Review

Rapid PCR of STR Markers: Applications to Human Identification

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Abstract

Multiplex PCR with fluorescently labeled primers has been an essential method for the amplification of short tandem repeats used in human identify testing. Within the STR workflow of extraction, quantitation, amplification, separation, and detection, multiplex PCR is commonly identified as the bottleneck in the process. The time requirement of up to three hours to complete 28-30 cycles of multiplex PCR for STR genotyping is the greatest amount of time required for a single step within the process. The historical use of commercially available thermal cyclers and heat stable polymerases may have given the impression that large multiplex will always require long PCR cycling times to ensure that all of the varying sized targets (typically 100-400 bp) can be amplified in a balanced manner throughout the multiplex. However, with the advent of improved polymerases and faster thermal cyclers the time required for the amplification of large STR multiplexes is no longer on the order of three hours, but as little as 14 minutes. Faster amplification times can be performed while retaining the balance and integrity of large multiplex PCRs by implementation of alternate polymerases and thermal cyclers. With the reduction in PCR cycling times there has also been an impact on the development of integrated and microfluidics devices which employ the use of reduced or rapid thermal cycling protocols as part of their integration. Similarly, PCR inhibitor resistant polymerases can also reduce overall STR processing times for reference samples by eliminating the need for DNA extraction and purification that is additionally implemented within the development of integrated DNA typing devices.

Keywords: STR; PCR; multiplex PCR; rapid PCR; rapid DNA; direct PCR

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# Introduction

The forensic DNA typing community currently has a core set of STR markers that are widely used to generate STR profiles for use in databases and casework applications [1]. Currently, the process to generate these profiles is typically conducted in eight to 10 hours using traditional laboratory methods. This process includes: DNA extraction, quantitation, multiplex PCR amplification, and fragment separation and detection. Over recent years, significant efforts have been made to enable the rapid generation of STR profiles to include rapid PCR, direct PCR, microfluidic chip based amplification and detection, and integrated microfluidic devices for rapid sample to profile processing as alternative approaches.

Many of the commonly used commercial multiplex STR typing kits are not optimized or intended for rapid PCR thermal cycling. Current protocols for kits such as IdentiFiler and PowerPlex 16 require approximately three hours for amplifying a multiplex containing 15 STR loci plus amelogenin [2, 3]. In recent years, both Thermo Fisher (previously known as Life Technologies and/or Applied Biosystems) and the Promega Corporation have developed STR genotyping kits which require less than three hours for amplification, and can additionally perform direct PCR amplification of samples which eliminates the extraction and quantitation steps within the genotyping process.

GlobalFiler Express and PowerPlex Fusion simultaneously amplify 24 markers in approximately 40 min and 85 min, respectively [4, 5]. These commercial improvements in the reduction of time required for PCR amplification have further advanced the ability to develop rapid DNA typing protocols as well as instrumentation focused on STR genotyping.

Additional advances have been made in the area of microfluidics for PCR amplification as well as separation and detection. Integration of all of the methods of the forensic DNA process has been a challenging effort, but has shown to be possible in recent years. Several parallel efforts have been made to develop a system which will incorporate all of the forensic steps and utilizes a simple swab in- answer out workflow.

# Alternative DNA Polymerases

Alternative DNA polymerases to the commonly used AmpliTaq Gold have enabled the development of rapid PCR protocols. The processivity of a DNA polymerase refers to the number of nucleotides (nt) incorporated before dissociation. Work from Pomerantz and O’Donnell describes conditions by which the *Escherichia Coli* polymerase III processivity was increased when associated with a sliding clamp and replisome subunits from less than 10 nt (<20 nt/s) to greater than 50 kb (1000 nt/s) [6]. Researchers have also identified a means to increase the processivity of a DNA polymerase by covalently linking a non-specific double-stranded DNA binding protein to the polymerase domain [7]. This has led to the expression of commercial ‘fusion’ DNA polymerases that exhibit higher processivities [8, 9]. This approach demonstrated a 16 to 32 fold increase in polymerase efficacy, but it also demonstrated the ability to amplify DNA with shorter extension times, thus shortening overall PCR thermal cycling times [7]. Additionally, the use of alternate polymerases or mutants of the Taq DNA polymerase has demonstrated the ability to increase resistance to inhibition found in whole blood and crude soil samples 10 to 100 fold when compared to wild-type Taq, which is strongly inhibited by the addition of 0.1 to 1% volumes of whole blood [10]. The ability to increase the processivity of a DNA polymerase as well as increase resistance to inhibitory factors makes alternate DNA polymerases ideal for rapid and direct PCR protocols.

Additional studies with bacteriophage ɸ29 DNA polymerase has proven to be yet another unique enzyme which has two distinctive properties, high processivity and a faithful polymerization coupled to strand displacement [11]. The ɸ29 DNA polymerase has been constructed to be chimeric by fusing DNA binding domains to the C terminus of the polymerase. De Vega et al. has demonstrated that the addition of Helix-hairpin-Helix (HhH) domain increases DNA binding of the polymerase without hindering the replication rate [11]. The Helix-hairpin-Helix motif was additionally examined in five proteins containing either the Stoffel fragment of Taq polymerase or the Pfu polymerase [12]. From this work, the ability to express hybrid proteins led to enhanced DNA-binding properties and higher processivities than that of TaqDNA polymerase [12]. Additional work examining the impact of DNA polymerases in biotechnology can be found Frontiers [13].

