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Ultrahigh speed direct PCR, A method for obtaining STR genotypes in 6 minutes

Bruce McCord, Georgiana Gibson-Daw, Patricia Albani, Karin Crenshaw, Mallory Baud, Dide Bolens, Ate Kloosterman, Roberta Marriot.

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PI Bruce McCord, Florida International University

Overview

The goal of this project was to develop rapid, direct screening methods for forensic DNA typing capable of amplification rates as fast as 6 minutes. The project utilized high speed thermal cyclers and examined a variety of rapid polymerases with a goal to produce multiplex PCR amplifications of STRs faster than ever reported. Analysis was performed by standard capillary based methods as well as through the use of a specially modified microfluidic system (dual laser Agilent 2100 with heat plate) capable of 80 second high resolution separations. The project goal was to push the technology to permit high speed direct analysis of human DNA with no extractions. Over the course of these experiments, we ultimately achieved multiplexed STR amplifications as fast as 6.5 minutes using extracted DNA, and 8.5 minutes using direct PCR of diluted saliva with no extraction. The results of this project can be used to develop quick screening of suspects at ports of entry or for identification in criminal casework.

Introduction

It is often important to rapidly screen suspects who may have been involved in a crime, especially when time is of the essence or when large numbers of samples may need to be quickly processed. These situations create a need for rapid screening of DNA samples. While current DNA typing methods provide the best biometric information yielding identity, kinship and geographical origin, they are not sufficiently fast to permit identification of a suspect's DNA in real time. Rapid polymerase chain reaction (PCR) procedures [Vallone 2008, Vallone 2009, Giese 2009] can greatly speed the processing time of forensic samples [Verheij 2012] because of the high processivity of newly designed fast polymerases, particularly when combined with newly developed rapid thermocyclers and microfluidics. The decrease in processing time and minimal use of reagents can lead to improved processing of forensic samples [Vallone 2008, Vallone 2009, Aboud 2013]. However, the current generation of rapid PCR systems are expensive to utilize, and most require an extraction step prior to analysis [Mapes 2016].

Small volume microfluidic systems have also been produced which can be rapidly heated and cooled [Belgrader 1999, Giordano 2001]. This technology includes lab on a chip based systems in which amplification is performed along with separation and detection in a single integrated device [Easley 2006, Liu 2007, Liu 2011] with total times ranging from 20 to 90 minutes [Khandurina 2000, Hopwood 2010, Estes 2012, Lounsbury 2013]. While much research has been done on improving the instrumentation used in PCR amplification, cycling conditions and the use of newly developed faster polymerases [Butss 2014, Romsos 2015], little work has focused on the actual optimization of the amplification chemistry. We have found that the speed of these analyses can be greatly increased by utilizing a smaller set of PCR amplicons along with new fast polymerases and fast thermocyclers [Aboud 2013]. Specially engineered enzymes [Wang 2004, Giese 2009, Kermekchiev 2009], high speed thermal cyclers (capable of running 28 cycles in 16 minutes) and microfluidic chip electrophoresis [Woolley 1998, Shi 2003, Goedecke 2004, Shi 2006, Hopwood 2010] permit the development of rapid and direct PCR. When used for sample screening, such a system could rapidly triage different suspects, excluding those not involved in criminal activities. Furthermore, such a system could be useful in situations such as mass disasters where it is necessary to search for close relatives or to analyse evidence that is comingled.

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There were 3 different segments to this project In the first part of this project we used Y chromosomal short tandem repeat (Y-STR) typing to develop a rapid method for screening male DNA samples. The markers selected for this project show a higher polymorphism rate than other Y-STRs and so are known as rapidly mutating Y-STRs (RM Y-STRs) [Ballantyne 2012]. These loci should prove particularly useful in sample screening where related individuals are present [Redd 2002]. The second part of this study was to develop a rapid and direct screening method for determination of a short STR multiplex. The autosomal multiplex we developed included the STR loci D5S181, D13S317, D7S820, CSF1PO, D16S539, Penta D and Amelogenin with amplicon sizes between 106 and 454 bp. This multiplex set was specifically designed to be run on microfluidic chips as well as larger fluorescent genotyping systems [Aboud 2013]. Here our aim was to utilize experimental optimization procedures to produce a rapid and robust multiplex PCR that would permit high speed DNA amplification using a relatively low reaction volume. This process was performed using both extracted and non extracted samples. Lastly to further increase the speed of direct PCR of non-extracted samples, we created a set of 8 miniSTRs for use with capillary based systems.

