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Interrogation of short tandem repeats using fluorescent probes and melting curve analysis: A step towards rapid DNA identity screening

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Abstract

Current forensic DNA profiling methods rely on the analysis of samples at specialised laboratories with an average turnaround time of several days. The ability to rapidly determine a partial profile of short tandem repeats at the point-of-arrest would be of great benefit to police forces around the world, for example enabling a suspect to be rapidly included or excluded from an investigation. We have developed a homogeneous PCR method for the interrogation of STR loci utilising fluorescent oligonucleotide probes and melting curve analysis. Alleles of the D18S51, TH01 and D8S1179 loci were differentiated and identified on the basis of target length and probe melting temperature. Assay performance was evaluated by comparing melting peak data with the AmpFlSTR® SGM Plus® system. The method is compatible with direct analysis of unpurified buccal swab samples, enabling a partial STR profile to be generated within 1 h.

Keywords: Short tandem repeats (STRs); Fluorescent probe; HyBeacon; Melting curve analysis; Real-time PCR

1. Introduction

In the UK, forensic DNA profiling commonly utilises ten short tandem repeat (STR) loci and the gender marker Amelogenin [1]. The method currently consists of DNA extraction, a 28-cycle PCR using fluorescent dye-linked primers and capillary electrophoresis-based size separation of amplified products. The requirement for multiple processes and instruments means that STR profiling is predominantly confined to specialist laboratories with the consequential restriction on the turnaround times that can ultimately be achieved. It is possible for laboratories to generate forensic profiles in as little as a single working day for urgent cases. However, it is extremely difficult to rapidly analyse *ad-hoc* urgent samples alongside a high-throughput routine forensic service. Furthermore, if the time from sample collection to arrival at the laboratory is taken into account, it is clear that a rapid and portable system capable of forensic DNA analysis would be a valuable addition to existing procedures.

Assay contamination is an important factor to consider when developing forensic tests that could be employed at the point-of-screening (e.g. at the point-of-arrest), particularly when amplified products must be transferred between instrumentation [2–4]. A homogeneous system that enables target amplification and detection to be performed in a single reaction vessel will prevent cross-contamination and reduce test duration, permitting forensic analysis outside of specialised laboratories [5,6]. Test duration and complexity may be reduced further if unpurified saliva or swab samples are exploited.

We have developed a homogeneous method for STR analysis, employing a fluorescent oligonucleotide probe and melting curve analysis. HyBeacon probes are included in PCR assays and emit greater amounts of fluorescence when hybridised to complementary target sequences than when single-stranded [7,8]. The stability and melting temperature of hybridised probes depend on the degree of homology between probes and their target sequences. Increasing the reaction temperature above the melting temperature (T_m) of the probe causes probe/target duplexes to dissociate and the level of fluorescence emission to decrease.

The D18S51 (GenBank Accession No. X91254), TH01 (GenBank Accession No. D00269) and D8S1179 (GenBank

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Accession No. AF216671) STR loci were used as model systems to determine the ability of HyBeacon probes combined with melting curve analysis to differentiate and identify repeat alleles on the basis of target length and T_m. The D18S51 and TH01 loci are among the least complicated STRs used for forensic profiling, consisting of multiple AGAA and AATG tetranucleotide repeats, respectively. Whilst D18S51 alleles have been reported to range from 5 to 31.2 repeats, only the complete repeats between 11 and 21 exhibit frequencies greater than 0.01 (determined using the ALlele FREquency Database) [9–11]. As such, detection of published D18S51 partial repeats, such as 13.2, may not be required at the point-of-screening. Analysis of only the eleven common D18S51 targets permits detection of 97.8% of the alleles worldwide (based on weighted averages of published frequency data). Similarly, the 6, 7, 8, 9, 9.3 and 10 repeat alleles of TH01 are the most common, covering approximately 99.8% of alleles worldwide [10,11]. The sequence reported for the 9.3 repeat allele is $[AATG]_6AT-G[AATG]_3$ and comprises an internal partial repeat [12,13]. Analysis of the common D8S1179 alleles possessing between 8 and 17 repeats (excluding partial repeats) permits detection of 99.9% of worldwide alleles [10,11]. D8S1179 alleles have been reported to possess both TCTA and TCTG sequence repeats [12,14,15].