# Rapid PCR Protocols for STR Typing

## Rapid PCR Protocols for STR typing: Greater than one hour

The use of standard PCR thermal cyclers within laboratories and modified PCR thermal cycling protocols have reduced the time required for PCR. Alternative polymerases to the commonly used AmpliTaq Gold in combination with optimized thermal cycling protocols have been demonstrated to shorten the time required for PCR. The developmental validation of Expressmarker 16 demonstrated that with the use of Hot-Start Q Taq, STR results could be obtained within 100 minutes [14]. A method dubbed T-Go-Fast reduced PCR thermal cycling time for Yfiler in about half to 110 minutes [15]. This reduction was employed with the Yfiler kit using a GeneAmp PCR System 9700 and involved using an alternate PCR mastermix, AmpliTaq Gold Fast Mastermix, and Up(2x) polymerase. Choung et al. demonstrated a reduction from 3.5 hours to as little as two hours in PCR cycling time for IdentiFiler [16]. In this work, an examination of IdentiFiler primers utilizing small reductions of time in each step of PCR (denaturation, annealing, and extension) with AmpliTaq Gold and the use of a GeneAmp 9700 thermal cycler further reduced PCR thermal cycling times by 60 minutes to 90 minutes for generation of complete STR profiles [16]. Several rapid PCR protocols have managed to reduce the time involved for PCR thermal cycling even further to one hour [15-17]. Bahlmann et al. initially examined three unique alternate polymerases for use with the PowerPlex S5 chemistry and the GeneAmp PCR System 9700 and Veriti thermal cyclers [17]. The final protocol generated complete 4plex STR profiles in one hour with HotStart Taq Plus. Development of in-house assays has also proven to perform with rapid PCR thermal cycling protocols. A recent study examined a new 26plex Y-STR assay for forensic applications which allowed for rapid PCR with the use of the Q5 High Fidelity DNA polymerase on a standard GeneAmp PCR System 9700 [18]. In this work, complete profiles were generated down to 125 pg of DNA input in one hour. The rapid Y-STR assay was then used to successfully generate haplotype data for the Chinese Han population. Thermal cycling time was reduced with PowerPlex 16 HS from three hours to one hour by employing the use of SpeedSTAR HS polymerase with the Veriti thermal cycler [19]. The stutter percentages calculated were slightly higher than found in the commercial developmental validation of the PowerPlex 16 HS chemistry, while the mean peak height ratios were above 78% for all samples tested.

## Rapid PCR Protocols for STR Typing: Less than one hour

Further goals in developing rapid PCR protocols have been to decrease the time required for amplification further to less than one hour. Vallone et al. performed rapid PCR with alternate polymerases and generated IdentiFiler profiles in less than 36 minutes using a GeneAmp PCR System 9700 and a two enzyme cocktail including SpeedSTAR and PyroSTART [20]. In this work, artifacts were observed as was incomplete adenylation for the 60 samples genotyped. Further work added Premix Ex Taq to the cocktail previously optimized for rapid thermal cycling of the IdentiFiler chemistry and reduced all occurrences of the previously observed incomplete adenylation and artifacts, while maintaining the rapid speed of amplification [21]. Five unique alternate polymerases in addition to AmpliTaq Gold (Phusion, AB77, AB95, AB-1, and AB-3) were tested with a rapid PCR protocol and generated full STR profiles in 46 minutes with the Veriti thermal cycler [22]. In this work, Phusion, AB77 and AB95 all produced PCR products which were non-adenylated and thus one basepair short of the commercial allelic ladder for the IdentiFiler and SGM Plus kits. AB-1 and AB-3 were found to be the best candidate polymerases for STR amplification.

Another modification in establishing faster thermal cycling protocols has been to reduce amplification thermal cycling parameters from a three-step protocol to a two-step thermal cycling protocol. Profiler Plus was the first commercial STR kit primers to have a manufacturer’s recommended 3-step thermal cycling profile modified into a 2-step profile [23]. This work evaluated three polymerases and focused on the SpeedSTAR HS buffer and polymerase to optimize a 2-step thermal cycling profile on the Bio-Rad C-1000 thermal cycler. Using a combination of cycling protocol changes and more efficient thermal cycler instrumentation and PCR reagents, the amplification of Profiler Plus profiles was achieved in less than 26 minutes. Additional work was performed to optimize a 2-step thermal cycling protocol for the amplification of IdentiFiler with the use of SpeedSTAR HS and the Bio-Rad C-1000 [24]. Results from this work were similar to Laurin and Fregeau [23], in that both Profiler Plus and IdentiFiler profiles could be produced in less than 26 minutes of amplification.

An evaluation of the differences between previously described 2-step [24] and 3-step [21] protocols across six unique commercial thermal cyclers demonstrated that concordant IdentiFiler protocols can be produced in as little as 14 minutes with the use of SpeedSTAR HS DNA polymerase and the Streck Philisa thermal cycling platform which has increased heating and cooling rates [25]. Artifacts observed within the 3-step protocols were greatly reduced across all thermal cyclers testing by employing the 2-step thermal cycling protocol. Assay conditions between the 2-step and 3-step protocols were robust enough to routinely amplify 250 pg to 500 pg of template DNA. The work in developing rapid PCR protocols and the evaluation of various PCR thermal cyclers may support validation for typing single-source samples in a databasing laboratory. An evaluation of the use of 2-step or 3-step PCR protocols for use with low template DNA (LT-DNA) was performed with IdentiFiler and IdentiFiler Plus commercial STR kits [26]. It was shown that the 2-step PCR protocol overall generated better STR typing results from LT-DNA samples compared with the 3-step protocol with no significant difference between the peak height ratios or stutter percentages between the two protocols.

