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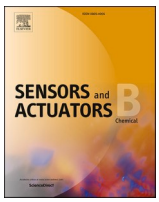

QueSTR probes: Quencher-labeled RNase H2-dependent probes for Short Tandem Repeat genotyping

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ABSTRACT

Forensic Short Tandem Repeat (STR) genotyping is almost exclusively performed by capillary electrophoresis (CE) in specialized laboratories. As an alternative to CE, and to enable miniaturized lab-on-a-chip STR profiling, we developed the QueSTR probes, a hybridization-based genotyping assay that relies on the recognition and cleavage of an RNA:DNA duplex by the RNase H2 enzyme. For each STR allele to be genotyped, a matching DNA probe containing one RNA moiety is designed. After performing asymmetric STR PCR, a hybridization curve analysis indicates the matching probe(s), and thus indicates the allele(s) present in the sample. Accurate genotyping of 13 samples was obtained using the QueSTR probes for three CODIS core loci (D16S539, D7S820, and TH01). A probe corresponding to the TH01 9.3 allele was included to demonstrate accurate genotyping, even in the presence of a partial repeat. The QueSTR probes are a valuable option to miniaturize STR genotyping in lab-on-a-chip devices that cannot harbor a CE analysis.

1. Introduction

Short Tandem Repeats (STRs) are short nucleotide sequences repeated multiple times in a head-to-tail fashion, which account for approximately 3% of the total genome [6]. STR profiling is commonly used for the identification of individuals, quality control of advanced therapy medicinal products (ATMPs), and animal and plant species authentication. Although generally known as "neutral" genetic markers, STRs can also have a functional role in pathologies, hinting at a possible role for STR profiling in diagnostics [10]. The repeat number of about 20 highly variable STR loci is determined to perform forensic DNA profiling [2].

Capillary electrophoresis (CE) is currently by far the most widely used method to perform STR amplicon size analysis [3]. CE requires bulky, sophisticated, and expensive instrumentation. There is an increasing need for faster and more portable analysis methods, e.g. for reference DNA profiling at police stations or mass disaster victim identification [1]. We recently described a hybridization-based STR genotyping assay, STRide probes, which is suited for miniaturization in the form of a small microarray [9]. STRide probes are labeled with a 6-carboxyfluorescein (FAM) fluorophore attached to a cytosine residue at the terminus of the probe, as shown in Supplementary Fig. S1. Their working mechanism relies on the intrinsic quenching properties of deoxyguanosine nucleotides on fluorescein derivatives, e.g., FAM [7]. Only when the cytosine residue of the probe to which the fluorophore is attached, hybridizes to its complementary guanine residue in the sample, quenching will occur. Although STR genotyping proved to be accurate and sensitive, STRide probes suffer from a design restriction [9]. The presence of a guanine residue in the sample, a few nucleotides away from the repeat region, is required. This might not only prevent successful assay design for some STR loci, it also hampers uniform probe design across loci. In this work, we present the QueSTR probes, which are quencher-labeled STR genotyping probes of which the design is not constrained by the context sequence. The goal of this research is to provide a detailed view into the working mechanism of the QueSTR probes. To demonstrate their potential, probes are designed and validated for three CODIS core STR loci: D16S539, TH01, and D7S820.

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2. Material and methods

2.1. Samples

Three commercially available reference DNA samples (9947a, 9948 (OriGene, Rockville, MD, USA), and 2800M (Promega, Madison, WI, USA)), 5 buccal swabs (samples 1–5), and 5 blood samples (samples 6–10) retrieved from anonymous, healthy volunteers were used to obtain the results presented in this paper. Ethical approval was obtained from the ethical review board of Ghent University Hospital, all volunteers signed the informed consent. The reference samples were used without further sample preparation. The buccal swabs were submerged in 200 μL of nuclease-free water and vortexed for 10″, followed by an incubation step of 15′ at room temperature. The resulting DNA extract, which has a concentration of about 0.1 ng DNA per μL, was used for downstream analysis. The blood samples were obtained by a finger puncture using a 21G Minicollect® Lancelino safety lancet with a penetration depth of 2.4 mm (Greiner Bio-One, Kremsmünster, Austria) and collected in a K3E K3EDTA Minicollect® collection tube (Greiner Bio-One, Kremsmünster, Austria). Next, DNA extraction of the blood samples was performed using the DNeasy® Blood and Tissue kit (Qiagen, Helden, Germany) according to the manufacturer's instructions.