The examination of a large number of tandem repeats requires a probe able to identify the full range of alleles on the basis of T_m, occasionally requiring differentiation of targets, such as the TH01 9.3 and 10 repeat alleles, which vary in length by only a single nucleotide. A long probe of at least 84 nucleotides is required to detect the full range of common D18S51 alleles; however, melting curve analysis using long probes does not permit reliable differentiation and identification of STR alleles since targets of similar length exhibit extremely small melting temperature differences (ΔT_m). The difficulty comes particularly in the distinction between high numbers of repeats since a length polymorphism results in a considerably reduced ΔT_m with oligonucleotides of 40–80 bases compared with conventional probe systems of approximately 20 nucleotides.

Differentiation of long STR alleles is improved by limiting the number of repeats available for probe hybridisation. We have achieved this by employing non-fluorescent blocker oligonucleotides which act to reduce the effective target length, permitting the use of shorter probes. The fluorescent probe and blocker oligonucleotides used for STR analysis comprise repetitive and non-repetitive sequence components (Fig. 1). The non-repetitive portions of the oligonucleotides function as an anchor and are required to ensure the specificity of hybridisation, preventing slippage along the STR target and the generation of broad and noisy melting peaks.

2. Methods

Buccal swab samples were collected from donors whose STR profiles had been previously determined using the Applied Biosystems AmpFlSTR® SGM Plus® PCR amplification kit and ABI PRISM® 3100 Genetic Analyzer. Appropriate consent was obtained and studies carried out in accordance with the Data Protection Act 1998. DNA was extracted from swabs using protocols and reagents from the QIAamp DNA Blood Mini Kit (QIAGEN, Crawley, UK). DNA quantification was performed using a QUANT-IT™ PicoGreen® dsDNA assay kit (Invitrogen Ltd., Paisley, UK) and a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Leicestershire, UK).

PCR volumes were 10 μl, containing 2 μl purified DNA (between 1 and 10 ng/μl concentration), 1× QIAGEN PCR buffer, 0.5 units HotStarTaq polymerase (QIAGEN, Crawley, UK), and 1 mM dNTPs (0.25 mM each—GE Healthcare, Amersham, UK). D18S51 assays also contained 1 μM forward primer, 0.1 μM reverse primer, 150 nM probe and 225 nM non-fluorescent blocker oligonucleotide. TH01 and D8S1179 reactions utilised 0.1 μM forward primer, 1 μM reverse primer, 75 nM fluorescent probe and 375 nM blocker. The sequences of primers, probe and blocker oligonucleotides are detailed in Table 1. The TH01 probe included a 5′ trimethoxystilbene oligonucleotide cap to increase melting temperature [16,17]. Asymmetric PCR was utilised to generate an excess of the target strand such that probe hybridisation was favoured over annealing of amplified sequences. 10 μl chill-out liquid wax (Bio-Rad Laboratories Ltd.; Hertfordshire, UK) was added to each utilised well of Microseal white 384-well PCR plates (Bio-Rad).

Amplification of target sequences was performed using a 384-well Tetrad thermal cycler (MJ Research). Following an initial denaturation to activate the hotstart enzyme (95 °C, 15 min), targets were amplified using 45 cycles comprising denaturation (95 °C, 15 s), primer annealing (55 °C, 30 s) and extension of products (72 °C, 30 s). Following amplification, reactions were incubated at 72 °C for 2 min prior to a denaturation (95 °C, 1 min) and cool (20 °C, 5 min).

Melting curve analysis was performed by heating PCR plates from 35 to 80 °C, at 0.1 °C/s, using a LightTyper instrument (Roche Diagnostics, Lewes, UK). Fluorescence emission was monitored continuously to measure the stability

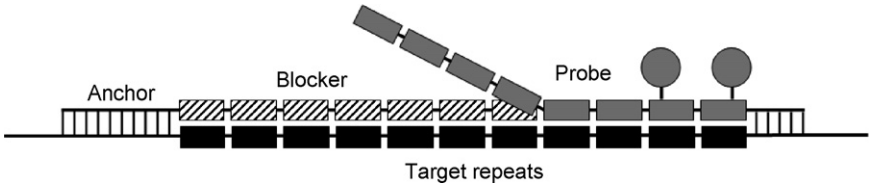

Fig. 1. Analysis of short tandem repeats using a fluorescent probe and blocking oligonucleotide. D18S51 Probe, blocker and target sequence repeats are represented by grey, striped and black boxes, respectively. Both probe and blocker oligonucleotides possess anchor components that hybridise to flanking non-repetitive sequence to prevent slippage. A blocker possessing 7 D18S51 repeats prevents the full length of the probe from hybridising to a target allele possessing 11 repeats.

