Rapid typing of STRs in the human genome by HyBeacon® melting†

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A new method based on DNA melting has been developed for the rapid analysis of STRs in the human genome. The system is based on homogeneous PCR followed by fluorescence melting analysis and utilises a HyBeacon® probe combined with a PCR primer-blocker oligonucleotide. The use of blockers of different length permits identification of the full range of common D16S539 repeats enabling detection of 99.8% of known alleles. The interrogation of STRs can be carried out on standard genetic analysis platforms and could be applied to other loci to form the basis of a bespoke high-throughput system for use in forensic analysis, particularly as fluorescent genetic analysis platforms are now available for high-resolution melting. This methodology may be suitable for rapid forensic DNA analysis at the point-of-arrest or in a custody suite where it is important to identify an individual from a small group of suspects/detainees.

Introduction

Short tandem repeats (STRs) of 2–6 bases of DNA occur frequently in the human genome. Examples are $d(GATA)_n$, $d(TTCT)_n$ and $d(TCTTA)_n$.[1] Many of these loci are polymorphic, with the number of repeats varying between individuals and populations. This allows STRs to be used for human identification,[2,3] forensic science,[4,5] disease-related linkage analysis,[6,7] and paternity/kinship testing.[8,9] The existing widely used technique for STR analysis involves DNA extraction and amplification using 5′-fluorescently labelled PCR primers followed by electrophoretic separation and analysis. The procedure is carried out in a laboratory environment and requires specialised equipment and skilled technicians.[10] As a result it is a time-consuming process.[11] There is a parallel requirement for a simple and robust method of STR analysis for use at the scene-of-crime and custody suites where rapid and portable analysis is required. Such a system does not need to be as informative as the electrophoretic system but it should be capable of identifying an individual from a small group of detainees.

We are currently developing a quick and effective fluorescence melting method to determine the length of STR alleles from extracted DNA and unpurified saliva samples. The STR profiling method is based on HyBeacon® probes, which are single-stranded oligonucleotides labelled with fluorescein on some of the heterocyclic bases of the DNA sequence. HyBeacons have been used successfully in fluorescence-based mutation and SNP analysis.[12–16] In the single-stranded HyBeacon® probe the fluorophores interact with one another and also with the DNA bases resulting in almost complete fluorescence quenching (Fig. 1a). When the probe hybridizes to its complementary target (Fig. 1b), normally a PCR product, the fluorophores move away from each other and from the DNA bases due to the rigidity of the duplex (Fig. 1c). This leads to the fluorescence enhancement which forms the basis of the HyBeacon® melting analysis.

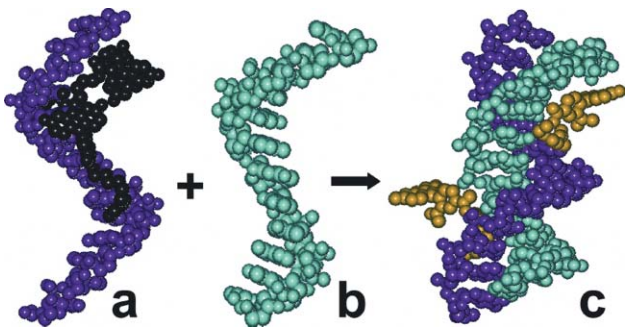

Fig. 1 STR HyBeacon® principle: (a) Single-stranded HyBeacon® probe with fluorescein dTs separated by around 6 bases. The fluorophores interact with each other and fluorescence is quenched. (b) Complementary DNA strand. (c) Duplex formed between HyBeacon® probe and its complementary target. In the rigid duplex the fluorophores are much further apart leading to enhanced fluorescence.

Analysing STR loci with HyBeacon® probes could form the basis of a fluorescence melting method for forensic profiling provided that sufficient resolution can be achieved. A potential problem is the small difference in melting temperature (Tm) between very similar long repeat DNA sequences, *e.g.* 12 and 13 repeats of d(GATA) at the D16S539 locus. This problem can be circumvented by employing non-fluorescent blocker oligonucleotides to reduce the effective length of the target sequence available to the HyBeacon® probe, thus increasing the difference in Tm between STR repeats of similar length. The simplest system comprises two PCR primers, a HyBeacon® probe and a blocker oligonucleotide. This "bimolecular" approach, in which the blocker and primer are

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in separate oligonucleotides (ONTs), has recently been evaluated and shown to permit reliable analysis of forensic loci.[17]

However the bimolecular approach to blocking is not efficient and inappropriate full length probe hybridization occurs, resulting in undesirable additional melting peaks which complicate sample analysis. The new STR system described here solves this problem by utilizing a "unimolecular" blocking strategy with blocker and amplified target located on the same DNA strand (PCR product), ensuring that the blocking of the STR target is intramolecular, and therefore efficient. This requires a system comprising a single oligonucleotide containing both blocker and PCR primer components (hairpin blocker/primer) in conjunction with a HyBeacon® probe and an unlabelled reverse primer (Fig. 2a). The human D16S539 locus, which is used in forensic profiling panels, is employed here as the principal model system.