A summary of the STR assay, polymerase, thermal cycler and corresponding cycling times for the references in Sections 3.1 and 3.2 can be found in Table 1. The number of PCR cycles for the times listed ranged from 28-32 (specific details are described and may be found in the corresponding reference).

# Direct PCR Protocols for STR Typing

## Commercial Direct PCR Kits for STR Typing

In recent years direct PCR STR kit formulations have shown the ability to bypass the extraction and quantitation stages in the forensic DNA workflow within reference laboratories to reduce the time required for sample throughput. Direct PCR kits such as PowerPlex 18D, IdentiFiler Direct, GlobalFiler Express, Investigator STR Go!, and ESSplex SE Go! have been commercially developed to meet this demand [27-29]. An internal validation of GlobalFiler Express recently demonstrated that both blood and buccal samples from FTA and non-FTA substrates can produce complete STR profiles with a 40 minute PCR [30,31]. Additionally, recent STR kits such as PowerPlex 16 HS, PowerPlex Fusion, IdentiFiler Plus, and PowerPlex ESI/ESX 16/17 Fast may be used to amplify extracted DNA *or* amplify DNA directly from blood or saliva samples in less than the three hours [32-35].

Direct PCR is advantageous for reducing processing time for single source samples such as blood or saliva on substrates within databasing laboratories. There has been examination of commercial kits and their ability to directly amplify targeted DNA collected on various collection substrates. Wang et al. reported the ability for the IdentFiler Direct chemistry to directly amplify DNA from a 1.2 mm blood punch on FTA paper as well as a 1.2 mm buccal punch taken from the Whatman EasiCollect which is also an FTA paper substrate [36]. A comparison of three commercially available STR kits (IdentiFiler Direct, IdentiFiler Plus, PowerPlex 18D) was performed evaluating the generation of complete profiles with the use of one or two 1.2 mm punches from the Whatman EasiCollect device [37]. Myers [37] found that IdentiFiler Direct and PowerPlex 18D performed similarly yielding ~96 % complete profiles on the first pass. Similarly, IdentiFiler Plus yielded a first pass success rate of 94 %, but about 15 % of the profiles displayed a “minus A” peak at one or more loci. Additionally, there was no observed difference between one or two punches in performance of IdentiFiler Direct or PowerPlex 18D [37].

An evaluation of PowerPlex 21 was performed testing 45 mock crime scene samples with a success rate of 80% generating complete profiles [38]. It was determined that employing direct PCR of appropriate crime scene samples would suggest a savings of 3 to 4 hours of hands on time for laboratory personnel.

### Modification of Commercial STR kits for Direct PCR

In addition to the testing of commercialized direct PCR kits, several laboratories have evaluated direct PCR protocols in combination with commercial STR kits which were not initially intended for direct PCR. In 2008, Park et al. tested primers from IdentiFiler, YFiler, and PowerPlex Y in combination with Direct-N-Elute elution buffer, AnyDirect Buffer, and HotStarTaq for 2.0 mm blood and saliva punches [39]. They reported that in many cases there was poor amplification with the saliva samples, but that blood punches routinely amplified and generated a compete STR profile. The Direct-N-Elute buffer is a pre-treatment solution in which the elution mix is then transferred into the PCR reaction. Similarly, Thermo Fisher and the Promega Corporation have developed Prep-N-Go, SwabSolution, and PunchSolution (respectively) for this same pre-treatment of samples prior to PCR with STR kits which have not been optimized for direct PCR [40-42].

Profiler Plus and NGM STR chemistries have been evaluated for use with direct PCR protocols for both punches and swab cuttings [43]. Linacre et al. evaluated SGM Plus and PowerPlex 16 for direct PCR from fabric, swabs, and trace/touch evidence [44]. Complete and partial profiles were generated from both the work produced by Templeton [43] and Linacre [44], with an observed decrease in time required for sample generation due to the elimination of the extraction, quantitation, and normalization steps in the forensic DNA typing process.

A summary of the STR assay, substrate, and thermal cycler for the direct PCR references can be found in Table 2.

### Rapid Direct PCR Protocols for STR Typing

Another source of increased speed for amplification results from performing direct PCR with the implementation of rapid polymerases, thermal cyclers, and optimized thermal cycling protocols. Verheij et al. examined the amplification of four commercial STR kits (SGM Plus, IdentiFiler, SEfiler, NGM) combined with the PIKO thermal cycler and Phusion Flash polymerase [45]. Direct amplification was performed in as little as 47 minutes using several on sample types (whole blood, saliva, semen, and mock caseworks samples). Products amplified with the method reported in Verheij et al. were non-adenylated, thus needed a modified allelic ladder to be properly analyzed. Additional work has been performed examining a rapid wire-based sampling method for rapid-direct PCR employing the use of an in-house 5plex assay, Piko thermal cycler, and Phusion polymerase to amplify DNA from hot wires used to collect blood clots and postmortem tissue samples [46].

More recent work has shown the ability to perform rapid-direct PCR in less than 16 minutes through the use of the Streck Philisa thermal cycler, Z-Taq polymerase, and Any Direct F Buffer to amplify a MP7 assay containing 6 STRs and Amelogenin [47]. Additionally, the use of the SpeedCycler2 thermal cycler amplified the MP7 assay in 19 minutes with the same buffer and polymerase cocktail. The PCR amplicons were analyzed on a Bioanalyzer 2100 and sized using an in-house macro. A concordance rate of 99.5% was observed for a total of 18 samples. A total processing time of 25 minutes was observed to include PCR, separation, and liquid handling.