Table 1
Primer, probe and blocker sequences used for analysis of D18S51, TH01 and D8S1179 loci, where 5, F, P and X represent 5′ trimethoxystilbene, fluorescein dT, 3′ phosphate and 3′ amino C7, respectively

Target	Oligonucleotide	Sequence
D18S51	D18F	TGCCACTGCACTTCACTCTGA
D18S51	D18R	GTGTGGAGATGTCTTACAATAACAGTTG
D18S51	HYBD18	TTCTTTCTTTCTTTCTTTCTTTCTTFCTTTCFGAGACP
D18S51	D18BL7	CTTTCCTCTCTCTTT(TTCT)$_7$P
D18S51	D18BL10	CTTTCCTCTCTCTTT(TTCT)$_{10}$X
D18S51	D18BL14	CTTTCCTCTCTCTTT(TTCT)$_{14}$X
TH01	TH01F	GGCTTCCGAGTGCAGGTCA
TH01	TH01R	GGTGATTCCCATTGGCCTG
TH01	HYBTH01	5TGGFGAATGAAFGAATGAATGAATGAATGAP
TH01	TH01BL3.3	ATGAATGAATGAATGAGGGAAATAAGGGP
TH01	THO1BL2.1	GAATGAATGAGGGAAATAAGGGAGGAACP
D8S1179	D8F	CGGCCTGGCAACTTATATGT
D8S1179	D8R	GCCTTAATTTATTTACCTATCCTGTAGA
D8S1179	HYBD8	TCTATCTATCTATCTATCTATCTATCFATCTAFTCCCCP
D8S1179	D8BL5	GTATTTCATGTGTACATTCGTA(TCTA)$_5$X
D8S1179	D8BL8	GTGTACATTCGTA(TCTA)$_8$X
D8S1179	D8BL11	GTGTACATTCGTA(TCTA)$_{11}$X

of probe hybridisation and determine the identity of amplified target sequences. Melting peaks were constructed by plotting the negative derivative of fluorescence with respect to temperature ($-$d(Fluorescence)/dT) on the y-axis) against temperature (x-axis). The T_ms of melting peaks were obtained with version 1.1 of the LightTyper software.

Unpurified buccal swabs were analysed using a Roche LightCycler instrument, performing target amplication and melting curve analysis in a single reaction vessel. Steriswab (Medical Wire & Equipment Co., Corsham, UK) samples were vortexed in 200 μl of tissue culture water (Sigma–Aldrich, Gillingham, UK) to release buccal cells. 2 μl of expressed swab was utilised directly in PCR assays without extraction of DNA. LightCycler PCR mastermixes were as described above, using TaKaRa Z-Taq PCR buffer and polymerase (Lonza, Wokingham, UK), additional $MgCl_2$ (3 mM total) and 10 ng/μl BSA (Roche Diagnostics, Lewes, UK). Following an initial denaturation (95 °C, 2 min), targets were amplified using 50 cycles comprising denaturation (95 °C, 5 s), primer annealing (55 °C, 10 s) and extension of products (72 °C, 10 s). Following amplification, reactions were incubated at 72 °C for 2 min prior to a denaturation (95 °C, 15 s) and cool (35 °C, 30 s). Melting curve analysis was then performed by heating reactions from 35 to 75 °C, at 0.1 °C, monitoring fluorescence continuously.

Table 2
Probe T_ms and acceptance ranges for D18S51 repeat alleles

Allele	Blocker	N	Mean T_m (°C)	S.D.	Lower limit	Upper limit
11	D18BL7	16	46.39	0.27	45.58	47.20
12	D18BL7	29	52.82	0.19	52.24	53.40
13	D18BL7	57	57.08	0.25	56.33	57.83
14	D18BL7	12	60.35	0.20	59.74	60.96
15+	D18BL7	91	62.00	0.35	60.94	63.06
14	D18BL10	18	46.73	0.14	46.30	47.16
15	D18BL10	11	52.93	0.20	52.32	53.53
16	D18BL10	24	57.08	0.18	56.53	57.62
17	D18BL10	15	59.99	0.31	59.07	60.90
18+	D18BL10	39	62.23	0.41	61.00	63.45
18	D18BL14	8	46.78	0.37	45.68	47.87
19	D18BL14	25	52.99	0.27	52.18	53.80
20	D18BL14	12	57.24	0.19	56.68	57.80
21	D18BL14	8	60.31	0.26	59.52	61.10
22+	D18BL14	8	62.71	0.34	61.68	63.73

The mean T_ms of probe melting peaks are presented, where N is the number of peaks investigated and S.D. is the standard deviation. 15+, 18+ and 22+ represent the melting peaks generated when the entire length of the probe was permitted to hybridise.