Results and discussion

Unimolecular STR assay design

The unimolecular blocker/primer ONTs comprises (from 5′ to 3′) a five base GC rich stem, several d(TATC) blocking repeats complementary to the amplified D16S539 target sequences, an anchor sequence to clamp the blocker to the start of STR repeat region and prevent slippage, a hexaethylene glycol (HEG) PCR stopper[18] and a primer (Fig. 2a). PCR extension from blocker/primer oligonucleotides results in the $d(TATC)_n$ blocker and its amplified target sequence being part of the same DNA strand. The interaction between the blocker and STR target is therefore intramolecular and is kinetically and thermodynamically favoured over the alternative bimolecular system (ESI Fig. S1). This unimolecular approach is the basis of the Scorpions system that is widely used in genetic analysis[19,20] and permits the blocker to compete favourably with the HyBeacon® probe to sequester a predetermined number of STR repeats. The blocker cannot hybridize to its complement until the target sequence has been amplified so it does not delay the progression of *Taq* polymerase. Blocker oligonucleotides increase the difference in Tm between D16S539 alleles by reducing the number of repeats available to bind to the HyBeacon®. In the case of the unimolecular blocker D16BS7 (Table 1), the d(TATC) regions form stable duplexes with seven d(GATA) target repeats, leaving the HyBeacon® probe to bind to the remaining repeats (Fig. 2b). Thus, only four of the d(TATC) repeats in the HyBeacon® are permitted to hybridize to a D16S539 allele possessing eleven repeats of d(GATA). Reducing the number of repeats interrogated by the fluorescent probe provides excellent Tm differentiation between STRs of different lengths.

The HyBeacon® probe consists of a defined number of repeats of TATC (in the case of D16FL1 this is 8 repeats). This is followed by a sequence of 5 bases at the 3′ end (GC rich stem in Fig. 2a) which are complementary to the 5′ end of the blocker primers D16BSN (where N = number of TATC repeats). When blocker and probe are hybridized correctly to the STR target these GC-rich components create a 5-mer mini-duplex that holds the two strands together at the correct location (Fig. 2b). This prevents imprecise hybridization (probe slippage) which can lead to broad melting peaks and induce an extra peak due to the full length probe hybridizing to the target (discussed in section on trimethoxystilbene cap below).

HyBeacon® probe D16FL1 and blocker/primers D16BS4/7 evaluation in STR analysis

PCR products were amplified from small quantities of synthetic DNA templates 10R-14R (sequences in Table 1), yielding Tms of 33, 40.1, 45.7, 50.2 and 53.5 °C respectively, using the combination of the D16FL1 probe and D16BS7 blocker (Fig. 3). A 12/13 repeat heterozygote amplified directly from human saliva was then analysed and gave the same Tms as the 12 and 13 repeat samples from the synthetic templates (Fig. 3 inset). These results demonstrate that the ΔTms between adjacent repeats (up to 7 °C between 10 and 11 repeats and 3.3 °C between 13 and 14 repeats) are adequate to characterise these D16S539 alleles. It is not possible to interrogate the full range of D16S539 alleles using a single blocker oligonucleotide since the melting temperatures of the 8 and 9 repeat alleles would be below the detection limits of the LightCycler® instrument (*i.e.* below 30 °C) and the Tm of 10R was only just above 30 °C. Thus the 7 repeats blocker is suitable for analysing 11 to 14 repeats, and the shorter D16S539 alleles can be identified using the D16BS4 oligonucleotide, which possesses only four blocking repeats.

We then demonstrated that the full range of common D16S539 alleles[11] can be identified from purified genomic DNA using the D16FL1 probe along with D16BS4 and D16BS7 blocker/primer oligonucleotides in a two-tube format. The D16BS4 blocker

Table 1 Oligonucleotide (ONT) sequences; **5** = fluorescein dT, HEG = hexaethylene glycol, **P** = 3′-phosphate, **Y** = trimethoxystilbene