2.2. CE genotyping

As a reference method, all samples from the healthy volunteers were STR-genotyped using CE. PCR amplification was performed using the AmpFlSTR® Identifiler® Plus PCR amplification kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol with an input of 1 ng of extracted DNA. After amplification, CE was performed using the ABI3130xl Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA). The obtained electropherograms were analyzed by the GeneMapper ID-x 1.2 software (ThermoFisher Scientific, Waltham, MA, USA). The obtained true genotypes for all samples can be found in Supplementary Table S1.

2.3. Asymmetric PCR amplification

Similar to the STRide probe assay [9], amplification of the targeted loci was performed asymmetrically. Asymmetric PCR, a method in which one primer is added in excess, results in an excess of the corresponding amplicon strand. This is preferred for hybridization-based methods, as the presence of the other amplicon strand competitively inhibits probe hybridization. Asymmetric amplification was performed as described previously [9]. More specifically, singleplex asymmetric PCR was performed in a volume of 50 μL containing dNTPs (ThermoFisher Scientific, Waltham, MA, USA) at 200 μM each, 1X Qiagen PCR buffer, $MgCl_2$ at a concentration of 0.5 mM, 1.3U HotStarTaq enzyme (Qiagen, Helden, Germany), and a set of primers (Integrated DNA Technologies, Newark, NJ, USA) per STR as listed in Supplementary Table S2. An input of 1 ng of DNA was used for all types of samples. Thermal cycling consisted of an initial denaturation step of 15 min at 95 °C, followed by 60 amplification cycles of 95 °C for 60 s, 59 °C for 60 s, and 72 °C for 80 s

2.4. QueSTR probe genotyping

After amplification, aliquots of 8.5 μL PCR product were dispensed in a qPCR plate. To each aliquot, 15 mU of RNase H2 enzyme (Integrated DNA Technologies, Newark, NJ, USA), and a specific STR probe (Table 1) was added, resulting in a final probe concentration of 0.15 μM. The total volume per well was 11.5 μL. Probes were acquired from Integrated DNA Technologies (Newark, NJ, USA) and from Biolegio (Nijmegen, The Netherlands). Hybridization was performed using a LightCycler 480 (Roche, Basel, Switzerland). During an initial denaturation step of 95 °C for 2′, the 'hot start' RNase H2 enzyme was activated. Subsequently, the samples were cooled down to 40 °C at a ramp rate of 0.01 °C/s. Fluorescence intensity was continuously measured during hybridization, resulting in 10 acquisitions per degree. By plotting the first derivative of the fluorescence intensity versus temperature, hybridization peaks were constructed. The hybridization temperature is in this research defined as the temperature at which hybridization is initiated, and was calculated as the maximum of the second derivative of the fluorescence intensity versus temperature, similarly to C_q determination in qPCR analysis.

Table 1
QueSTR probe sequences (FAM-labeled nucleotides are underlined; the ribonucleotide is indicated with an 'r'; Q represents Black Hole Quencher® 1).

Locus	Sequence (5′ – 3′)	Range of alleles
D16S539	TTTGTCTTTCAATGA(TATC)$_n$TATC(TATC)$_4$CrAC-Q	N = 4–8
TH01	CTGTTCCTCCCTTATTTCCCT(CATT)$_n$CATT (CATT)$_4$CACCrATG-Q	N = 1–5
TH01 9.3	CTGTTCCTCCCTTATTTCCCT(CATT)$_3$CATCATTCATT (CATT)$_4$CACCrATG-Q	N/A
D7S820 (long anchor)	TATTTAGTGAGATAAAAAAAAACTATCAATCTGT (CTAT)$_n$CTAT(CTAT)$_4$CGTrTA-Q	N = 3–7
D7S820 (short anchor)	AAACTATCAATCTGT(CTAT)$_n$CTAT(CTAT)$_4$CGTrTA-Q	N = 3–7

3. Results and discussion

3.1. QueSTR probes design

To develop STR genotyping probes of which the design is not constrained by the context sequence of the STR region, we exploited the capability of the RNase H2 enzyme to specifically recognize and cleave an RNA:DNA duplex [8]. QueSTR probes are DNA oligonucleotide probes containing one RNA unit. The probes can be divided in three regions: an anchor region, a repeat region, and a sensor region, as shown in Fig. 1A. The anchor region, which is relatively long and complementary to the region directly next to the repeat region, ensures correct alignment between probe and sample by preventing slippage of the probe. The repeat region hybridizes complementary to the STRs in the sample, and is functionalized with a fluorophore, e.g., 6-carboxyfluorescein (FAM). The sensor region is a relatively short region, directly next to the repeat region, which contains a ribonucleotide and is terminally functionalized with a quencher. This quencher suppresses the fluorescent signal of the fluorophore. A set of probes was designed for each locus, with varying repeat numbers, encompassing the most common alleles occurring within the population.

