtained with 15sec) was unknown, the 10sec denaturation was selected for further study because it performed as well as or better than 15sec with regard to peak height and other profile quality criteria.

Therefore, a 10sec denaturation was next evaluated with a 50sec annealing/extension step in comparison to the 60sec annealing/extension step previously tested, using a large data set (n=88) for both in an effort to reduce skewing due to small sample size; fast profiles from these 88 samples were compared to those obtained using standard PCR. Full profiles were obtained from 93%, 91% and 94%, respectively, with an average of 96% of alleles detected from both of the fast protocols compared to 98% from standard. Fast PCR's slightly increased rate of dropout and decreased profile completeness were likely a result of reduced peak heights obtained from fast PCR (967rfu for the 50sec data set and 675rfu for 60sec) compared to standard PCR (1303rfu). It should be noted however, that fast PCR was performed on a different thermal cycler and detected on a different 3130xl Genetic Analyzer than the standard

PCR data, which could account for the differences seen in peak height. Inter-locus peak balance was acceptable from all methods, with average CVs of LPH:TPH ranging from 0.247 for standard PCR to 0.258 (50sec) and 0.261 (60sec) fast PCR protocols, which is considerably lower than that observed by Identifiler fast PCR (typically >0.300). PHR <50% were infrequent, occurring in 1% of fast PCR profiles (50sec data set only; none observed in the 60sec data set) compared to 3% of standard profiles. Overall, first pass success rates (that is, the percentage of profiles passing after the first pass/round of testing) were highest from the 50sec fast PCR data set (92%), compared to 91% for 60sec fast PCR and standard PCR (dropout and PHR <50% were the only reasons for sample failure). Thus, the 50sec annealing/extension step was selected for the optimized Identifiler Plus fast PCR protocol, which had a total run time of 49min.

3 μ l PowerPlex 16 HS fast PCR protocol: Optimization of the 3 μ l PowerPlex 16 HS PCR protocol also began with assessing different final extension lengths to prevent the formation of -A. Similar to the other fast PCR protocols, -A was present using the 1min and 5min final extensions, but was eliminated using 10min. Furthermore, from the first round of testing with the 10min final extension step, full profiles were obtained from all samples, no signs of NSA were present, average peak heights were 1256rfu, PHR <50% (17% of samples) were limited to 0.25ng samples, but inter-locus imbalance was higher than desired (average CV of LPH:TPH was 0.438). Next, 2-step PCR cycling was evaluated using annealing/extension temperatures of 58°C, 60°C and 62°C in an effort to improve profile quality. Low-level NSA was observed using both of the lower temperatures, but not at 62°C; however, profile quality decreased using 2-step PCR, most notably via allelic dropout (11% of samples) despite increased average peak heights (1476rfu), increased occurrences of PHR <50% (28% of samples) and decreased inter-locus balance (average CV of LPH:TPH of 0.474). Therefore, 2-step was not pursued further, and development of 3-step cycling continued with an assessment of increasing ramp rates from 29% and 23% to 100% for the annealing and extensions, respectively. Compared to the use of manufacturer's recommended ramp rates, use of 100% ramp rates exhibited allelic dropout from one sample (a 0.25ng replicate; 6% of samples), despite increased average peak heights (1580rfu), decreased occurrences of PHR <50% (11% of samples; also limited to 0.25ng) and similar inter-locus balance (average CV of LPH:TPH of 0.446); however, increased average instances of called stutter per sample and pull-up >20% accompanied these higher peak heights. Use of 100% ramp rates was further tested in conjunction with reduced annealing times (15sec compared to 30sec). Even though full profiles were obtained from all samples using the shorter annealing time, a significant reduction in average peak height was observed (890rfu) and occurrences of PHR <50% doubled (22% of samples; limited to 0.25ng), while occurrences of stutter and pull-up declined; inter-locus peak balance was not significantly affected (average CV of LPH:TPH of 0.422). Since peak heights were still at an acceptable level using 100% ramp rates and 15sec annealing, these were assessed next with shorter extension times (20sec and 15sec, compared to 30sec). Extension times less than 30sec resulted in a significant increase in allelic dropout and reduction in peak heights, such that full profiles were only obtained from 73% (15sec) and 82% (20sec) of samples. Thus, the 30sec extension was maintained.