ONT ID	Sequence
D16FL1	TATCTATC5ATCTATC5ATCTATC5ATCTATCGCCGC**P**
PRIM2	ACGTTTGTGTGTGCATCTGTAAGCATGTATC
D16BS4	GCGGC$(TATC)_4$CACCTGTCTGTCTGTCTGTA-HEG-GATCCCAAGCTCTTCCTCTT
D16BS7	As D16BS4 but with $(TATC)_7$
D16FL4	**Y**CAATGA5ATCTA5CTATCTATCTATCTATCTATCTATC**P**
D16BL5	$(TATC)_5$CACCTGTCTG-HEG-GATCCCAAGCTCTTCCTCTT
D16BL7	$(TATC)_7$CACCTGTCTG-HEG-GATCCCAAGCTCTTCCTCTT
D16BL10	$(TATC)_{10}$CACCTGTCTG-HEG-GATCCCAAGCTCTTCCTCTT
10R	CCATTTACGTTTGTGTGTGCATCTGTAAGCATGTATCTATCATCCATCTCTGTTTTGTCTTTCAATGA$(TATC)_{10}$CACC TGTCTGTCTGTCTGTATTGATCTAGGGAAGAGGAAGAGCTTGGGATCTGCCTTTGTTTGCTCCCATATAAAAT
11R	CATTTACGTTTGTGTGTGCATCTGTAAGCATGTATCTATCATCCATCTCTGTTTTGTCTTTCAATGA$(TATC)_{11}$CACCT GTCTGTCTGTCTGTATTGATCTAGGGAAGAGGAAGAGCTTGGGATCTGCCTTTGTTTGCTCC
12R	As 10R but with $(TATC)_{12}$
13R/14R	As 11R but with $(TATC)_{13}$ and $(TATC)_{14}$