3. Results

A D18S51 probe consisting of eight tandem repeats of TTCT (Table 1) was used in combination with blocker oligonucleotides comprising 7, 10 and 14 repeats to analyse PCR amplified target alleles. These will be referred to as D18BL7, D18BL10 and D18BL14, respectively. The D18BL7 blocker, for example, permits only 4 of the probe repeats to hybridise to an 11 repeat allele (Fig. 1). DNA samples were analysed in a three-tube format to detect the full range of common D18S51 alleles on the basis of peak T_m. Melting curve analysis in the presence of the D18BL7 enables detection and identification of 11, 12, 13 and 14 repeat alleles (Table 2 and Fig. 2). The 14 repeat allele is also detected, along with 15, 16 and 17 repeat alleles, using D18BL10 (Fig. 2). Finally, D18BL14 is used to detect 18, 19, 20 and 21 repeat alleles. Analysis with each of these blocker oligonucleotides also results in an additional, higher T_m, ''common peak'' arising from full length probe hybridisation to targets with 15+, 18+ and 22+ repeats, respectively. Melting peak T_ms (Table 2) were obtained from 134 DNA samples analysed on five 384 well plates prepared on different working days, thereby accounting for sample-to-sample and run-to-run sources of variation. Acceptance criteria for the identification of repeat alleles were defined using the mean peak T_ms ± three standard deviations (i.e. 99.7% confidence interval). Whilst the 14 repeat D18S51 allele can be detected using both the D18BL7 and D18BL10, identification is restricted to the D18BL10 test to prevent