Reduced denaturation time (10sec and 5sec, compared to 15sec) was assessed next, but as seen with a reduction in extension time, a reduction in denaturation time also resulted in significant increases

in allelic dropout and decreases in peak height. Therefore, 15sec denaturation was maintained. Next, a reduction in initial activation (1min versus 2min) was assessed. With this reduction, full profiles were still obtained from all samples, while average peak height (914rfu), inter-locus balance (average CV of LPH:TPH of 0.414) and occurrences of PHR <50% (no occurrences) were not effected in a negative manner. The 1min initial activation was then tested with a 25°C final hold in comparison to 4°C, from which full profiles were obtained, a significant increase in average peak height was observed (1888rfu), accompanied by increased occurrences of pull-up (not exceeding 20% of the true allele), but not significant changes in inter- or intra-locus balance. The implementation of the 25°C final hold completed the development of the PowerPlex 16 HS fast PCR protocol, resulting in a 51min amplification protocol. It should be noted that the desired level of inter-locus peak balance (CV of LPH:TPH $\leq$ 0.350) could not be achieved.

This fast PCR protocol was tested using 89 samples, and the resulting profiles were compared to those obtained using standard PCR. Full profiles were obtained from 96% of fast profiles and 93% of standard profiles, with an average of 98% and 99% of alleles detected, respectively. Peak heights were slightly lower from fast PCR (average of 1308rfu) compared to standard (average of 1449rfu), but were still on the high end of the desired range. Inter-locus peak imbalance was higher than desired using fast and standard PCR (average CVs of LPH:TPH of 0.416 and 0.372, respectively). PHR <50% were infrequent, occurring in 2% of fast PCR profiles compared to 4% of standard profiles. Overall, first pass success rates were highest from fast PCR (94%), compared to 90% for standard PCR; dropout and PHR <50%, were the only reasons for sample failure.

Validation of low volume, fast PCR protocols using KAPA2G

See Figures 1-3 for representative profiles from fast PCR protocols utilizing KAPA2G.

Determination of the optimal range of input DNA: The optimal DNA input range was determined to be 0.375-1.50ng for each of the five fast PCR protocols based on the analyses below (Figure 4).

For all five fast PCR protocols, average percent alleles detected and percent full profiles increased as template amount increased, such that full profiles were obtained from nearly all samples when $\geq$ 0.188ng DNA was amplified (Figure 5). Though the sensitivity range (i.e., the range in which full profiles were obtained from the majority of all samples) was determined to be 0.188-3.00ng for all but 3 μ l Identifier fast PCR (0.375-3.00ng), data from 0.188-3.00ng is discussed further for each of the five fast PCR methods for comparison purposes. Allele peak height and balance is summarized in Figure 6. As expected, average allele peak height increased as DNA input increased. Reproducibility of peak height was measured via average coefficient of variation per DNA input, which was $\leq$ 0.350 when $\geq$ 0.188ng DNA was amplified using each of the four fast PCR protocols (except for 0.188ng with a 6 μ l Identifier amplification), indicating acceptable levels of reproducibility.

None of the five methods tested were able to result in the desired level of general inter-locus balance (CV of LPH:TPH $\leq$ 0.350) for all DNA input amounts. Nearly all methods exhibited CVs >0.350 at 0.188ng and 3.00ng, while PowerPlex 16 HS CVs were >0.350 for all but 3.00ng. Intra-locus balance was measured via heterozygote

Figure 1: Representative Identifier Profiles from Fast and Standard PCR.

Profiles were obtained using each of the validated Identifier/KAPA2G fast PCR reaction volumes (A-C) and 6 μ l Identifier standard PCR (D). Allele calls, allele size (bases) and peak height (rfu) are displayed for each allele.

Figure 2: Representative Identifier Plus Profiles from Fast and Standard PCR.