The thermal cycler used for the STR amplifications was the Philisa from Streck (Nebraska, US). The polymerases used were Z-Taq and Omnitaq. The use of these along with buffer optimization and addition of enhancers such as the PEC-1 (PCR enhancer cocktail 1) permits the rapid amplification of DNA straight from saliva with no extraction steps being necessary. By using off the shelf instruments and commercially available enzymes it was possible to create a procedure that acts as a quick, highly informative sample screening process that also retains sufficient DNA for later manual processing using standard STR or Y-STR kits.

Rapid microfluidic analysis of a Y-STR multiplex for screening of forensic samples

A Y-STR multiplex was developed and named RM4 (Rapidly mutating 4 loci) and it consisted of 4 sets of primers which anneal to separate 5 loci: DYS526a and DYS526 b, DYS570, DYS576 and DYS626. After Optimization the multiplex was amplified in a final PCR master mix volume of 9 µL, to which 1 ng of DNA was added. The master mix was made up of 2 X Z-Taq buffer (Takara Bio), 0.2 mM each dNTPs (Takara Bio), TAMRA fluorescently labelled primers at concentrations of 0.72 µM DYS526, 0.19 µM DYS570, 0.48 µM DYS576 and 0.60 µM DYS626 (IDT), 0.0625 Units/10µL of Z-Taq polymerase (Takara Bio). DNA used for this experiment was control DNA 2800, 9948 and 20 extracted DNA samples donated by volunteers.

Amplification was performed on the Philisa thermocycler (Streck) in 16min 11s. Optimal conditions included heat denaturation at 95^{o}C for 5 s, followed by 29 cycles of 98^{o}C for 5 s, 62^{o}C for 9 s, 72^{o}C for 12 s. Master mix variations tested focused on the amount of Z-Taq and primers used as well as the addition of BSA. These were as follows: A) 0.3 µL Z-Taq +2 µL primer mix, B) 0.2 Z-Taq +2 µL primer mix, C) 0.2 µL Z-Taq + 1.5 µL primer mix, D) 0.3 µL Z-Taq + 1.5 µL primer mix, E) 0.25 µL Z-Taq +2 µL primer mix, F) 0.25 µL Z-Taq +2 µL primer mix + 0.5 µL BSA, G) 0.25 µL Z-Taq + 2 µL primer mix + 0.5 µL BSA. All samples were analysed on the ABI 310 using the following conditions: POP4, Capillary length to detector: 36 cm, module GS STR POP4 (1 mL), injection voltage 15 kV, injection time 5 s, run temperature 60^{o}C, run voltage 15 kV, Figure 1.

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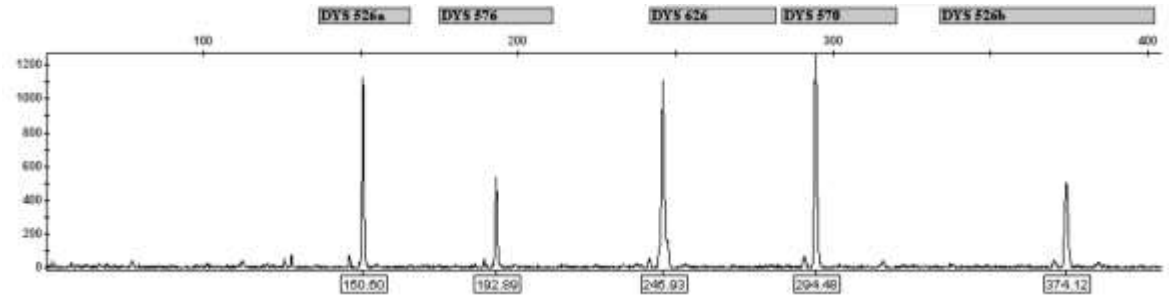

Figure 1. Rapid Y Multiplex run with extracted DNA at an input concentration of 1ng. Run on ABI 310