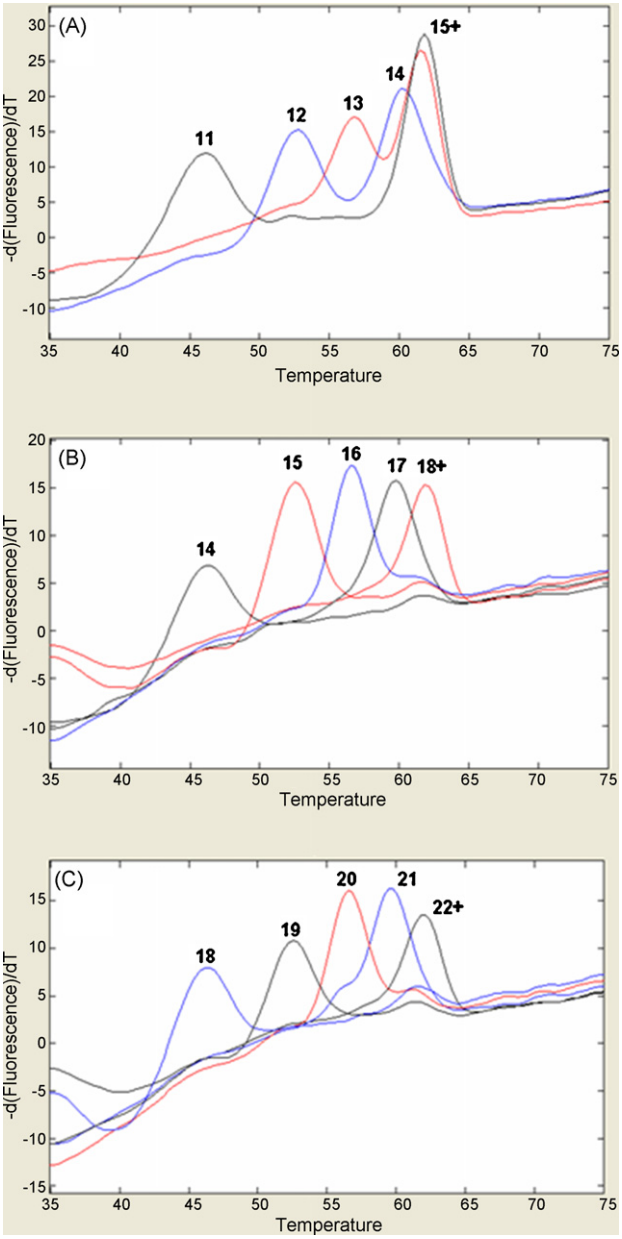

Fig. 2. D18S51 melting peaks generated with PCR amplified targets and a LightTyper instrument. The probe in combination with (A) D18BL7, (B) D18BL10 and (C) D18BL14 blockers detects the full range of common repeat alleles. The highest T_m melting peaks 15+, 18+ and 22+ arise from full length probe hybridisation.

miscalls associated with the 15+ "common peak". Sample analysis demonstrated detection of a 22 repeat D18S51 allele (confirmed with SGM plus), but since the full length of the probe is permitted to hybridise, target alleles exceeding 22 repeats will generate melting peaks of equivalent T_m.

A TH01 probe possessing 6.1 repeats was used along with blocker oligonucleotides comprising 2.1 and 3.3 repeats (TH01BL2.1 and TH01BL3.3, respectively—Table 1) to analyse PCR amplified targets. The junction between the probe and TH01BL3.3 corresponds to the position of the nucleotide deletion in the 9.3 repeat allele [12]. The combination of the 6.1 repeat probe and TH01BL2.1 permitted reliable detection and identification of the 6 and 7 repeat alleles, whereas melting curve analysis using TH01BL3.3 enabled efficient detection of 8, 9, 9.3 and 10 repeat alleles (Table 3). DNA Samples were analysed in a two-tube format, using TH01BL2.1 and TH01BL3.3, to detect and identify the full range of common TH01 alleles. The range of peak T_ms used for identification of 6, 7, 8 and 9 repeat alleles were based on the mean T_ms ± three standard deviations (Table 3). The temperature range employed to type 9.3 and 10 repeat alleles was determined using the mean T_ms ± two standard deviations (i.e. 95.4% confidence interval). A narrower T_m range was required to differentiate the 9.3 and 10 repeat melting peaks due to the reduced ΔT_m between them (Table 3).

Table 3
Probe T_ms and acceptance ranges for TH01 repeat alleles

Allele	Blocker	N	Mean T_m (°C)	S.D.	Lower limit	Upper limit
6	TH01BL2.1	57	45.78	0.30	44.88	46.68
7	TH01BL2.1	37	51.72	0.39	50.55	52.89
8+	TH01BL2.1	128	57.38	0.60	55.58	59.18
8	TH01BL3.3	48	50.33	0.20	49.73	50.93
9	TH01BL3.3	53	54.73	0.22	54.07	55.39
9.3	TH01BL3.3	45	58.25	0.24	57.77	58.73
10	TH01BL3.3	8	59.18	0.14	58.90	59.46

The mean T_ms of probe melting peaks are presented, where N is the number of peaks investigated and S.D. is the standard deviation. 8+ represents the "common peak" arising from incomplete blocking and full length probe hybridisation to alleles possessing 8 repeats and more.

A D8S1179 probe consisting of eight tandem repeats of TCTA (Table 1) was used in combination with blocker oligonucleotides comprising 5, 8 and 11 repeats (referred to as D8BL5, D8BL8 and D8BL11, respectively). Whilst D8S1179 alleles have been reported to comprise both TCTA and TCTG elements, the TCTG repeats are restricted to alleles possessing 13 repeats and more [12,14,15]. Furthermore, TCTG repeats appear to be located only in the second, third and fourth repeat positions (5′ to 3′; see GenBank Accession No. AF216671 and

Table 4
Probe T_ms and acceptance ranges for D8S1179 repeat alleles

Allele	Blocker	N	Mean T_m (°C)	S.D.	Lower limit	Upper limit
8	D8BL5	10	38.2	0.24	37.5	38.9
9	D8BL5	10	45.2	0.16	44.7	45.7
10	D8BL5	17	50.3	0.40	49.0	51.5
11	D8BL5	10	53.4	0.24	52.7	54.2
11	D8BL8	10	37.7	0.23	37.0	38.4
12	D8BL8	19	45.3	0.43	44.0	46.6
13	D8BL8	30	49.9	0.26	49.1	50.7
14	D8BL8	29	53.4	0.31	52.5	54.4
14	D8BL11	33	38.0	0.39	36.8	39.1
15	D8BL11	20	45.1	0.31	44.2	46.1
16	D8BL11	10	50.0	0.10	49.7	50.3
17	D8BL11	10	53.3	0.26	52.5	54.0

The mean T_ms of probe melting peaks are presented, where N is the number of peaks investigated and S.D. is the standard deviation. The 11 and 14 repeat alleles are reliably detected with two of the blocker oligonucleotides, however, identification of these alleles is limited to analysis with D8BL8 and D8BL11, respectively.