Profiles were obtained using the validated Identifier Plus/KAPA2G low volume, fast PCR method (A) and Identifier Plus standard PCR (B). Allele calls, allele size (bases) and peak height (rfu) are displayed for each allele.

Figure 3: Representative PowerPlex 16 HS Profiles from Fast and Standard PCR.

Profiles were obtained using the validated PowerPlex 16 HS/KAPA2G low volume, fast PCR method (A) and PowerPlex 16 HS standard PCR (B). Allele calls, allele size (bases) and peak height (rfu) are displayed for each allele.

Figure 4: Optimal DNA Input Ranges for Fast PCR Protocols Using KAPA2G.

An optimal range (grey) was determined for each amplification based upon the evaluated criteria (n=5 or 6 samples per DNA input; those with n=5 had a sample removed due to injection failure).

peakheight ratios, averaging >82% for all ≥ 0.375 ng amplifications. Instances of PHR <50% were most frequent when 0.188ng DNA was amplified, but on average occurred less than once per sample.

Figure 5: Sensitivity of Fast PCR Protocols Using KAPA2G.

Sensitivity is displayed as percent alleles detected and percent full profiles (n=5 or 6 per DNA input; those with n=5 had a sample removed due to injection failure).

Figure 6: Peak Height Summary for Fast PCR Protocols Using KAPA2G.

Average peak height, reproducibility of peak height per allele, inter-locus peak balance and intra-locus peak balance/imbalance are displayed for each DNA input (n=5 or 6 per DNA input; those with n=5 had a sample removed due to injection failure).

Various artifacts were observed above the analysis threshold, but stutter (n-4) was the most prevalent type (Figure 7). Average percent stutter ranged from 10-19% and demonstrated a slight increase for the 0.375ng samples compared to 0.750-3.00ng. This was expected given

that peak heights were lower at 0.375ng compared to higher inputs; thus, any stutter peaks that met the 75rfu analysis threshold at 0.375ng were a larger percentage of the true allele peak. Instances of stutter increased as DNA input increased and tended to be much less prevalent from PowerPlex 16 HS than the Identifier/Identifier Plus profiles. Unacceptably high stutter peaks (>20%) occurred occasionally, but were limited to profiles obtained using 3.00ng DNA (data not shown). Other forms of stutter (n+4 and n-8) did occur above threshold on occasion, but were nearly always limited to 1.50ng and 3.00ng amplifications. Furthermore, average percent stutter for these two forms of stutter (<4% for all) was much lower than that of n-4 stutter. Unacceptably high n-8 stutter (>2 occurrences in a single profile) occurred was limited to a single 3.00ng Identifier Plus amplification (data not shown).

Pull-up peaks were the next most abundant artifact that was detected above threshold, but were nearly always limited to amplification of ≥ 1.50 ng DNA and were more frequent for Identifier Plus and PowerPlex 16 HS. Unacceptably high pull-up (>20%) was limited to 3.00ng amplifications using Identifier (6µl) and Identifier Plus (data not shown). A single occurrence of -A (2.7% of the true allele) was present in a 3µl Identifier amplification using 3.00ng DNA (data not shown). Elevated baseline was limited to 1.50ng and 3.00ng and was

Figure 7: Artifacts for Fast PCR Protocols Using KAPA2G.

Average percent stutter and pull-up are displayed, as well as average number of detected stutter, pull-up and elevated baseline per profile (n=5 or 6 samples per DNA input; those with n=5 had a sample removed due to injection failure).

unacceptably high (occurrences at >3 loci) at 3.00ng for all five PCR methods (data not shown). No signs of non-specific amplification were noted.

Stochastic threshold: Stochastic thresholds were determined for each fast PCR method and were compared to those of standard PCR (Figure 8). Fast PCR stochastic were as good as or better (85-125rfu) than those from standard PCR (120-270rfu).

Precision: Precision of allele sizing was assessed based on multiple injections of positive control 9947A amplified using each of the four fast PCR methods and compared to that of standard Identifier. All assessments indicated acceptable levels of precision per manufacture recommendations (standard deviation <0.15; Figure 6).