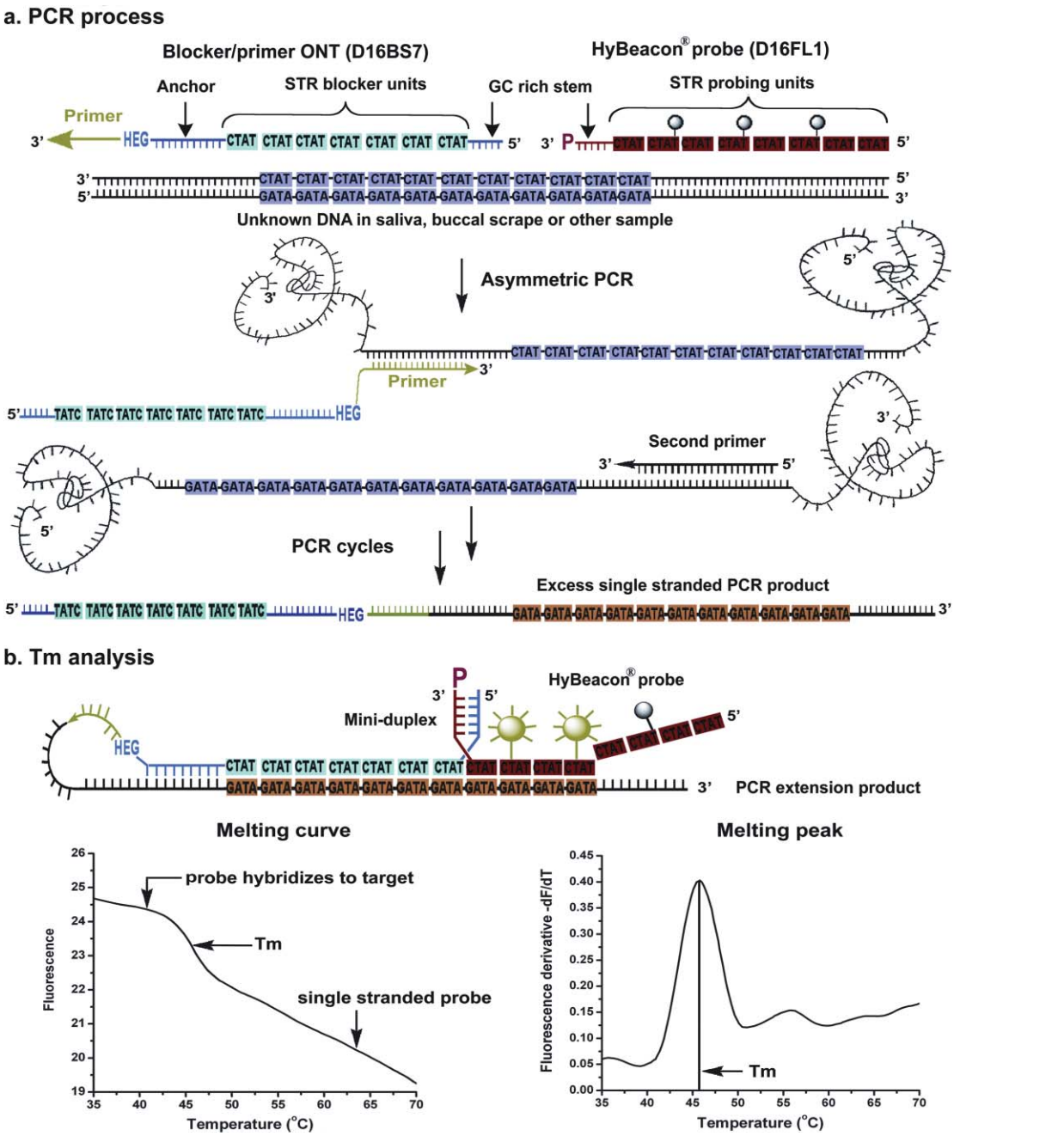

Fig. 2 Schematic of the STR HyBeacon® system. PCR amplification and Tm analysis are performed in a single tube. (a) The probe system is composed of a hairpin blocker/primer oligonucleotide (*e.g.* D16BS7) and HyBeacon® probe (*e.g.* D16FL1). The 5′-end of the hairpin blocker/primer oligonucleotide (D16BS7) comprises a GC-rich stem followed by 7 repeats of d(TATC) and a 20-mer anchor sequence to locate the blocker at the start of the STR repeat region. The HyBeacon® probe (D16FL1) has 8 repeats of d(TATC), three internal fluorescein dT nucleotides, a five base GC-rich zone that is complementary to the GC-rich unit of the hairpin blocker/primer and a phosphate group at the 3′ end (P) to prevent extension of the probe during PCR. Asymmetric PCR amplification produces an excess of the target DNA strand. (b) Hairpin blocker construct after PCR extension and its interaction with the STR HyBeacon®. Fluorescein dT moieties in double-stranded regions become considerably more fluorescent upon hybridization. The green circle indicates increased fluorescence in double-stranded DNA relative to single-strand (grey circle). The melting curve is obtained by monitoring fluorescence emission and heating the tube from 35 to 70 °C. At low temperature the HyBeacon® probe binds to its target and as a result the fluorescence is high. When the probe becomes single stranded at higher temperature the HyBeacon® is almost non-fluorescent. The first derivative of the melting curve (melt peak, –dF/dT) is used to determine the melting temperature.

permits identification of 8, 9, 10 and 11 repeat alleles and the D16BS7 blocker is used to identify 11, 12, 13 and 14 repeats (Fig. 4). Analyses were carried out on 44 separate extracted DNA samples (Table 2). A third blocker oligonucleotide with a greater number of repeats may be required to interrogate longer uncommon D16S539 alleles in a forensic setting since the above system does not reliably differentiate the 14 repeats from rare alleles possessing 15 repeats and greater.

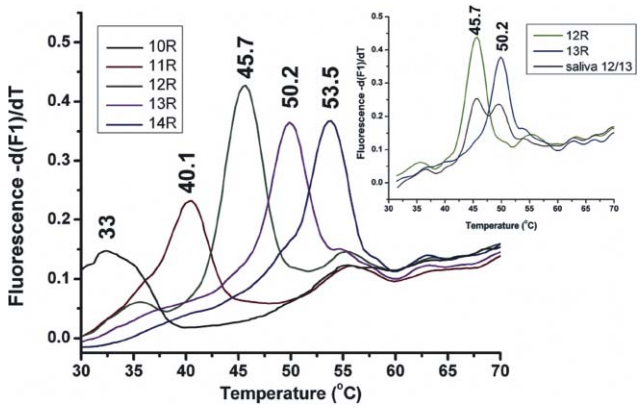

Fig. 3 HyBeacon® probe D16FL1 with blocker/primer D16BS7 was used to analyse PCR amplicons from the synthetic templates 10R-14R. Inset shows the analysis of unpurified saliva possessing 12 and 13 repeat D16S539 alleles in comparison with the 12R and 13R synthetic targets.