A summary of the STR assay, substrate, and thermal cycler for the rapid-direct protocols can be found in Table 2.

# Additional Rapid PCR Protocols (non-STR related)

A brief sampling of work which demonstrates additional non-STR related rapid PCR protocols is described below. The work summarized below represents specific references often cited within articles in this review.

Real-Time PCR protocols have made an effort to become faster at detection. Liquid blood has been used in the past to generate ultrafast PCR allowing for the amplification of HIV RNA from blood in 555 seconds (9.25 min) with the use of *Thermococcus kodakaraensis* polymerase and the optimization of both electrical and chemical systems [48]. A term called “Hyper PCR” was used to describe a thermal cycler with a SYBR assay and SpeedSTAR HS polymerase used to identify adenovirus in 17 minutes (“Hyper PCR” in 10 min 36 sec and melt curve analysis in 6 min) and tested this protocol on 147 clinical samples with results obtained in considerably less time than conventional real-time PCR and a correct identification of all viral culture-positive samples [49]. Similarly, an ultra-fast thermal cycler was used to amplify synthetic SARS respiratory pathogenic targets in less than three minutes [50]. Lagally et al. demonstrated 280 nL PCR using touchdown PCR in 15 minutes for 30 cycles amplifying a 136 bp M13/puC16 cloning vector and a 231 bp human DNA fragment [51]. Each of these advancements within the clinical diagnostics community has the potential to influence the technologies and chemistries adapted for forensic genotype testing in a rapid manner.

Efforts have also been made to try to merge direct PCR with RT-PCR by using 0.5 mm buccal punch with the commercially available Quantifiler Trio chemistry, which successfully demonstrated that direct RT-PCR is possible with this chemistry [52].

Melt curve analysis and hybridization assays have shown to be successful in rapid amplification and analysis of DNA samples in less than 15 minutes [53]. Similarly, the use of HyBeacon probes for rapid genetic analysis has also been a common method of rapid genetic detection [54, 55], allowing for point-of-screening testing to be performed in less than an hour [56].

Recently, extreme PCR was demonstrated using single-copy genes from human genomic DNA with either the use of Klentaq1 polymerase or KAPA2G Fast DNA polymerase to amplify DNA fragments (45 bp to 102 bp fragments) in as little as 15 to 60 seconds in 35 cycles of PCR [57]. This work employed the use of a prototype instrument to thermal cycle samples in addition with increasing primer and polymerase concentrations 10- to 20-fold above typical concentrations in efforts to match the kinetics of primer annealing and polymerase extension with the faster thermal cycling [57].

# Rapid PCR on a Chip

Reduction of PCR volume and miniaturization of the thermal cycling component was a main focus of the microfluidic community to begin the design of lab-on-a-chip devices to attempt to integrate the forensic DNA typing process onto a microfluidic platform. The standard practice for on-chip PCR has been to amplify DNA within a single stationary chamber that is cycled through the three steps of thermal denaturation, annealing and extension [58-61]. New techniques have been developed to transfer the DNA solution through sequential heated chambers to avoid the need for changing the temperature of a single chamber [62, 63]. Additional methods for PCR amplification on a microfluidic chip platform include using hot water circuits and melt curve analysis to produce results in less than 3.5 minutes [64, 65]. The LightCycler instrument was shown to amplify a 300 bp fragment of λ-DNA with Z-Taq polymerase in less than 522 seconds (8.7 min) [66]. Neuzil et al. designed, fabricated and tested a real-time PCR chip capable of conducting one cycle of PCR in 8.5 seconds, which corresponds to 40 cycles of PCR in 5 minutes and 40 seconds [67]. This work corresponds to a heating rate of 175 °C/sec and a cooling rate of -125°C/sec.

Belgrader et al. report one of the first efforts of rapid STR typing using a battery-powered thermal cycler and the AmpFlSTR Blue STR system [68]. In this work, the miniature analytical thermal cycler instrument (MAT-CI) contained a PCR microchip, a solid-state optics system for real-time fluorescent detection, 13 NiCd batteries, and an electronic controller for computer interfacing. With this device the heating capabilities were 25% greater than a standard GeneAmp 9600 thermal cycler, reducing PCR thermal cycling times from 2.6 hours on a GeneAmp 9600 to 60 minutes with the MAT-CI [65]. Further work by Belgrade et al. demonstrated the ability to detect a single marker target in as little as 7 minutes with the Advanced Nucleic Acid Analyzer previously described [69].

Similar to Belgrader et al., a custom thermal cycler was designed by NetBio Inc. which allows for the amplification of Profiler Plus ID PCR amplification kit primers with the use of SpeedSTAR HS DNA polymerase and Takara’s 1x Fast Buffer I in 17.3 minutes [70]. The incorporation of SpeedSTAR HS and Takara’s Fast Buffer I in the PCR mastermix with the custom designed biochip, provides a decrease of 127.8 minutes in time required for conventional thermal cycling using TaqGold.