Precision of allele sizing was assessed based on positive control 9947A amplified using the 3µl fast PCR methods that were developed for each of the three primer sets tested. All assessments indicated

Figure 8: Stochastic Thresholds for Fast PCR Protocols Using KAPA2G.

These were established for each amplification method by determining the point at which heterozygous loci will not be mistaken for homozygous loci due to dropout of a single allele (n=510 to 595 loci for each amplification method). ID = Identifier, ID+ = Identifier Plus, HS = PowerPlex 16 HS, F = Fast PCR, S = Standard PCR.

acceptable levels of precision per manufacture recommendations (standard deviation <0.15; Figure 9).

As was seen previously with the comparison of various 3µl Identifier fast PCR protocols [13], nearly all assessments indicated more precise allele sizing between injections compared to intra-injection precision. Furthermore, all amplification methods exhibited satisfactory precision for allele sizing.

Stutter Assessment: For the detailed stutter analysis, n-4 stutter was the most frequent type of stutter observed and accounted for 82% (3µl PowerPlex 16 HS) to 96% (6µl Identifier) of all stutter peaks. Percent stutter (n-4) varied more by locus than amplification method (Figure 10).

Figure 9: Precision of Allele Sizing for Fast PCR Protocols Using KAPA2G.

Precision was assessed via standard deviation for each allele from 9947A positive control DNA (for each amplification method, n=9 for each of the 25 alleles for PowerPlex 16 HS and 26 alleles for Identifier Plus/Identifier).

Maximum observed stutter for each fast PCR method exceeded the locus specific stutter thresholds supplied by vendors for each primer set processed under their recommendations (i.e., standard PCR, using 25µl reaction volumes) (Figure 11). Unless otherwise modified by the user in the GeneMapper® ID Panel Manager, the vendor-specific thresholds will be used by GeneMapper® ID during analysis and due to the increase in percent stutter for fast PCR, more stutter peaks will be called using these fast PCR methods compared to standard. Thus, laboratories desiring to implement fast PCR (or other amplification methods with stutter thresholds different than those supplied by the vendor) should be aware of this issue.

Implementing global stutter thresholds of 20% for all loci is not an uncommon practice for reference samples [8]. Maximum stutter

limits per locus were calculated via the sum of average percent stutter plus three standard deviations (a more conservative approach adds two standard deviations [11]), which never exceeded 20% for any locus or amplification method. It should be noted, however, that the maximum observed percent stutter (n-4) was occasionally (0.05-0.18% of samples) above 20% for all Identifier and Identifier Plus fast PCR methods, ranging from 21-25%, but never exceeded 20% for PowerPlex 16 HS. Half of the profiles exhibiting n-4 stutter >20% had stutter peaks that corresponded with pull-up from another

Figure 10: Average Percent Stutter (n-4) for Fast PCR Protocols Using KAPA2G.

Stutter is displayed by amplification method and locus (n=1592 to 2044 stutter peaks per method). D2S1338 and D19S433 are Identifier and Identifier Plus loci, while Penta D and Penta E are PowerPlex 16 HS loci. ID = Identifier, ID+ = Identifier Plus, HS = PowerPlex 16 HS, F = Fast PCR.

Figure 11: Maximum Observed Stutter (n-4) for Fast PCR Protocols Using KAPA2G Compared to Standard PCR.

Maximum observed stutter was nearly always higher than the vendor-specified, locus specific stutter thresholds for standard, full volume PCR reactions of each primer set. ID = Identifier, ID+ = Identifier Plus, HS = PowerPlex 16 HS, F = fast PCR.

locus, 33% were from allele 25 at D18S51 and the remaining 17% were from loci with low-level peak heights (<140rfu), which are subject to stochastic effects. Thus, a global 20% n-4 stutter threshold would work well for any of the developed fast PCR methods.