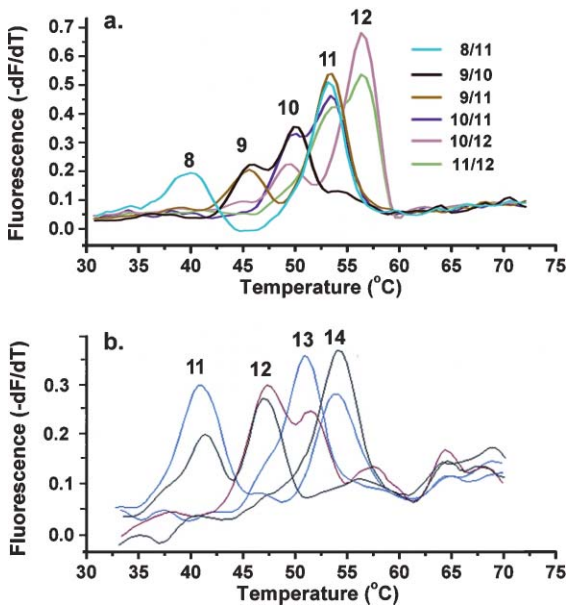

Fig. 4 Interrogation of D16S539 alleles from purified genomic DNA using the D16FL1 probe with (a) D16BS4 and (b) D16BS7 unimolecular blockers. This two-tube format permits reliable detection and identification of the common D16S539 alleles possessing between 8 and 14 repeats. 4a: 8/11, 9/10, 9/11; 10/11; 10/12; 11/12. 4b: 12/13 in red; 13/13, 11/14 in blue; 14/14, 11/12 in black.

STR analysis using a HyBeacon® probe with a trimethoxystilbene (TMS) caps

Unimolecular assay design. A HyBeacon probe (D16FL4) was also designed with a TMS cap at the 5′ end (Fig. 5). This was evaluated as an alternative to the previous system, using trimethoxystilbene to stabilise the blocker-target duplex instead of a mini-duplex. Trimethoxystilbene is known to increase duplex stability by stacking interactions.[21,22] TMS has two further advantages; (i) it acts as an additional fluorescence quencher in the single stranded HyBeacon® and (ii) it produces sharper melting curves. The latter point is particularly significant, as it facilitates discrimination between peaks with similar Tm's. The phenomenon is currently the focus of a detailed investigation using a range of chemical modifications. The D16FL4 HyBeacon® also has six bases of mixed sequence at the 5′ end adjacent to the TMS which firmly anchor the probe to its target, eight STR repeat units with two fluorescein dTs and a phosphate group at 3′ end as a PCR stopper (Fig. 5). The blocker/primer ONTs used (D16BL5/7/10) were similar to D16BSN but lacked the GC-rich stem. With the trimethoxystilbene HyBeacon®, a $(TATC)_5$ blocker unit was used and this gave a large ΔTm between eight and nine STR repeats.

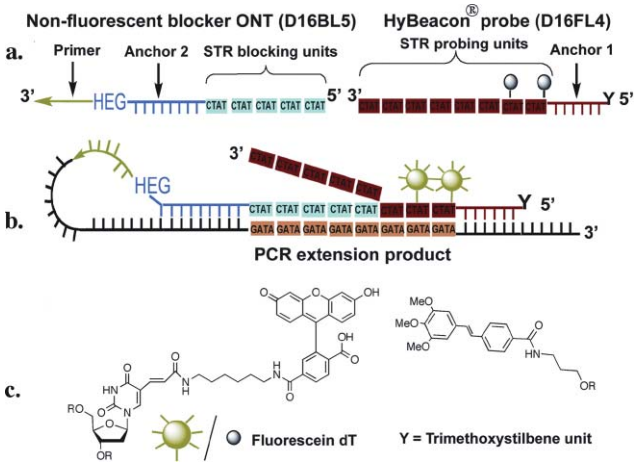

Fig. 5 Schematic of the HyBeacon® probe D16FL4 and blocker/primer D16BL5 system. (a) Probe and blocker/primer. (b) Probe and blocker hybridized to target. Green and dark grey circles represent the unquenched and quenched fluorescein dT moiety respectively. (c) Structures of trimethoxystilbene unit (Y) and fluorescein dT.

Table 2 The mean Tms (°C) of D16FL1 and D16FL4 HyBeacon® probe melting peaks, where N is the number of samples interrogated and SD is the standard deviation. Number of replicates depended on sample availability

Allele	D16FL1/Blocker	N	Tm	SD	Allele	D16FL4/Blocker	N	Tm	SD
8	D16BS4	7	39.6	0.12	8	D16BL5	10	41.9	0.24
9	D16BS4	8	45.5	0.35	9	D16BL5	28	48.8	0.12
10	D16BS4	9	50.0	0.31	9	D16BL7	28	35.0	0.17
11	D16BS4	25	53.5	0.31	10	D16BL7	16	42.5	0.14
11	D16BS7	25	41.4	0.34	11	D16BL7	17	49.1	0.10
12	D16BS7	21	47.3	0.40	12	D16BL7	21	53.7	0.10
13	D16BS7	20	51.3	0.51	12	D16BL10	21	34.5	0.21
14	D16BS7	8	54.2	0.25	13	D16BL10	10	42.0	0.31
					14	D16BL10	8	48.3	0.17