STRBase [12]). It was, therefore, possible to position the variable repeats within the blocker oligonucleotides (Table 1) to prevent nucleotide mismatches upon probe hybridisation. DNA samples were analysed in a three-tube format to detect the full range of common D8S1179 alleles. Melting curve analysis in the presence of the D8BL5 blocker permits reliable detection and identification of 8, 9 and 10 repeat alleles. The D8BL8 blocker is used to detect 11, 12 and 13 repeat alleles and D8BL11 enables identification of 14, 15, 16 and 17 repeat alleles. Analysis with D8BL5 and D8BL8 oligonucleotides also generates additional, higher T_m, melting peaks arising from probe hybridisation to alleles with 11+ and 14+ repeats, respectively. Additional melting peaks were not observed with the D8BL11 blocker, but would be generated in the presence of rare alleles comprising 18 repeats and more. Acceptance criteria for the identification of D8S1179 repeat alleles were based on mean melting peak T_ms ± three standard deviations (Table 4). The blocker oligonucleotides appeared to efficiently limit probe hybridisation to the TAGA repeat elements of target sequences, thereby avoiding nucleotide mismatches with CAGA target repeats and the generation of melting peaks of reduced T_m that could cause alleles to be miscalled.

With the exception of D18S51 17/18+ and TH01 9.3/10 allele combinations, the T_m acceptance criteria employed to type DNA samples, using exported LightTyper data, enabled all combinations of common D18S51, TH01 and D8S1179 alleles to be reliably characterised; for example permitting samples of TH01 type 6/9.3 to be clearly differentiated from 6/10. Although the ΔT_ms between D18S51 17 and 18+ and TH01 9.3 and 10 melting peaks were insufficient for the generation of two discrete melting peaks using the LightTyper software, samples of 17/18+ and 9.3/10 type could be identified by visual examination since they resulted in melting peaks of increased height and intermediate T_m. D8S1179 melting peaks were sufficiently separated such that all allele combinations could be reliably identified.

Forensic DNA analysis performed outside of specialised laboratories may require the use of unpurified samples since DNA extraction can be a time-consuming process, requiring additional equipment such as water-baths and centrifuges. Target amplification and melting curve analysis performed with a Roche LightCycler instrument demonstrates that STR analyses using unpurified buccal swab samples yield melting peak data that is comparable with tests that utilise purified DNA (Fig. 3). A partial profile of STRs may be generated within an hour directly from unpurified swab samples.

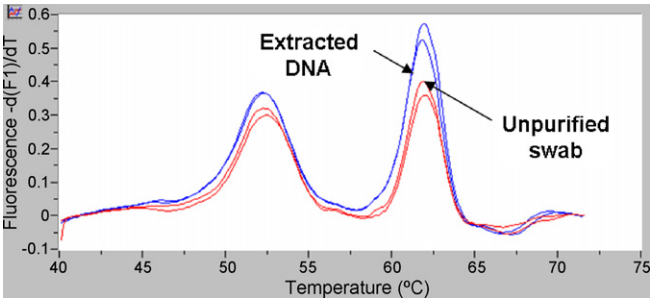

Fig. 3. D18S51 melting peaks obtained with purified DNA and unpurified buccal swab samples. The 12 and 15+ repeat alleles were detected and identified using the HYBD18 probe and D18BL7 blocker.

The D18S51, TH01 and D8S1179 alleles identified through melting curve analysis were in concordance with SGM plus profiling data. Only one DNA sample yielded a discordant combination of TH01 alleles, generating an 8/8 type by melting curve analysis and a 5/8 call using SGM Plus. The rare 5 repeat allele is not detected using TH01BL2.1 or TH01BL3.3 and is reported to exhibit a worldwide frequency of less than 0.002 (ALFRED) [10,11]. Clearly a reporting strategy for samples presenting a single melting peak should not exclude the presence of a rare undetected allele (see Section 4).