Both n+4 and n-8 stutter occurred significantly less than n-4, often not occurring at some loci (data not shown). Since occurrences

Stutter Type	Identifiler (3µl)	Identifiler (5µl)	Identifiler (6µl)	Identifiler Plus (3µl)	PowerPlex 16 HS (3µl)
n+4					
Average	3.82% _a	2.97% _{a,b}	3.17% _{a,b}	2.43% _b	2.75% _b
95% CI	[3.09%, 4.56%]	[2.53%, 3.40%]	[2.26%, 4.08%]	[2.09%, 2.78%]	[2.45%, 3.05%]
Calculated Maximum	10.60%	8.62%	10.40%	7.25%	9.17%
Observed Maximum	9.01%	12.20%	8.13%	9.49%	16.60%
n-8					
Average	2.52% _{c,d}	2.05% _{d,e}	2.24% _{c,d,e}	1.77% _e	2.60% _c
95% CI	[2.01%, 3.03%]	[1.80%, 2.30%]	[1.55%, 2.93%]	[1.58%, 1.97%]	[2.38%, 2.82%]
Calculated Maximum	8.52%	6.18%	8.99%	4.98%	7.65%
Observed Maximum	10.90%	10.90%	10.40%	7.55%	8.88%

Table 7: Average Percent Stutter for n+4 and n-8 Stutter.

Average percent stutter (n+4 and n-8) is displayed for each low volume, fast PCR method.

of n+4 and n-8 were often low for individual loci, these types of stutter were averaged across all loci. Average n+4 percent stutter ranged from 2% to 4%, whereas average n-8 percent stutter ranged from 2% to 3% (Table 7). Maximum allowable n+4 and n-8 stutter were calculated across all loci as opposed to individual loci. However, it should be noted that observed maximum n+4 exceeded calculated maximums for three fast PCR protocols (5µl Identifiler, 3µl Identifiler Plus and 3µl PowerPlex 16 HS), while observed maximum n-8 exceeded calculated maximums for all five protocols. These discrepancies could arise from the fact that all stutter peaks of the same type were grouped together since there was not enough data to calculate these values for individual loci. Given that many databasing laboratories allow a 20% n+4 stutter threshold, and the maximum observed n+4 stutter was 16.6%, it appeared reasonable to apply the same 20% global stutter threshold to n+4 stutter for all five fast PCR protocols. Many laboratories don't provide specific allowances for n-8 stutter; therefore, based upon the data obtained from this study, global stutter thresholds for n-8 stutter were set to 12% for the Identifiler fast PCR protocols and 10% for Identifiler Plus/PowerPlex 16 HS.

Averages in each row that share subscripts are not statistically different at $\alpha=0.05$ according to the Tukey HSD procedure. Not enough data existed to calculate average percent stutter by locus for these two types of stutter.

Automation (Large Sample Sets): Each of the five fast PCR protocols were tested using 86-89 buccal samples (swab cuttings or Buccal DNA Collector™ punches) with known profiles (Table 8 for a summary of profile quality). Full profiles were obtained from $\geq 97\%$ of samples for each fast PCR method, with an average of $\geq 98\%$ alleles detected; all profiles exhibited concordant allele calls and no unexplained alleles were present. Inter-locus peak balance was assessed via the average CV of LPH:TPH and was <0.350 for all fast methods except with the PowerPlex 16 HS primer set, but it should be noted that inter-locus peak balance was also lower than desired using standard PowerPlex 16 HS (3µl amplification; data not shown). Intra-locus peak balance was assessed via average PHR and occurrences of PHR $<50\%$. All methods exhibited average PHR between 85.8% and 86.8%, and all but 6µl Identifiler exhibited at least one profile with one or more loci exhibiting PHR $<50\%$, though the latter differences in occurrence of profiles with PHR $<50\%$ was not found to be statistically significant for the five fast protocols ($p=0.45$ using Pearson's Chi-squared test). Furthermore, if a larger sample size had been tested, 6µl Identifiler likely would have exhibited a similarly low percentage of profiles with at least one locus with a PHR $<50\%$. Lastly, first pass success rates were

$\geq 95\%$ for each of the five fast PCR protocols. It should be noted that all samples exhibiting dropout with a fast PCR method also exhibited dropout when amplified using standard PCR (except for one sample amplified using 6µl Identifiler fast PCR). On the other hand, PHR $<50\%$ were generally not reproducible across any of the PCR methods.