A modified Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) equipped with a heat plate and denaturing polymer also was used in these experiments and compared with a standard ABI 310 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA)). In the device electrophoresis takes place on a glass microchip with a 1.8 cm separation channel. Up to 12 samples may be analyzed in series, with microfluidic separations of all four loci in under 80 seconds. The system utilizes a single excitation wavelength of 532 nm, emission wavelengths of 575 nm and 685 nm and a voltage of 1500 V for sample detection. The two fluorescent dyes utilized in these experiments were TAMRA and LIZ. DNA amplicons were labelled with TAMRA dye and GeneScan LIZ 500 Size Standard was used as an internal lane standard in the red channel for sizing precision(26). The chip was prepared by pipetting 15 µL of the denaturing polymer from the Agilent high resolution DNA reagents kit and then pressure was applied with a syringe for 4 minutes to allow the polymer to flow through all areas of the chips channels. 15 µL of the polymer were then added to the G and ladder wells. Sensitivity studies were also completed with input levels ranging from 2 ng-0.5 ng. These gave useable profiles, however the 0.25 ng sample showed low threshold peaks (< 150 RFU). That said, all peaks were still present even at 150 pg of input DNA.

Mixtures of male and female DNA were tested and profiles of the 9948 male DNA were successfully produced for all ratios of female to male DNA from 0.25:1 up to 40:1. The peak balance remained consistent throughout all ratios tested however the peak heights did decrease slightly when more than a 20:1 ratio of female to male DNA was used. Test of up to 40:1 female to male DNA still produced DNA profiles. Figure 2 shows the profile generated for the 40:1 ratio of female to male DNA. Results showed a standard deviation of 0.14 and 12 for peak size and height respectively.

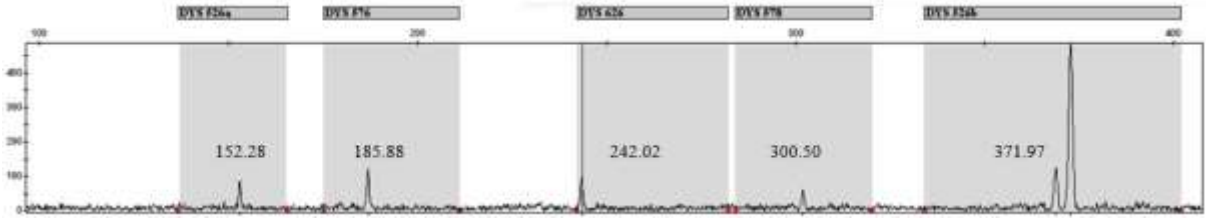

Figure 2. 40:1 ratio of female 9947 DNA to male 9948 DNA. Run on ABI 310.

The above data shows the successful creation of a 4 loci Y-STR multiplex that can be used to screen crime scene samples for male DNA. We have also demonstrated how the combination of a high speed thermal cycler (Streck) and a rapid polymerase (Takara) permits the decrease of amplification time for the Y-STR multiplex to under 17 minutes. This can then be coupled with a microfluidic separation device to further reduce analysis time to under 25 min per profile after sample extraction. This procedure should prove useful for screening sexual assault casework and exclusion/presumptive identification of male suspects.

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Optimization of ultrahigh speed multiplex PCR for forensic analysis.

A custom multiplex (MP7) was assembled from 7 Loci chosen from a subset of standard forensic STR loci containing 4 and 5 base repeat motifs. This 7 locus multiplex has a power of discrimination of 1 X 106 or greater, which is sufficient to identify a potential suspect, once identified a full laboratory analysis would be required using standard genotyping methods to confirm a match. The loci were selected based on their size and relatively low level of sequence variants (improved electrophoretic resolution) and included D5S818 (D5), D13S317 (D13), D7S820 (D7), D16S539 (D16), CSF1PO (CSF), and Penta D, as well as the Amelogenin locus. The Amelogenin (AM) locus was labelled with Liz fluorescent dye, while the other six of the loci were labelled with the TAMRA fluorescent dye (IDT). These loci were chosen based on size, primer melting temperature, and low levels of variant alleles. The multiplex was designed to be compatible with a two-channel microfluidic chip based system (modified Bioanalyzer) as well as the Life technologies capillary based DNA sequencers.