Evaluation of TMS capped HyBeacon®. DNA samples were analyzed in a three-tube format to detect the full range of common D16S539 alleles using the D16FL4 TMS-capped HyBeacon® and three different blocker/primers. Melting analysis using D16BL5 blocker/primer enabled identification of 8 and 9 repeat alleles, D16BL7 was used to identify 9 to 12 repeats and D16BL10 was employed to characterise 12 to 14 repeats (Table 2 and Fig. 6). Sixty separate DNA samples were used for this analysis. We were unable to obtain human DNA samples of 8/10, 8/13, 8/14, 10/10, 10/14 and 13/14 alleles. Ten of the DNA samples had STR profiles determined previously using the Applied Biosystems AmpFlSTR® SGM Plus® PCR amplification kit on an ABI PRISM® 3100 Genetic Analyzer and every allele identified with the D16FL4 probe was in accordance with SGM Plus data and also with melting curve analyses using the D16FL1 HyBeacon®. In addition, all available homozygous and heterozygous combinations of the common 8, 9, 10, 11, 12, 13 and 14 repeat alleles of D16S539 were differentiated and identified in this study. A comparison of the Tms of the D16BLN/D16FL4 system (TMS) and D16BSN/D16FL1 system (with a GC-rich mini-duplex) (Table 2) indicates that the TMS HyBeacon® probe/target gives an increase in Tm of up to 9.2 °C.

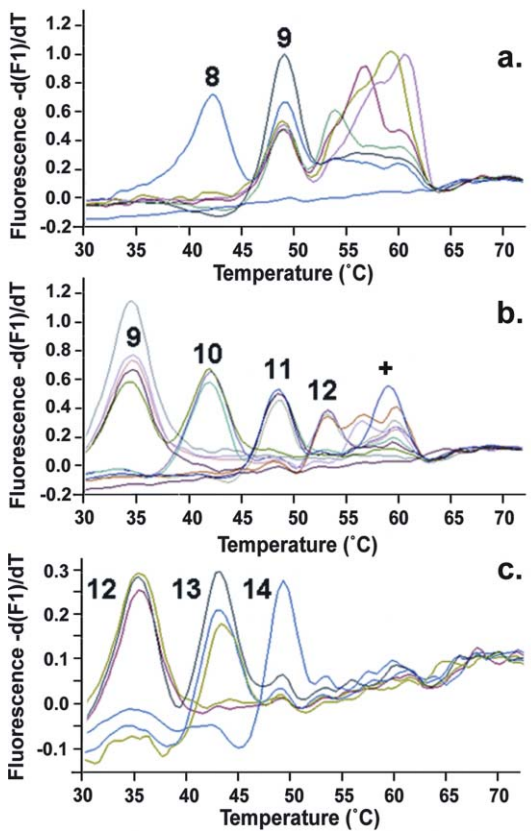

Fig. 6 Interrogation of D16S539 alleles using the D16FL4 probe with (a) D16BL5, (b) D16BL7 and (c) D16BL10 unimolecular blockers, detecting the full range of common alleles possessing between 8 and 14 repeats. a Resolves 8 and 9 repeats, b resolves 9 to 12 repeats and c resolves from 12 to 14 repeats. An individual sample appears in one or more of these panels and can be unambiguously genotyped. Full length probe hybridization to unblocked repeats generates an additional melting peak (+) but does not compromise detection or identification of D16S539 alleles.

The combination of D16FL4 TMS-capped probe and D16BL7 unimolecular blocker reliably identifies 9 to 12 repeat alleles, but also detects 13 repeat targets and full length probe hybridization with melting peaks at approximately 57 °C and 60 °C respectively (Fig. 6b). These higher melting peaks, which are due to hybridization of the full HyBeacon® to the target and displacement of part of the blocking oligo, are clearly differentiated and do not compromise identification of target alleles (also see ESI Fig. S2). The additional peaks can be avoided by the simple modification introduced into the D16FL1/D16BS4/S7 probe-blocker system (Fig. 2b), the five base mini-duplex. Combined with using a molar excess of blocker this mini-duplex inhibits inappropriate full length probe hybridization and therefore prevents generation of undesirable melting peaks (ESI Fig. S1). The TMS capped and mini-duplex intramolecular probe/blocker systems, although different in detail, both offer an effective method for STR analysis.

Although unimolecular blocker/primers are not essential for the interrogation of all STR loci or alleles, certain combinations of probes and blockers do benefit from analysis by the unimolecular approach. Unimolecular blocking can reduce the amount of probe that may hybridize along its full length to inappropriate unblocked repeats, compared with equivalent bimolecular blockers. A strategy that utilizes an excess of a high Tm bimolecular blocker is possible, but this is likely to reduce the efficiency of PCR with certain targets, so is not a simple alternative to the unimolecular system. Unimolecular oligonucleotides do not reduce the efficiency of PCR since blockers cannot compete for the template and inhibit the early stages of PCR. As mentioned previously, the benefit of unimolecular blocking was observed with the D16FL1 probe and D16BS4 (ESI Fig. S1). The advantage of unimolecular blocking has also been demonstrated in the analysis of the TH01 STR locus (ESI Fig. S3).