Furthermore, it should be noted that a low level ($\sim 100\text{rfu}$), unexplained artifact was observed at Amelogenin ($\sim 108\text{b}$) for 1.1% of 3µl Identifiler and 5µl Identifiler fast amplifications (a single sample for both amplification volumes, Figure 12). This artifact was not reproducible, and at no other time during fast PCR development or validation was this artifact observed.

Contamination Assessment: All negative amplification controls ($n=44$) were free of contamination, and no signs of contamination due to the amplification process were identified in any other sample, including positive controls.

Lot-to-Lot Variation: Lot-to-lot variation of KAPA2G™ Fast Multiplex PCR Kit was assessed for three different lots of the master mix (Table 9 for a summary of profile quality). Full profiles were obtained from all samples ($n=5$) and positive amplification controls ($n=2$) using each lot. Average peak heights were significantly higher from lots 2 and 3 than lot 1, whereas no significant difference in intra-locus (average PHR and instances of PHR $<50\%$) or inter-locus

Figure 12: Fast PCR Artifact at Amelogenin.

A low-level artifact ($\sim 108\text{b}$) was identified in one 3µl Identifiler amplification (top) and one 5µl Identifiler amplification (bottom). These fast amplifications were from different samples.

Profile Assessment	Identifiler (3µl) ^a	Identifiler (5µl) ^b	Identifiler (6µl) ^c	Identifiler Plus (3µl) ^c	PowerPlex 16 HS (3µl) ^d
Full Profiles Detected	99%	99%	98%	97%	98%
Alleles Detected					
Average	98.90%	99.00%	98.10%	98.00%	99.40%
95% CI	[96.7%, 100%]	[97.0%, 100%]	[95.4%, 100%]	[95.5%, 100%]	[98.4%, 100%]
Peak Height (rfu)					
Average	827	961	579	1106	1295
95% CI	[811, 843]	[943, 979]	[567, 591]	[1076, 1136]	[1263, 1326]
Observed Maximum	3418	3897	2757	7955	7162
Observed Minimum	119	81	78	76	75
Inter-Locus Balance: CV of LPH:TPH					
Average	0.294	0.301	0.306	0.264	0.416
95% CI	[0.282, 0.306]	[0.291, 0.311]	[0.295, 0.317]	[0.252, 0.276]	[0.408, 0.424]
Intra-Locus Balance: PHR					
Average	0.866	0.864	0.861	0.868	0.858
95% CI	[0.861, 0.872]	[0.858, 0.870]	[0.855, 0.867]	[0.862, 0.874]	[0.852, 0.864]
PHR <50% ^e	0.90%	1.80%	0.00%	0.90%	2.70%
First Pass Success Rate	98%	97%	98%	95%	95%
Failure Reasons					
Dropout	1.20%	1.10%	2.30%	3.40%	2.30%
PHR <50% ^f	1.20%	2.20%	0.00%	1.10%	3.40%

Table 8: Profile Summary from Automation Assessment.

Profile summaries are displayed for each of the five low volume, fast PCR protocols.

^an=86; ^bn=89; ^cn=87; ^dn=88; ^ePercent of all heterozygous loci; ^fPercent of samples

(average CV of LPH:TPH) balance were observed for the three lots. Despite lower peak heights using Lot 1, high quality, full profiles were obtained from all three lots.

Storage Conditions: Storage conditions were assessed via 12 combinations of number of thaws and length of 4°C storage prior to use. Full profiles were obtained for all samples (n=5) and positive amplification controls (n=2). Peak heights were significantly different for many of the storage conditions (Table 10), but general trends with regard to number of thaws and length of 4°C storage were not substantiated for the majority of data subsets (Figure 13). Peak height variation due to different amplification and detection runs on the same instrument is a widely accepted concept. However, in this particular case, it was unknown whether this additional variation in peak height influenced the data such that apparent trends appeared to exist for the 3-thaw and immediate use data sets, when in fact such trends did not exist, or if additional trends existed but appeared not to. Either way, peak height values were acceptable from all data sets. Intra- and inter-locus peak balance were assessed via PHR and CV of LPH:TPH, respectively, and did not exhibit significant differences between storage conditions. Therefore, all storage conditions tested were suitable for use.