The optimized process used to obtain ultrafast PCR amplification times was as follows: 3 µL of Master Mix X with1 µL of 50 ng DNA (K562, Promega) to give a total volume of 4 µL. This master mix included 1 µL 10 X Z-Taq buffer (Takara Bio), 0.8 µL 2.5 mM each dNTPs (Takara Bio), 4.9 µL Primer mix made up of 10 µM TAMRA fluorescently labelled primers 3.25 µL D5S818, 3.25 µL D13S317, 6 µL D7S820, 4 µL D16S539, 3.5 µL CSF1PO, 9 µL Penta D and 4 µL Amelogenin. (IDT), 1 µL 25 mM MgCl2 and 0.75 Units/10µL of Z-Taq polymerase (Takara Bio). Amplifications were performed on a Philisa thermocycler (Streck) in just under 6.5 min. Optimal conditions were 24 cycles of heat denaturation at 98^{o}C for 2 s, followed by a combined annealing elongation step of 62^{o}C for 7 s. All PCR products were analysed using an ABI 310 under the same run conditions as previous samples, Figure 3.

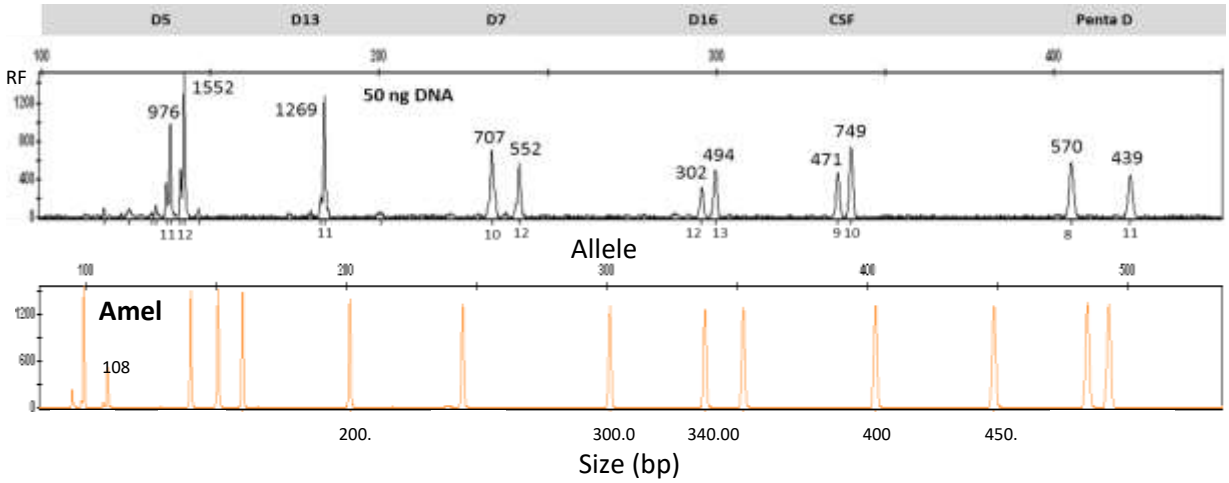

Figure 3. Profile generated from 50 ng of the K562 control DNA standard. The top lane shows the 6 STR loci labelled with TAMRA dye, while the bottom lane shows the orange lane with the ILS Liz 500 and the Amelogenin locus labelled with Liz. Overall peak balance was 68.4%. Samples analysed using ABI 310).

Sensitivity studies were performed to show the process would work with lower concentrations of input DNA, using a series of dilutions using the K562 control DNA. Input levels ranging from 100 ng-2 ng gave useable profiles however samples below 2 ng of input DNA showed low threshold peaks indistinguishable from the noise, for this as with all previous experiments the calling threshold was set at 100 RFUs, Amelogenin while not shown here was successfully amplified in every sample. As expected peak height was observed to decrease with decrease of input DNA and thus, the recommended minimum input level for this procedure was >2 ng of DNA, Figure 4.