A molar excess of blocker oligonucleotide was utilised to prevent inappropriate full-length probe hybridisation to unblocked target sequences. Whilst the TH01BL2.1 did not completely prevent full-length probe hybridisation, the T_m of the small 8+ ‘‘common peak’’ did not compromise detection or identification of 6 and 7 repeat TH01 alleles (Table 3). Inappropriate full-length probe hybridisation did not occur in D18S51 or D8S1179 assays or TH01 analyses using TH01BL3.3.

4. Discussion

The combination of a fluorescent probe and non-fluorescent blocker oligonucleotides has enabled efficient detection and identification of D18S51, TH01 and D8S1179 repeat alleles. The D18S51 and D8S1179 assays clearly discriminated between the common alleles, comprising 11–21 and 8–17 repeats, respectively, with all T_m data within the defined acceptance criteria of the means ± three standard deviations. All STR alleles may be reliably identified by melting peak T_m with the exception of a 17/18+ D18S51 repeat combination (which can be determined visually).

For TH01, the 6, 7, 8 and 9 repeat targets are clearly distinct from all other alleles (with all T_m data within the acceptance criteria of mean ± three standard deviations) and the possibility of them being miscalled is therefore low. The 9.3 and 10 repeat alleles differ in length by only a single nucleotide (with 29 versus 30 probe nucleotides hybridised, respectively) and generate melting peaks separated by approximately 1 °C. Differentiation of these targets is potentially aided through base-stacking interactions [18] between the probe and blocker when hybridised to the 10 repeat allele, increasing the T_m to a greater extent than expected from the addition of a single nucleotide. The T_m acceptance criteria of 9.3 and 10 repeat alleles overlap if based on the mean ± three standard deviations. The 9.3 and 10 repeat alleles in this study were, therefore, differentiated using a narrower range of melting peak T_ms comprising the mean ± two standard deviations (increasing the possibility of miscalling melting peaks). All common TH01 alleles will be reliably identified by melting peak T_m with the exception of a 9.3/10 repeat combination (which can be determined by visual examination).

Whilst the D18S51, TH01 and D8S1179 assays described here identify the common 11–21, 6–10 and 8–17 repeat alleles,

respectively, they will not characterise the rare alleles outside of these ranges [12]. A DNA sample that yields a single melting peak could therefore also possess an undetected allele, such that a suspect presenting only an 8 repeat TH01 allele could be of 8/8 or <6/8 type. Forensic DNA analysis performed at the point-of-screening would take into account the rare STR alleles and an individual could be assigned a series of possible profiles.

At the point-of-screening, analysis software would automatically combine the results of the multiple blocker assays enabling individuals with minimal training to interpret STR profiles. Software would detect and identify STR alleles on the basis of melting peak T_m, but would also consider melting peak heights and/or areas to remove any fluorescence noise (e.g. small spurious peaks) that may be generated through localised melting of low-T_m areas. Since the D18S51 17/18+ and TH01 9.3/10 allele combinations could be identified ''by-eye'', additional methods of data analysis, such as Principal Component Analysis (PCA), may be included to type samples by the shape of melting curves without detection of discrete melting peaks. Furthermore, real-time PCR instruments such as the Rotor-Gene 6000 (Corbett Research) and LightCycler 480 (Roche Diagnostics), are capable of standard melting curve analysis and high resolution melt investigations. STR melting peaks and normalised HRM data may be used in combination to genotype forensic samples by two independent methods, increasing the confidence of analysis.

The D18S51, TH01 and D8S1179 assays described here utilise separate primer, blocker and probe oligonucleotides. The probe and blocker oligonucleotides must compete for hybridisation to amplified target repeats. Hybridisation of the blocker is favoured over probe annealing by including a molar excess; however, inappropriate full length probe hybridisation might still occur, due to incomplete blocking, thus complicating melting curve analysis of STR targets. Blocker hybridisation occurs by a bimolecular method since the blocker and target repeats are located on different DNA strands. If required, the blocking efficiency could be improved if blocker and amplified target repeats were located on the same DNA strand, employing an oligonucleotide comprising both blocker and primer components [19]. Intramolecular hybridisation of the blocker would be thermodynamically favoured over probe annealing such that inappropriate full-length probe hybridisation may be prevented. This approach has been exploited for the STR locus D16S539 which is used routinely in forensic analysis. Detection and differentiation of D16S539 alleles was achieved through intramolecular hybridisation of blocker oligonucleotides that were attached to the 5′ end of a primer [data in preparation for publication]. The D16S539 assay is currently performed in a three-tube format and was developed to identify only the common STR alleles, with published frequencies exceeding 0.01, enabling detection of 99.8% of target alleles, respectively [10,11].