Conclusions

Low volume, fast PCR development using KAPA2G™ Fast Multiplex PCR Kit, various forensic STR primer sets, and non-fast thermal cyclers was quite successful, resulting in amplification times of 43-51min for 3µl reactions on a Veriti thermal cycler and 51min for 5-6µl reactions on a 9700. These protocols are robust for buccal samples, exhibiting first pass success rates of ≥95% and 100% allele concordance compared to standard PCR. Percent stutter does increase using fast PCR as compared to that of standard PCR [1,7,13], but use

Profile Assessment	Lot 1	Lot 2	Lot 3
Peak Height (rfu)			
Average	577	787 _a	852 _a
95% CI	[534, 620]	[725, 849]	[793, 911]
Observed Maximum	1892	2936	2748
Observed Minimum	138	129	215
Inter-Locus Balance: CV of LPH:TPH			
Average	0.379 _b	0.370 _b	0.331 _b
95% CI	[0.320, 0.438]	[0.338, 0.402]	[0.289, 0.373]
Intra-Locus Balance: PHR			
Average	0.854 _c	0.857 _c	0.851 _c
95% CI	[0.828, 0.880]	[0.836, 0.878]	[0.829, 0.873]
PHR <50%	3.8% _d	1.3% _d	0.0% _d

Table 9: Lot-to-Lot Variation of KAPA2G™ Fast Multiplex PCR Kit.

Profile summaries are displayed for each of the five low volumes, fast PCR protocols. Full profiles were obtained from all samples and positive amplification controls. Averages in each row that share subscripts are not statistically different at α=0.05 according to one-way ANOVA analysis and/or the Tukey HSD procedure.

of a 20% global stutter filter [8] or modification of the locus specific stutter thresholds in GeneMapper® ID should prevent excessive stutter peaks from being called by the software, each of which are acceptable for reference samples. Furthermore, the protocols developed in this study improved upon many of the previously reported downfalls of fast PCR. Though a low-level (~100rfu) artifact was observed in 1.1% (one sample) of 3µl and 5µl Identifiler fast amplifications at the Amelogenin locus, it was not reproducible and

Length of 4°C Storage	1-Thaw	2-Thaw	3-Thaw
Peak Height ^a			
Immediate Use	780 _{b,c} [745, 815]	959 _{d,e} [919, 999]	1138 _a [1075, 1201]
One Week	671 _b [645, 697]	1068 _b [1023, 1113]	871 _{c,d} [838, 904]
Two Weeks	837 _{c,d} [795, 879]	630 _b [606, 654]	832 _{c,d} [812, 852]
One Month	837 _{c,d} [811, 863]	891 _d [833, 949]	732 _{b,c} [710, 754]
PHR ^f			
Immediate Use	0.856 [0.850, 0.861]	0.868 [0.865, 0.870]	0.832 [0.830, 0.840]
One Week	0.858 [0.851, 0.865]	0.849 [0.839, 0.860]	0.840 [0.829, 0.850]
Two Weeks	0.859 [0.853, 0.864]	0.851 [0.845, 0.856]	0.868 [0.863, 0.873]
One Month	0.854 [0.848, 0.860]	0.854 [0.844, 0.863]	0.863 [0.853, 0.869]
CV of LPH:TPH ^g			
Immediate Use	0.370 [0.338, 0.402]	0.319 [0.268, 0.369]	0.383 [0.333, 0.457]
One Week	0.350 [0.302, 0.398]	0.357 [0.316, 0.398]	0.370 [0.329, 0.428]
Two Weeks	0.356 [0.305, 0.406]	0.359 [0.302, 0.416]	0.388 [0.332, 0.430]
One Month	0.347 [0.295, 0.400]	0.354 [0.299, 0.410]	0.385 [0.329, 0.424]

Table 10: Variation Due to Storage Conditions of KAPA2G™ Fast Multiplex PCR Kit.