The method described here may not be suitable for the analysis of all short tandem repeats since many loci exhibit common partial repeat alleles and possess complicated structures with multiple repeating regions of different sequence.[2] Repeating sequences that are self complementary, such as d(CAG), may also be unsuitable if the formation of stable hairpin structures prevents efficient target detection and identification. However, additional fluorescent probe assays have been successfully developed, using bimolecular blockers, to interrogate D18S51, D8S1179 and TH01 short tandem repeats.[17] The combination of four STR tests will permit the generation of a partial STR profile outside of specialized laboratories, for example at the point-of-arrest, to rapidly include or exclude an individual from an investigation. The unimolecular blocking strategy may also provide further benefits when STR tests are multiplexed.

Analysis of PCR-amplified STR targets using electrophoretic methods has shown that additional products (referred to as shadow bands or stutter bands) may be generated through slippage events during PCR. Single stutter products, four nucleotides shorter than the main product, are typically observed with tetranucleotide repeats with a frequency of less than 10%.[23] Stutter peaks have not been observed during melting curve analysis of STR targets with fluorescent probes and non-fluorescent blockers. Stutter products would be expected to result in melting peaks with a height less than 10% of that observed for a genuine peak and would be hidden within the fluorescence noise.

Conclusions

STRs at the human D16S539 locus have been analyzed in a homogeneous format using a fluorescent HyBeacon® probe in conjunction with a blocker oligonucleotide chemically attached to a primer *via* a PCR stopper. Intramolecular hybridization of the blocker oligonucleotide prevents inappropriate full length probe hybridization and permits clear differentiation of STR alleles. The use of two or three blocker oligonucleotides of different length enables identification of the full range of common D16S539 alleles comprising 8 to 14 repeats, with reported allele frequencies exceeding 0.01, enabling detection of 99.8% of worldwide alleles.[24] This methodology is suitable for the analysis of other STR loci (see THO1 locus in ESI Fig. S3) and with such developments could permit rapid forensic DNA analysis to be performed at the point-of-arrest or in custody suites. Investigations are also under way to extend the scope of STR HyBeacons by exploring their use on mixed and contaminated samples such as those encountered in a forensic environment. With further development it is conceivable that melting curve analysis of fluorescent probes could also form the basis of a bespoke high-throughput system for STR analysis in non-forensic applications, particularly as genetic analysis platforms are now becoming available for high-resolution melting.

Experimental

Oligonucleotide synthesis

Standard DNA phosphoramidites, solid supports and additional reagents were purchased from Link Technologies Ltd and Applied Biosystems Ltd. Phosphate-on synthesis columns (for 3′-phosphate addition), hexaethylene glycol phosphoramidite (spacer 18) and fluorescein dT phosphoramidite were purchased from Link Technologies Ltd, and trimethoxystilbene phosphoramidite[21] from Glen Research. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 0.2 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 25 sec and this was extended to 360 sec for all other monomers. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C.

Oligonucleotide purification

Purification of oligonucleotides was carried out by reversed-phase HPLC on a Gilson system using a Brownlee Aquapore column (C8), 8 mm × 250 mm, pore size 300 Å. The following protocols were used: Run time 30 min, flow rate 4 mL per min, binary system, gradient: Time in min (% buffer B); 0 (0); 3 (0); 5 (10); 21 (40); 25 (100); 27 (0); 30 (0). Elution buffer A: 0.1 M ammonium acetate, pH 7.0, buffer B: 0.1 M ammonium acetate with 50% acetonitrile pH 7.0. Elution of oligonucleotides was monitored by ultraviolet absorption. Oligonucleotides were then desalted using NAP-10 Sephadex columns (GE Healthcare), aliquoted into eppendorf tubes and stored at –20 °C.

Capillary electrophoresis (CE) conditions

Oligonucleotide purity was confirmed by high resolution capillary electrophoresis analysis. Analysis was carried out using a Beckman Coulter P/ACE(tm) MDQ Capillary Electrophoresis system with 32 Karat software and UV monitoring at 254 nm. A solution of an oligonucleotide (0.2–0.4 OD_{254}) in 100 μL of water was injected into the gel for 5–20 sec with an injection voltage of 10.0 kV and a separation voltage of 9.0 kV. A single stranded DNA 100-R Gel was used with Tris-borate buffer and 7 M urea (Kit No 477480). Representative CE-traces are shown in ESI Fig. S4.