The use of customized hydrogel micro solid phase encapsulating assay mix (µSPEAM) beads manufactured with the 5-dye PowerPlex ESI 17 PCR amplification mix allowed for a decrease in PCR thermal cycling time from 2 hours and 43 minutes to 1 hour and 50 minutes [71]. Due to the reduction in fluidic complexity, the Peltier single chamber approach outlined in Zhang et al. affords the user flexibility with the relative ease in which it can be assimilated within a larger integrated micro-Total Analysis System µTAS [72]. For highly specific multiplexed PCR a Peltier-based method was utilized for heating and cooling rather than noncontact heating mechanisms that offer higher ramp rates, such as hot air cycling [73], or infrared radiation (IR) exposure [74].

Infrared radiation exposure for heating and fan cooling has been examined thoroughly, which has led to the development of glass microchambers for IR-mediated PCR were shown to have the ability to reduce PCR cycle time to <10 seconds per cycle [74]. Further work exhibited the ability to successfully detect amplified PCR products within 15 cycles, giving a total reaction time of just over 200 seconds for 0.1 ng of a 500 basepair fragment of λ-phage DNA [75]. Examination of commercial STR typing kits has also been performed with IR-mediated PCR to include MiniFiler in 1.5 hours with TaqGold, IdentiFiler with PyroStart Fast PCR mastermix and SpeedSTAR HS polymerase in 45 minutes [76].

DNA extraction and amplification has been shown to be successful on a microfluidic chip platform yielding partial profiles for IdentiFiler with Phusion Flash and SpeedSTAR HS polymerases for a buccal swab in 42 minutes [77]. Solid phase extraction paired with IR-mediated PCR was shown to successfully amplify the p16 gene in 25 minutes [78]. Additionally, the combination of IdentiFiler primers with Phusion Flash and SpeedSTAR HS polymerases was shown to yield full profiles when the extraction component was removed in 33 minutes [79].

Several review articles have been compiled examining the advances in microfluidic chip PCR and microfluidic devices [72, 80-82] for further information on this topic. A summary of each STR assay, polymerase, and thermal cycling platform referenced in Section 6 can be found in Table 3.

# Rapid Separation and Detection of STRs on a Chip Platform

Separation and detection on a microfluidic chip was an additional effort of the microfluidic and forensic communities to attempt to integrate all aspects of the forensic STR typing process. In 1997, Schmalzing et al. described a microchip CE device fabricated using silica wafers which demonstrated a quadruplex STR chemistry (CTTv) could be separated and analyzed with high accuracy in less than two minutes [83]. Additionally, separation of a single locus could take place in 30 seconds with this platform. Further work from this laboratory has demonstrated the ability to separate 16 samples amplified with the PowerPlex 16 chemistry in 40 minutes using a glass micro-capillary array electrophoresis (µCAE) platform with 20 cm channels [84]. A 95.8 % success rate observed for 96 samples run using the glass µCAE platform [84].

PowerPlex 1.1 ladders were separated in 20 minutes using microchip separation while PowerPlex 16 ladders could be separated in a single channel in 35 minutes [85]. A 12 channel device with a 10 cm separation channel is described to separate and base-call a 9plex assay in less than 30 minutes with greater than 10 fold improved sensitivity from standard CE devices commonly found within forensic laboratories [86]. Plastic µCAE devices have shown the ability to separate 32 samples simultaneously for the CTTv ladder in 10 minutes on a 4.5 cm long channel [87].

An examination of the use of µCAE devices for forensic STR separation over several years. In 2006, the separation of PowerPlex 16 and Profiler Plus PCR products could be performed in less than 30 minutes (28 min or 20 min, respectively) with a final sample volume of 1 nL [88]. In comparison, the PowerPlex 16 and Profiler Plus chemistries required 34 minutes or 30 minutes, respectively for separation and detection on a commercial ABI 310 CE as reported from Yeung et al. [88]. Additional work has further shown the capabilities of µCAE devices and has demonstrated rapid (~30 minute) separation of high quality genomic and mitochondrial DNA sequencing analysis with 1 bp resolution, and with 99% accuracy of 96 samples simultaneously [89-91]. Yeung et al. in 2009 used a biotin primer modified STR 9plex on a 4 channel µCAE device with a 10 cm effective separation length and determined a limit of detection of 25 copies required to still generate a full STR profile [92]. The µCAE device was additionally reported to successfully genotype 47 single source buccal samples as well as 19 non-probative casework samples for PowerPlex 16 and PowerPlex Y STR chemistries [93]. All 96 lanes of the chip were filled simultaneously with a total run time of 28 minutes. In 2010, the same lab developed a compact, laser-induced fluorescence detection scanner capable of separation and detection in a 7 cm chamber for 96 samples simultaneously, and integrated 160 nL PCR into the separation and detection µCAE device [94].

Additional work examining the ability to integrate PCR and µCAE microfluidic systems has been performed with the majority of the current focus in clinical diagnostics [95-97]. A summary of the each STR assay, chip substrate, separation path length and time can be found in Table 4.

# Fully Integrated Devices

Integration of all of the processes of the forensic DNA process is a challenging goal, but has shown to be possible in recent years. Several parallel efforts have been made to develop a device which will incorporate all of the forensic workflow and utilizes a simple swab in, answer out process. Reported success rates and run time of the devices in sections 8.1, 8.2, and 8.3 are summarized in Table 5.

## ParaDNA System

The ParaDNA Screening, from LCC Forensics (Middlesex, UK) is a device which is a presumptive DNA test with a documented sensitivity of 62.5 pg of DNA [98]. This device utilizes direct PCR with fluorescent HyBeacon melt analysis of two STR loci (TH01 and D16S539) along with Amelogenin and produces a DNA Detection Score (percentage) based on the total change in fluorescence. This score is related to the ability that samples have to produce a STR profile. The ParaDNA device produces results in the form of a DNA detection score within 75 minutes, thus possibly allowing for improved submission policies within a forensic DNA laboratory.