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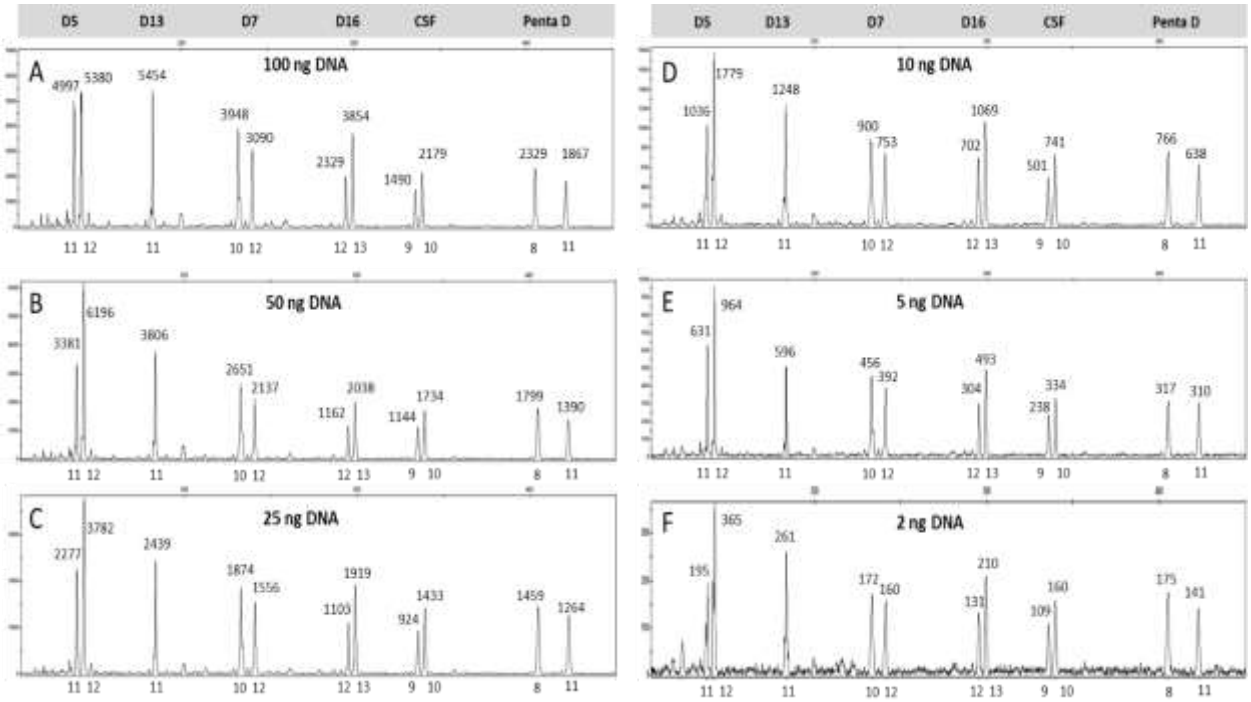
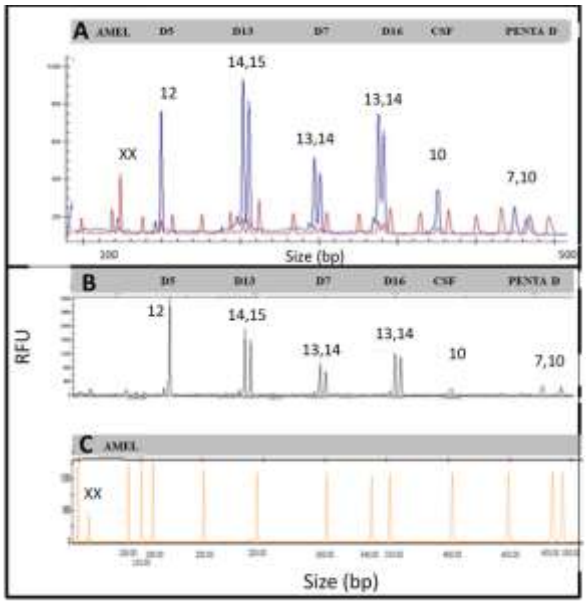


Figure 4. Profiles obtained from optimized method as the amount of input DNA (K562 control standard) was reduced from 100 ng to 2ng. Overall peak balances for the respective profiles are as follow: A) 76.0%, B) 67.1%, C) 70.4%, D) 71.7%, E) 76.8%, F) 71.5%. Scale in RFUs can be seen on the left of each electropherogram and is set to the largest peak height in order to improve visual determination of peak height and threshold. Samples analyzed using ABI 310.



To show the process works successfully for real world samples a study population of 30 volunteers was used to test the applicability of the developed method for the use with cheek swab samples. Of these nineteen cheek swabs were also examined using microfluidic electrophoresis on a modified Agilent 2100 Bioanalyzer, which included a heat plate to permit multichannel denaturing electrophoresis and detection with an excitation wavelength of 532nm, and detection at 575 and 670 nm. Run time for the chip separation was 80 seconds. The resulting electropherogram was compared to that obtained from the ABI 310 CE, Figure 5. Overall peak height ratio and balance for heterozygous loci were compared on each system resulting in an overall profile balance ratio of 82.4% +/- 6.6% (standard deviation) for the ABI310 while

Figure 5. A comparison of rapid PCR results using both ABI 310 and modified Agilent 2100.

the chip system had an overall profile balance ratio of 84.6 +/- 7.4. In addition, all allele calls were concordant between the two systems. This data demonstrates shows that either system may be used to obtain accurate allele calls from the PCR products.