Peak height, PHR and CV of LPH:TPH averages and 95% confidence intervals are displayed for each storage condition. Peak height averages that share subscripts are not statistically different at $\alpha=0.05$ according to the Tukey HSD procedure; intra- and inter-locus balance (PHR and CV of LPH:TPH) were not significantly different for any of the storage conditions using two-way ANOVA.

^an=192; ^fn=80; ^gn=7

Figure 13: Effect of Storage Conditions on Allele Peak Height.

Average allele peak height is displayed for the various storage conditions tested (n=7 samples per data set). Consistent trends with regard to peak height were only noted for the 3 Thaws data set (decrease as length of 4°C increased) and the Immediate Amp data set (unexpected increase as number of thaws increased).

no other non-specific amplification occurred in any of the other protocols. This is much improved compared to the NSA products that were previously reported by Vallone et al., [10]. Incomplete adenylation (-A) has been shown to often accompany STR profiles obtained using fast PCR [3,10,13], but was not problematic using the protocols developed here with 10min final extensions. Poor intra-locus peak balance has also been observed with other fast PCR protocols [13], but was not problematic using the protocols in this study and only occurred at one or more loci in $\leq 2\%$ of samples tested, which was less than that observed for standard PCR of the same samples.

Furthermore, this study demonstrated the ability to achieve robust, reliable, high quality STR profiles for reference samples using fast PCR without having to purchase costly reagents, supplies, or equipment. Utilizing low volume reactions, the cost of using a fast PCR polymerase (i.e., KAPA2G) outside of the standard forensic STR amplification is extremely low (\$0.06-\$0.12 / 3-6 μ l reaction) and would likely be offset by no longer having to purchase supplemental AmpliTaq Gold® DNA polymerase for standard Identifiler reactions (the limiting reagent in the Identifiler amplification kit), as well as obtaining more reactions per Identifiler Plus/PowerPlex 16 HS kit given that the entire tube of primer could be utilized (excess primers are thrown away under standard PCR conditions when the kits' master mixes are depleted). And for laboratories transitioning from full volume reactions (25 μ l) down to these low volumes, fast PCR reactions, the savings would be even greater. No additional supplies were needed for these protocols, including costly fast thermal cyclers.

All-in-all, this study has demonstrated the development of robust, low volume, fast PCR protocols for buccal samples to yield high quality STR profiles accompanied by a substantial reduction in amplification time with little (if any) increase in per sample costs.

Future Studies

Newer amplification kits than those included in this project are now commercially available - PowerPlex® Fusion and Fusion 6C Systems, GlobalFiler® and GlobalFiler® Express PCR Amplification Kits, and Investigator® 24plex QS and GO! Kits - and allow for amplification of more STR loci. Many of these newer kits have already incorporated fast PCR strategies to reduce amplification time substantially, without the use of fast thermal cyclers. It would be noteworthy to further assess the performance of these newer kits using low volume PCR reactions, as an extra cost-saving measure for DNA reference samples, to see how they would compare to the methods presented here.

Additionally, this study was specifically tailored for DNA reference samples, but it would be valuable to assess these protocols with evidentiary type samples (blood stains, cigarettes, differentials and other mixtures, etc.). Targets for DNA input would have to be re-evaluated using a human-specific quantification method, and STR profiles would need to meet stricter PHR thresholds (at least 60-70%) to allow for proper mixture interpretation.

Acknowledgements

We would like to thank Kapa Biosystems and Applied Biosystems for donating reagents for this study.

Conflicts of Interest

At the time this research was conducted, Catherine Connon and Aaron LeFebvre were employees of Cellmark Forensics, the sister lab to Bode Technology (now collectively known as Bode Cellmark Forensics). Reagents were donated for evaluation by manufacturers (Kapa Biosystems and Applied Biosystems), but did not result in bias.

Role of Funding Source

This project was funded by Cellmark Forensics, a LabCorp Specialty Testing Group. Additional reagents were donated by Kapa Biosystems and Applied Biosystems.

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