A concordance study between the ParaDNA Intelligence Test was conducted by Ball et al. in which 381 UK Caucasian samples were genotyped with the ParaDNA Intelligence Test and compared with SGM Plus [99]. The comparison demonstrated a 98.4% concordance between samples run with the ParaDNA Intelligence Test and conventional SGM Plus with standard laboratory equipment and manufacturer conditions. The ParaDNA Intelligence Test device allows non-expert users (using the same platform as described in Dawnay et al. [98]) to amplify five STRs and Amelogenin directly from human biological samples and evidence items collected from crime scenes.

## ANDE

The ANDE (accelerated nuclear DNA equipment) device, developed by NetBio Inc. (Waltham, MA), provides users with a fully integrated device to generate full STR profiles using the PowerPlex 16 chemistry within 84 minutes [100]. This device utilizes injection molded biochipsets and lyophilized reagents, with the ability to run five samples simultaneously on a disposable platform. Reagents have demonstrated a shelf life of 6 months at 22°C. The ANDE device contains an automated allele calling expert system and RFID sample tracking. Evaluation of the success of the CODIS core loci was at 85% for the 100 buccal swabs tested [100].

## RapidHIT 200

IntegenX Inc. (Pleasanton, CA) developed the RapidHIT 200 integrated device, which utilizes the PowerPlex 16HS chemistry denoted ‘PowerPlex 16 RapidHIT’ and produces profiles in less than 90 minutes [101-107]. The RapidHIT 200 allows for 5 to 7 buccal swabs to be run simultaneously. Success was defined as a full and concordant profile generated by the RapidHIT 200. There are various metrics within the literature for the measurement of success, whether measuring full profiles as success or number of total alleles called. Success rates ranged from 74% [105], 94% [106], 94.7% [102], 95% [104], to 95.3% [107]. Holland et al. observed 95.3% of samples run generate a full profile, with a concordance rate of 98.6% of alleles correctly called for those samples which produced a full profile by the instrument software for the PowerPlex 16 RapidHIT chemistry [107].

Alternate substrates to buccal swabs were also tested. Blood samples yielded a 94% success rate using the “Run Other” protocol [103]. Verheij et al. examined mock case work samples to include cigarette butts, cigar butts, drinking items, and chewing gum with success rates of 57%, 19%, 50%, and 54% respectively [104]. The developmental validation of the PowerPlex 16 RapidHIT yielded a success rate of 89% for producing full profiles with buccal samples using automated sample-to-profile processing with manual review of electropherograms [101].

Additionally, an upgraded RapidHIT 200 device is now available with the GlobalFiler Express chemistry and the ability to run 7 samples simultaneously [108]. Hennessy et al. demonstrate the ability to produce correct profiles for 150 reference buccal samples, as well as a sensitivity of 6,260 cells (37.5 ng) of sample input within the developmental validation for this device with the GlobalFiler Express chemistry [108].

##  Additional Integrated Devices

Hopwood et al. demonstrated a fully integrated device with the ability to obtain STR profiles from lysed buccal cells using a disposable plastic cartridge attached to a reusable glass microchip device without any manual interpretation [109]. This technology has the ability to process a single sample in under 4 hours, though currently the software is unable to routinely label all of the peaks due to a difference in the time base of the instrumentation and the NanoIdentity software [109].

While complete integration of the forensic workflow can be challenging, a few groups have made advances to integrate PCR and separation/detection. In 2011, Njoroge et al. reviewed microfluidic systems which were composed of two or more microdevices directed toward DNA analysis with a primary focus on the integration of various processing steps with microcapillary electrophoresis or microarrays [110].

Hurth et al. focused on integration of PCR and CE detection based on the design of the instrumentation from Hopwood et al. [109]; this employed a 3.5 hour PCR with 10 minutes CE [111]. Integration of PCR and µCE was demonstrated to take place in 60 minutes, with 45 minutes for PCR amplification of the PowerPlex 18 Fast chemistry and 15 minute CE separation on a 7cm array microchip [112]. This device has four channels which must run individually (due to the optics) and has an approximate 2 hour processing time (5 minute lysis, 45 minute PCR, 15 minute/lane CE) to include a half hour for sample and chip preparation and ≈90 minute runtime on the chip to process 4 samples. This platform provided a 73.3% success rate for correctly calling the CODIS 13 core loci. Additional work by Le Roux et al. focused on integration of liquid extraction (LE) with the existing PCR and µCE previously mentioned [113]. This work demonstrated sample to profile for both buccal samples and FTA samples with the PowerPlex 18 Fast chemistry with 100% concordance examining three donors.

Several versions of integrated PCR and CE devices have been reported [114-116]. In 2007, an assay with Amelogenin and 3 Y-STRs was reported with 4 samples using FastStart Taq polymerase [114]. This work utilized Basefinder 4.0 for raw data processing and required one and a half hours of processing time. In 2008 a 9plex assay (selection of PowerPlex 16 primers) using FastStart Taq polymerase was amplified, separated and detected on a microdevice with a 7 cm CE separation channel [115]. This process required 2 hours for PCR and 8 minutes for separation and detection. MegaBACE 1.2 was used for allele calling and resulted in a 2.5 hour total time for PCR, CE, and data analysis. Extraction was added into the system in 2011 for a total analysis time of three hours which includes 15 minutes for cell lysis, 45 minute DNA digestion and hybridization with biotin-labeled capture oligos, 40 minute of DNA template capture and washing, 40 minute of PCR amplification and 30 minutes of post-PCR cleanup prior to injection for CE [116]. This method can produce full 9plex STR profiles from 2.5 ng input standard DNA and obtain STR profiles from buccal swabs in about three hours.