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The above data demonstrates the successful development of an ultrafast amplification of a 7 Loci multiplex in 6.5 minutes. This was achieved through the use of a high-speed thermocycler and rapid polymerase and involved the optimization of cycling conditions, reagent concentrations and sample volume. Once developed, this method was tested with DNA samples extracted from cheek swabs of 30 volunteers. The sensitivity of the method was also tested, and showed that complete profiles could be generated using DNA quantities as low as 2ng. Overall, this method provides an ultrafast amplification that can speed up the processing of forensic cheek swabs while still leaving more than sufficient sample for later analysis and confirmation.

Development of Rapid Direct PCR amplification

Using the MP7 multplex previously described we worked to develop a rapid and direct PCR method to speed up the genotyping process even further by removing the extraction step. The initial procedure utilized a master mix with a 10 µL reaction that included : 0.2 X OmniTaq polymerase, primer mix (0.46 µM D5S818, 0.36 µM D13S317, 0.54 µM D7S820, 0.46 µM D16S539, 0.36 µM CSF1PO, 2.11 µM Penta D and 0.54 µM Amelogenin), 1.2 X Taq buffer, 3 mM MgCl2 0.1 X PEC-1, 0.31 mM each dNTP and 0.3 mg/mL BSA. 2µL of diluted to 10 % saliva was added to 8 µL of this master mix, giving a total volume of 10 µL. Amplifications were performed on a Philisa thermocycler (Streck) with an initial cycling time of 13.5 min using 32 cycles of 98ºC for 3 seconds followed by 62 ºC for 16 seconds. All PCR products were analysed using an ABI 310 (Applied Biosystems) by adding 1 µL of PCR product to a mixture of 12.5 µL HiDiTM Formamide (Applied Biosystems) and 0.5 µL internal lane standard (ILS). The ILS used throughout this paper was LIZ 500 (Applied Biosystems) which was labelled with the orange LIZ dye. This mix was then run on the ABI 310 under the same conditions as previous samples, Figure 6.

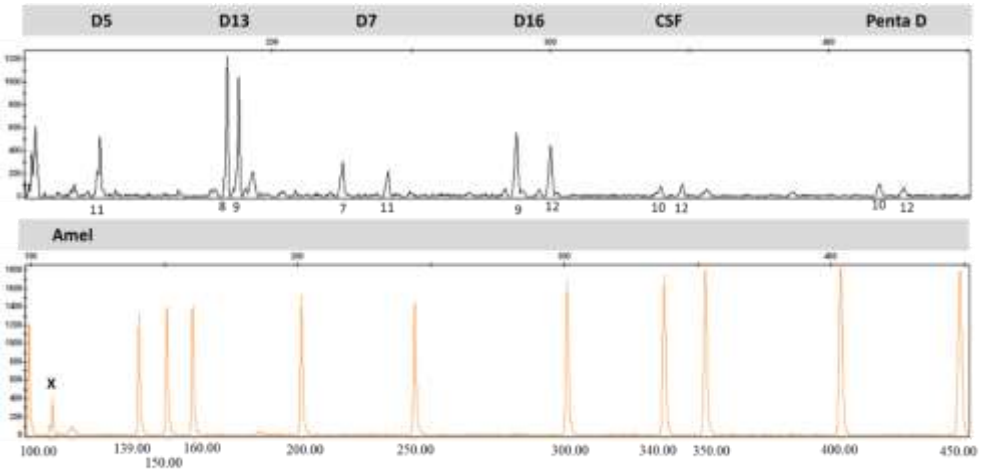

Figure6, initial profile generated from dilutes saliva following a 13.5 minute amplification. The top lane shows the 6 STR loci labelled with TAMRA dye, while the bottom lane shows the orange lane with the ILS Liz 500 and the Amelogenin locus labelled with Liz. This profile was the starting point for further optimization of amplification speed and peak balance.