In the current format, using only fluorescein-labelled probes, analysis of the four STR loci would require eleven separate reaction vessels (i.e. at least 22 μl of sample) to amplify, detect and identify the full range of required alleles. This is clearly unsuitable for forensic analysis, especially where sample volume (or concentration) is limited and a full forensic profile is required. We are currently in the process of developing a multiplex method of analysis, using probes labelled with different coloured dyes, which would reduce the required number of reaction vessels to three. The ultimate goal would be to reduce the method to a single-tube. Since buccal swab samples expressed in 200 μl of water yield high quality melting peak data, STR analyses could be performed without DNA extraction. Reagents provided in a freeze-dried or bead format, within reaction vessels, could permit individuals with minimal training to perform STR analysis at the point-of-screening simply by adding the sample.

For an STR locus with n measurable alleles, each with a known allele frequency, the probability that two randomly selected, unrelated individuals will possess identical combinations of alleles is given by:

$$p = \sum_{i=1}^{n} p_i^4 + 4\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2$$

where p_i and p_j are the allele frequencies (probabilities) of the ith and jth alleles, respectively. The probability that two randomly selected individuals will possess identical combinations of D18S51, TH01 and D8S1179 alleles is 9.74×10^{-5} (or 1 in 10,266). Adding D16S539 as a fourth locus in the panel would increase the probability of identity to 7.12×10^{-6} (or 1 in 140,503). Probabilities for each STR locus were determined using weighted averages of ALFRED allele frequency data [10,11] and calculations accounted for the groups of rare alleles that fall outside of the identification ranges for each locus. The determined probabilities may not be representative of any real population since allele frequencies vary widely between databases and populations, but it does demonstrate that a partial profile comprising three or four STR loci may be suitable for forensic identity screening. One application may be to rapidly include or exclude individuals from an investigation based on partial STR profiles, sending samples to specialised laboratories to obtain a full profile if required.

Melting curve analysis using fluorescent oligonucleotide probes may not be appropriate for the examination of sample mixtures unless additional methods of data analysis permit multiple STR profiles to be elucidated through investigations of melting peak heights and areas. The described method may also not be suitable for the interrogation of all STR loci. For example, analysis of loci possessing multiple repeating regions of different sequence will not be possible unless variable regions can be restricted to blocker oligonucleotides or neutralised using synthetic universal bases (such as inosine and 5-nitroindole) which hybridise equally to the four naturally occurring bases. This is clearly not possible with complex loci such as D21S11 which is reported to have a sequence motif of:

$[TCTA]_n[TCTG]_n[TCTA]_3TA[TCTA]_3TCA[TCTA]_2TC-CATA[TCTA]_n$ (see STRBase [12]).

Whilst melting curve analysis is able to detect and discriminate partial repeat alleles, successful analysis requires

that the sequence and location of partial repeats is consistent between alleles. With the exception of the 9.3 repeat TH01 allele, none of the partial repeat alleles of D18S51, TH01, D8S1179 or D16S539 loci have been reported to exhibit frequencies exceeding 0.001 [10,11] and have not been considered for STR analysis at the point-of-screening.

5. Conclusion

We demonstrate the proof-of-principle that melting curve analysis, using fluorescent oligonucleotide probes, will permit rapid interrogation of a small panel of STR loci outside of specialised laboratories. HyBeacon tests are compatible with unpurified saliva and buccal swab samples [8] and may provide forensic DNA evidence within 1 h using a real-time PCR instrument. The generation of a partial STR profile at the point-of-screening is not intended to be a replacement for full forensic profiling methods (such as SGM Plus), but will permit forensic DNA evidence to be rapidly generated before a sample could arrive at a specialised laboratory. The ability to rapidly determine a partial profile of short tandem repeats at the point-of-arrest would be of great benefit to police forces around the world, for example enabling a suspect to be rapidly included or excluded from an investigation. Further work is required to reduce test complexity (e.g. through multiplexing) and ensure that assays are sufficiently accurate and robust for forensic analysis.

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