Additional work has been performed to generate fully integrated devices which allow for the use of whole blood as a crude biological sample [117]. Though this work primarily focused on the ability to detect *Bacillus anthracis* and *Bordetella pertussis*, the integration of solid-phase extraction, followed by target sequence amplification by IR-PCR and microchip electrophoretic amplicon separation and detection was completed in less than 30 minutes and could possibly be modified to fit into the forensic discipline.

# Implementation

The establishment of rapid PCR protocols is one step in reducing the overall time required to generate a DNA profile. This method can be implemented in a laboratory setting for quick turnaround for a case, or in a systematic manner within a databasing laboratory for reference samples. An example of the implementation of a rapid DNA profiling service was performed in the Netherlands [118]. The use of direct PCR facilitated by Phusion Flash DNA polymerase and the use of the Piko cycler system with the AmpFlSTR SGM Plus STR genotyping chemistry, greatly reduced the time required to generate a complete STR profile. In this report, the procedure was evaluated in 15 cases which were submitted for this process. Twelve cases aimed to gain an investigative lead, one case provided potential to identify a victim of a homicide, one case hoped to obtain onus of proof prior to the custody release time, and the last case questioned if the victim could be placed at the suspect’s residence. For one of the 15 cases the results provided a name that resulted in an arrest of a suspect within 24 hours after the crime was committed. The implementation of rapid PCR protocols, direct PCR protocols, or integrated devices aims to provide the ability to greatly reduce thermal cycling times required for PCR amplification of STR loci as well as greatly reduce the overall forensic DNA typing process. This could be valuable for laboratories which need to produce a profile in a time-sensitive case for single-source samples, by employing either rapid protocols within the laboratory or by implementing the use of a fully integrated device for DNA genotyping.

In the realm of law enforcement, the inclusion of rapid PCR protocols in fully integrated devices is well suited to typing single source reference samples due to the absence of a DNA quantitation step required for casework applications. However, useful information may still be obtained for investigative leads by typing crime scene evidence. Generating DNA profiles in an automated fashion with the use of integrated devices has future applications at booking stations and in other field locations such as airports or border crossings. The ability to generate a DNA profile in less than two hours allows for many potential point-of-contact collection and STR typing scenarios.

# Funding and Disclaimers

This work was funded in part through interagency agreement between the National Institute of Justice and the NIST Office of Law Enforcements and between NIST and the FBI Biometrics Center of Excellence. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. Commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

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**Table 1 Summary of studies employing rapid PCR protocols.** The STR assay (or corresponding STR kit PCR primers), polymerase, thermal cycler for each reference and corresponding thermal cycling time required are listed below.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay/Primers** | **Polymerase** | **Thermal Cycler** | **Time (min)** | **Reference Number** |
| Expressmarker 16 | Hot start Q-Taq | Not Reported | 100 | 14 |
| Yfiler | AmpliTaq Gold Fast PCR Master Mix, UP(x2) | GeneAmp PCR System 9700 | 110 | 15 |
| IdentiFiler | AmpliTaq Gold | GeneAmp PCR System 9700 | 120 | 16 |
| PowerPlex S5 | HotStar Taq Plus | GeneAmp PCR System 9700 and Veriti | 60 | 17 |
| 26plex Y-STRs | Q5 High-Fidelity DNA Polymerase | GeneAmp PCR System 9700 | 60 | 18 |
| PowerPlex 16 HS | SpeedSTAR HS | Veriti | 60 | 19 |
| IdentiFiler | SpeedSTAR HS, PyroStart | GeneAmp PCR System 9700 | 36 | 20 |
| IdentiFiler | SpeedSTAR HS, PyroStart, Premix Ex Taq | GeneAmp PCR System 9700 | 36 | 21 |
| SGM Plus | AB-1 | Veriti | 46 | 22 |
| IdentiFiler | AB-1 | Veriti | 46 | 22 |
| ProFiler Plus | SpeedSTAR HS | Bio-Rad C-1000 | 26 | 23 |
| IdentiFiler | SpeedSTAR HS | Bio-Rad C-1000 | 26 | 24 |
| IdentiFiler | SpeedSTAR HS | Streck Philisa (and 5 additional platforms) | 14 | 25 |