The above data shows the amplification of a sample of 10% diluted saliva in 13.5 minutes.

The next goal of the project was to optimize the speed, sensitivity, and balance of this PCR protocol [Gibson-Daw 2018] to create an even faster direct method for the analysis of DNA samples. Experimental design software, Design Expert 10, was utilized to further optimize the PCR reaction using overall peak height as the main parameter. It was established that the best combination of the ratios of each primer:

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were 2.2 µl D5S818, 2.4 µl D13S317, 4.8 µl D7S820, 5 µl D16S539, 7.1 µl CSF1PO, 15.5 µl Penta D and 2.5 µl Amelogenin (each primers has a concentration of 10mM). These conditions were tested and optimized using a variety of PCR parameters. The denaturation step was kept at 98°C for 3 seconds during the entire experiments. However, the annealing/extension and the number of cycles were changed. Ultimately the conditions were optimized to provide a 98°C denaturation step for 3 seconds with a 62°C extension steop for 12 seconds with 31 cycles at a total run time of 11 minutes 31 seconds, Figure 7.

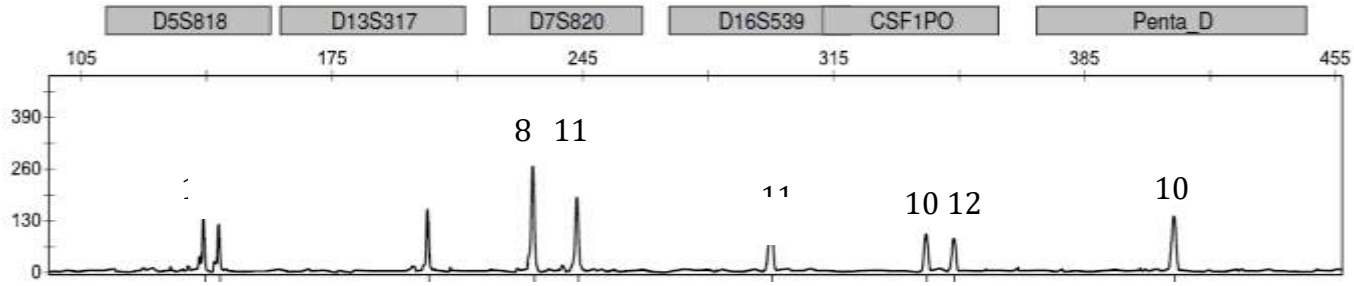

Figure 7. Optimization of direct PCR amplification of saliva using Omnitaq polymerase with a Streck Phillisa thermalcycler. Bottom panel shows an 11.31 min amplification.

As mentioned earlier, rapid direct PCR means that there is no extraction and no quantification. These experiments were initiated using 7% saliva , which was centrifuged to produce 1.4 µl of pelleted saliva and 20 µl of H2O. As the amplification speed was increased, we began to lose sensitivity. Further experiments indicated that 20-25% DNA concentration produced optimal results.

Experiments with MiniSTRs

Increasing the PCR reaction speed of the MP7 STR multiplex beyond 11 minutes resulted in allele loss and peak dropout, particularly for larger alleles. As a result, the last part of this project examined the potential for increasing amplification speed using a multiplex of 8 miniSTRs. A new set of primers were designed based on previously funded NIJ projects. The amplification of this multiplex was then optimized as part of a collaborative project with the University of Amsterdam. The multiplex included the following STR loci: D2S1338, D21S11, D10S1248, FGA, D8S1179, D7S820, D2S441, D18S51 and amelogenin. This permitted the system to be used in a 5 dye genetic analyzer with 4 lanes reserved for STRs and one additional lane for the allelic size standard. Two additional mini STR loci were designed for a 6 dye system but were not tested in this paper. Optimization was performed using experimental design software to reduce the overall amplification time. The procedure involved direct amplification of 70-100% saliva added to the PCR cocktail. Cycling conditions involved 30 cycles of 98 °C for 2 sec and 62 for 7 sec for a total amplification time of 7 minutes and 38 seconds.

Conclusions

The overall goal of this project was to develop a rapid PCR method that could amplify both Y and autosomal multiplexes in the quickest possible time. These goals were achieved with the use of the Philisa rapid thermocycler combined with rapid polymerases Z-Taq and Omnitaq and the addition of BSA and PEC-1.