A stable homoduplex is formed upon hybridization of a probe and a sample with the same repeat number. If this hybridization is performed in the presence of the RNase H2 enzyme, cleavage of the probe will occur in the sensor region, at the RNA moiety. This causes an increase of the fluorescent signal, as the quencher is removed from the fluorophore, as shown in Fig. 1B. If the probe and the sample do not share the same repeat number, a less stable heteroduplex is formed in which the probe or the sample DNA strand forms a bulged loop, as visualized in Fig. 1A. We reasoned that this difference in duplex stability could be exploited to perform STR genotyping. If hybridization is performed by controlled cooling of the reaction, in presence of the RNase H2 enzyme, a signal will occur at a lower temperature for mismatch probes, compared to matching probes.

3.2. Genotyping of the D16S539 locus

To demonstrate the functionality of the QueSTR probes, we designed probes for the D16S539 locus. Their sequences can be found in Table 1. The length of the sensor region was three nucleotides, identical to the

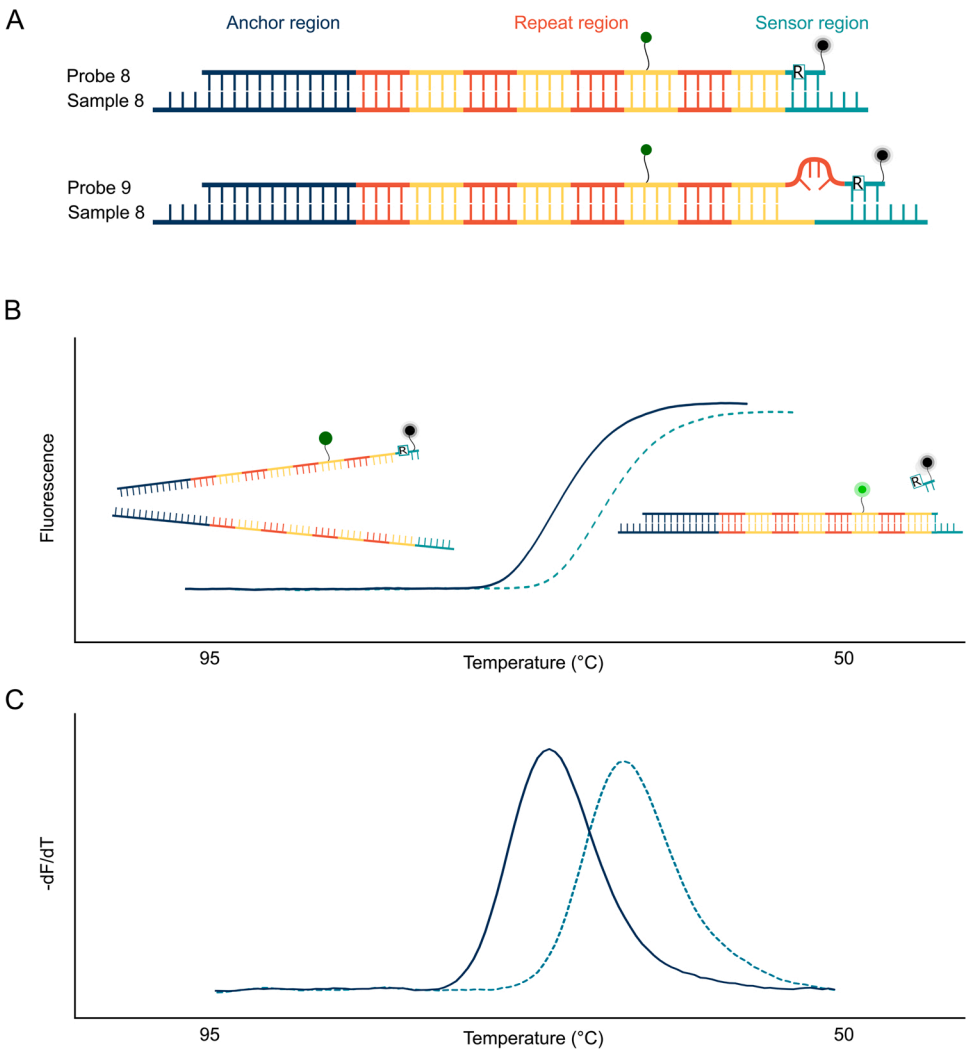

Fig. 1. QueSTR probes. (A) Probe design. All probes consist of three regions: an anchor region (blue), a repeat region (yellow-orange), and a sensor region (green). The sensor region contains one RNA moiety and is terminally functionalized with a quencher, while the repeat region is labeled with a FAM-fluorophore. If the probe and the sample share the same repeat number, a stable homoduplex will be formed (upper situation). Upon mismatch between probe and sample, a less stable heteroduplex will be formed (lower situation). (B) Hybridization curve analysis. Upon cooling down, hybridization of the sensor region to the sample will be recognized by the RNase H2 enzyme, resulting in cleavage of the probe and an increase of the fluorescent signal. Hybridization of a mismatch probe will result in a lower hybridization temperature (dotted green line), compared to matching probes (full blue line). (C) Hybridization peak calculation. The negative first derivative of the fluorescence as a function of temperature is calculated, which allows for more straightforward interpretation of the obtained data.