**Table 2 Summary of studies employing direct and rapid-direct PCR protocols.** The STR assay (or corresponding STR kit PCR primers), DNA capture substrate, and thermal cycler are summarized in Table 2. Thermal cycling times are not summarized as these were not reported in the work with the exception of references: 29, 30, 31, 35, 45, 47 (all 55 min or less).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay/Primers** | **Substrate** | **Thermal Cycler** | **Time (min)** | **Reference Number** |
| IdentiFiler Direct | FTA and EasiCollect | GeneAmp PCR System 9700 |  | 27 |
| PowerPlex 18D | FTA and Whatman 903 | GeneAmp PCR System 9700 |  | 28 |
| Investigator STR Go!; ESSplex SE Go! | FTA | GeneAmp PCR System 9700 | 48-55 | 29 |
| GlobalFiler Express | Fitzco Card, FTA, Bode Buccal Collector, Whatman EasiCollect | GeneAmp PCR System 9700 | 40 | 30 |
| GlobalFiler Express | Various Tested | GeneAmp PCR System 9700 | 40 | 30 |
| PowerPlex Y23 | FTA; Whatman 903, Bode Buccal Collector | GeneAmp PCR System 9700 |  | 32 |
| PowerPlex 21 | FTA | GeneAmp PCR System 9700 |  | 33 |
| PowerPlex Fusion | FTA and Whatman 903 | GeneAmp PCR System 9700 |  | 34 |
| PowerPlex ESI 16/17 Fast; PowerPlex ESX 16/17 Fast | FTA; Whatman 903, Bode Buccal Collector; OmniSwab | GeneAmp PCR System 9700 | 50 | 35 |
| IdentiFiler Direct | FTA and EasiCollect | GeneAmp PCR System 9700 |  | 36 |
| IdentiFiler Direct; PowerPlex 18 D; IdentiFiler Plus | EasiCollect | GeneAmp PCR System 9700 |  | 37 |
| PowerPlex 21 | Various Tested | Veriti |  | 38 |
| IdentiFiler, Yfiler, PowerPlex Y | FTA | GeneAmp PCR System 9700 |  | 39 |
| Profiler Plus and NGM | FloQSwabs | GeneAmp PCR System 9700 |  | 43 |
| SGM Plus and PowerPlex 16 | Various Tested | GeneAmp PCR System 9700 |  | 44 |
| SGM Plus, IdentiFiler, SEFiler, NGM | Various Tested | 24-PIKO | 47 | 45 |
| DQα, 4plex, Profiler Plus | Human Tissue on wire | GeneAmp PCR System 9700 |  | 46 |
| MP7 Assay (6 STRs and Amelogenin) | FTA | SpeedCycler2; Philisa | 16 | 47 |

**Table 3 Summary of studies performing PCR of STR markers on a chip.** The STR assay (or corresponding STR kit PCR primers), polymerase, thermal cycling mode, and total cycling time for each reference are listed below.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay/Primers** | **Polymerase** | **Thermal Cycling** | **Time (min)** | **Reference Number** |
| AmpFlSTR Blue | AmpliTaq Gold | Miniature Analytical Thermal Cycler Instrument | 60 | 68 |
| Profiler Plus | SpeedSTAR | Custom Thermal Cycler | 17.3 | 70 |
| PowerPlex ESI 17 | Not Reported | Custom hydrogel µSPEAM beads | 110 | 71 |
| MiniFiler | Taq Gold | Non-contact Infrared | 90 | 76 |
| IdentiFiler | SpeedSTAR | Non-contact Infrared | 45 | 76 |
| IdentiFiler | Phusion Flash and SpeedSTAR HS | Non-contact Infrared | 42 | 77 |
| IdentiFiler | Phusion Flash and SpeedSTAR HS | Non-contact Infrared | 33 | 79 |

**Table 4 Summary of studies performing separation and detection of STR amplicons on a chip.** The amplified markers, chip substrate, separation path length, and total cycling time for each reference are listed below.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay/Primers** | **Chip Substrate** | **Separation Path Length (cm)** | **Time (min)** | **Reference Number** |
| CTTv quadruplex | Silica Wafers | 100 mm and 300 mm folded channel | 2 | 83 |
| PowerPlex 16 | Glass | 20 | 40 | 84 |
| PowerPlex 16 | Glass | 11.5 | 35 | 85 |
| PowerPlex 1.1 | Glass | 11.5 | 20 | 85 |
| 9plex Assay | Glass | 10 | 20 | 86 |
| CTTv quadruplex | Plastic | 4.5 | 10 | 87 |
| CXR ILS | Plastic | 4.5 | 11 | 87 |
| PowerPlex 16 | Glass | 15.9 | 28 | 88 |
| Profiler Plus | Glass | 15.9 | 20 | 88 |
| PowerPlex 16 | Glass | 15.9 | 30 | 91 |
| 9plex Assay | Glass | 10 | 40 | 92 |
| PowerPlex 16 | Glass | 15.9 | 28 | 93 |
| PowerPlex Y | Glass | 15.9 | 28 | 93 |
| PowerPlex 16 | Glass | 7 | Not Reported | 94 |
| 9plex Assay | Glass | 7 | Not Reported | 94 |

**Table 5 Summary of integrated platforms performing STR typing.** The device, STR assay, reported success rate, and total run time are summarized for each reference are summarized for all fully integrated platforms. \*Additional substrate types are reported within this reference; only buccal swab success is listed within Table 5.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Device** | **Assay** | **Success (%)** | **Time (min)** | **Reference Number** |
| ParaDNA | Screening Assay |  n/a | 75 | 98 |
| ParaDNA | Intelligence Assay | 98.4 | 75 | 99 |
| ANDE | PowerPlex 16 | 85 | 84 | 100 |
| RapidHIT 200 | PowerPlex 16 | 89 | 90 | 101 |
| RapidHIT 200 | PowerPlex 16 | 94.7 | 90 | 102 |
| RapidHIT 200 | PowerPlex 16 | 94 | 90 | 103 |
| RapidHIT 200 | PowerPlex 16 | 95\* | 90 | 104 |
| RapidHIT 200 | PowerPlex 16 | 74 | 90 | 105 |
| RapidHIT 200 | PowerPlex 16 | 94 | 90 | 106 |
| RapidHIT 200 | PowerPlex 16 | 95.3 | 90 | 107 |
| RapidHIT 200 | GlobalFiler Express | 100 | 120 | 108 |