Gel purification of synthetic STR targets

Synthetic STR targets were purified by denaturing polyacrylamide gel electrophoresis. The crude oligonucleotide (10 OD) was lyophilized and redissolved in 100 μL of 50% formamide/water. The resulting solution was denatured at 95 °C for 5 min. and cooled to 0 °C immediately on an ice-bath before being loaded onto the preparative gel (pre-run gel of 13%/7 M urea, constant current 20 Amp for 1 h). The electrophoresis was performed for 3 h under the same conditions. The gel was removed from the plate, placed on plastic wrap, and put onto a UV fluorescent plate (TLC $silica_{254}$ plate, Merck) and visualized under UV light. The principal UV absorbing band was cut from the gel and eluted by the crush-and-soak method using water as eluent. The crushed gel in water was shaken (100 rpm) at 37 °C for 10 h and the gel was separated. The resulting oligonucleotide/urea supernatant was desalted on a NAP-10 Sephadex column (GE Healthcare) and quantified by UV_{260}. The purified oligonucleotides were obtained in yields of around 30%.

PCR amplification and fluorescence melting analysis

The human D16S539 locus used throughout this work (GenBank Accession No. G07925) is a component of forensic profiling panels such as the Applied Biosystem AmpFlSTR® SGM Plus® system. Target amplification and melting curve analysis was performed using a Roche LightCycler® 1.5 instrument. Interrogation of D16S539 alleles was performed with the HyBeacon® probes D16FL1 and D16FL4 which contains the duplex stabilizing end-cap trimethoxystilbene[21] (Table 1). Tests using the D16FL1 probes were performed in 10 μL reaction volumes, comprising 0.5 μM of the blocker/primer D16BS4 or D16BS7, 0.05 μM of reverse primer (PRIM2), 0.5 mM dNTPs, 75 nM of the probe, 4.0 mM of $MgCl_2$, 10 ng μL^{-1} BSA (Roche Diagnostics, Lewes, UK), 0.5 units of 5PRIME HotMaster Taq DNA polymerase (Flowgen, Nottingham, UK) in 1 × PCR buffer. Tests using the D16FL4 probe were performed in 20 μL reaction volumes comprising 1 μM of the D16BL5, D16BL7 or D16BL10 blocker/primer constructs (Table 1), 0.1 μM of reverse primer (PRIM2), 1 mM dNTPs, 75 nM probe, 3 mM $MgCl_2$, 10 ng μL^{-1} BSA, 1 × PCR buffer and 0.5 Units HotStarTaq (QIAGEN, Crawley, UK). Amplification of target sequences was achieved by asymmetric PCR using a 10 : 1 ratio of forward to reverse primer in order to

produce an excess of d(GATA)$_n$ target strand. A molar excess of blocker oligonucleotides was used to prevent/limit inappropriate full length probe hybridization. The quantity of template was 12 ng for synthetic templates, 1–5 ng μl^{-1} DNA for extracted samples (extracted from buccal swabs using QIAamp DNA Blood Mini Kits, QIAGEN, Crawley, UK) or 1 μL of saliva (diluted to 50% in water). The genotypes of extracted DNA and unpurified saliva samples were confirmed by the established SGM Plus® profiling method.

The PCR thermal cycling conditions used with D16FL1 probe were 94 °C for 2 min to activate the enzyme followed by 50 cycles of 94 °C/15 sec, 57 °C/30 sec and 70 °C/30 sec. Following amplification, reactions were heated to 95 °C for 1 min, annealed by cooling from 95 °C to 28 °C at 0.5 °C sec^{-1}, then maintained at 28 °C for 1 min. Fluorescence was then monitored during melting curve analysis using the continuous acquisition mode by heating to 80 °C at 0.1 °C sec^{-1}. The PCR thermal cycling conditions used with D16FL4 probe (and HotStarTaq polymerase) were 95 °C for 15 min to activate the enzyme followed by 50 cycles of 95 °C/10 sec, 55 °C/20 sec and 72 °C/20 sec. Following amplification, reactions were heated to 95 °C for 30 sec and cooled at 28 °C for 1 min. Melting curve analysis was then performed by heating reactions from 28 °C to 75 °C at 0.1 °C sec^{-1}.The fluorescence melting curve data was converted to the first derivatives giving the melting peaks (–dF/dT where F is fluorescence and T is temperature in °C). The total time of the amplification and melting curve analysis was on average 70 min and is dependent on the ambient temperature, *i.e.* ability of LightCycler® to cool to 28 °C. At least 45–50 cycles of PCR are required to generate sufficient target sequence for melting curve analysis. This number of cycles is commonly used in fluorogenic applications which use asymmetric amplification, in contrast to assays which employ equimolar concentrations of forward and reverse primers, and require fewer cycles. No contamination problems were encountered in this work.

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