A 4 locus Y-STR multiplex used for screening sexual assault samples for male DNA was amplified in less than 17 minutes by combining the Philisa rapid thermocycler with the use of the rapid Z-Taq polymerase and buffer. Next, a 7 locus autosomal STR multiplex was successfully amplified in 6.5 minutes also using the Philisa thermocycler and Z-Taq polymerase and buffer. This method was validated with a small test

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population of 30 volunteers and was shown to be sensitive down to 2 ng of input DNA. A rapid direct method was also developed for the MP7 multiplex In which saliva was added directly to the PCR reaction thus eliminating an extraction step and further reducing analysis time.

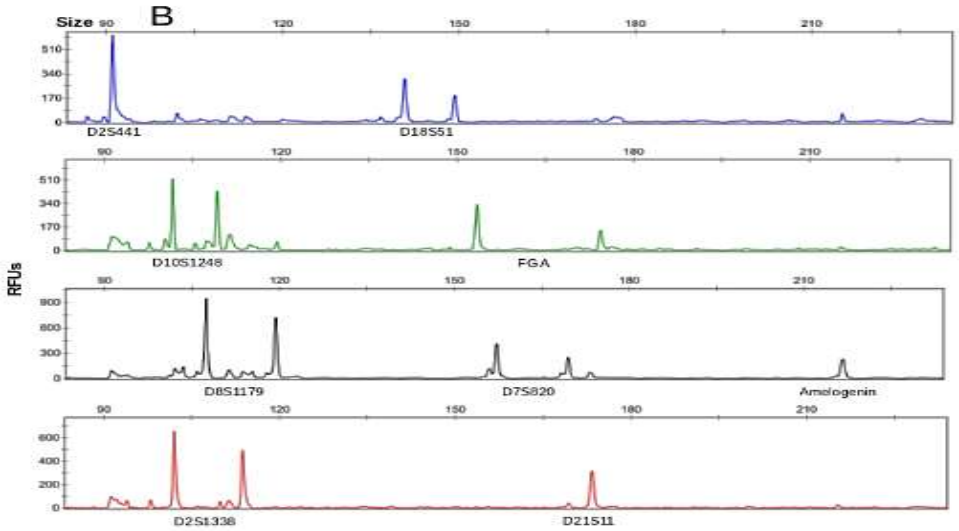

Figure 8. DNA profile obtained with rapid direct two step PCR, cycling condition M (30 cycles of 98 °C for 2 sec and 62 °C for 7 sec), input 100% saliva. Amplification time of 7 minutes and 38 seconds.

Table 1 Run times for various STR kits

STR kit	# loci	Extraction	Amplification time	Separation time	Overall run time (excluding transfer time)
Powerplex 16	16	2 hrs	89 min	0.5 hrs	3h 59 min
Global Filer	24	2 hrs	59.33 min	0.5 hr	3h 29.3 min
Powerplex Fusion 6c	24	2 hrs	42.42 min	0.5 hr	3h 12.4 min
Global filer Direct	24	n/a	24.40 min	0.5 hr	54.4 min
Rapid DNA	24	unk	unk	unk	90 min
2 dye 7 plex extracted	7	2hrs	6.5 min	0.5 hr - CE 80 sec Chip	2h 36.5 min 2hr 8 min
2 dye 7 plex direct	7	n/a	11.5 min	0.5 hr - CE 80 sec Chip	41.5 min 12.34 min
5 dye direct 8 Plex	8 mini STRs	n/a	7.5 min	0.5 hr.	37.5 min

Extraction time estimated based on robotic extraction data. Data on analysis times taken from manufacturer's user manuals. Direct kits (n/a) require no extraction. Rapid DNA systems such as Rapid HIT ID (unk) use a continuous process from sample in to result.

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For this rapid direct method amplification was achieved in 11 minutes 31 sec. An even further decrease in amplification time was achieved through primer redesign. By keeping the maximum STR length under 220bp, a more rapid direct amplification was achieved using 100% saliva added directly to the PCR tube in 7 minutes 38 seconds. **These methods demonstrate the fastest multiplexed STR amplifications yet reported in the literature, Table 1.** They could easily be implemented in the field as they require minimal equipment, reagents and sample handling, while providing robust and reproducible results with a good power of discrimination. Their use could be invaluable to produce fast results at border crossings, police stations and in situations in which rapid screening of potential suspect DNA is needed.

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