sensor region of the STRide probes previously developed for this locus by our group [9]. The sensor region should be kept as short as possible to maximally prevent hybridization of this region in case of a mismatch. After asymmetric amplification, probes and RNase H2 enzyme were added to aliquots of the PCR product. The hybridization curves, obtained by calculating the negative first derivative of the fluorescence as a function of the temperature, are shown in Fig. 2 for the three reference samples. The results obtained for the five buccal swabs and the five blood samples are shown in Supplementary Fig. S2. For all samples, the hybridization curves corresponding to the true alleles occur at the highest temperature. However, the temperature at which the true-allele probes hybridize, differs between alleles, as the hybridization temperature depends on the probe length. A signal is thus obtained at a higher temperature for probes with a higher repeat number. This is exemplified by reference sample 2800M, for which the peaks corresponding to true alleles 9 and 13 are separated from each other by about 1.5 °C, as these probes differ 16 nucleotides in length. Reference sample 9947a, on the other hand, is characterized by true alleles 11 and 12. The hybridization peaks corresponding to these alleles occur at about the same temperature, due to their similar length.

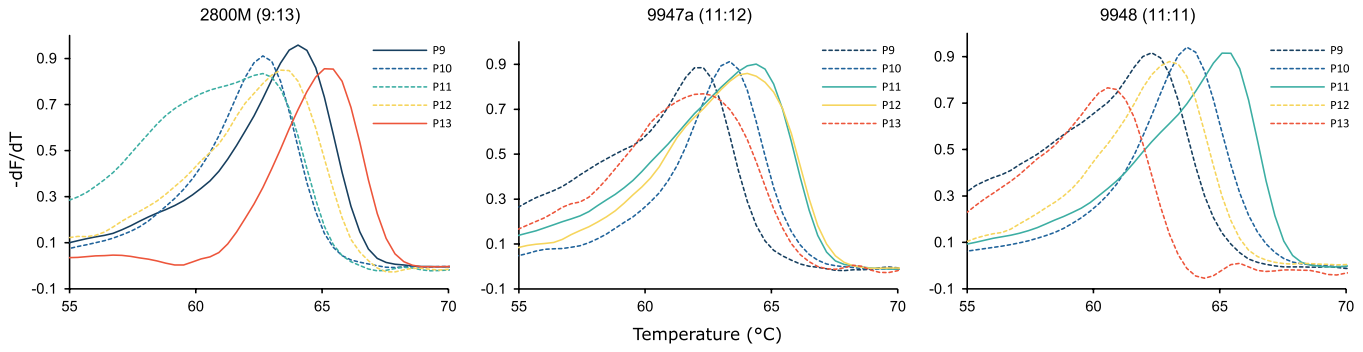

Fig. 2. Locus D16S539 hybridization profiles obtained for three reference DNA samples 2800M, 9947a, and 9948. Sample names and true genotypes are indicated above each graph. Hybridization curves of matching probes are represented as solid lines, those of mismatch probes are represented as dotted lines.

Hybridization of a probe and a sample with a different repeat number, results in a signal at a lower temperature, as the resulting duplexes are less stable. A bigger mismatch causes a more pronounced destabilization, resulting in a lower hybridization temperature. Therefore, probes differing only one repeat number from the true allele result in the hybridization peaks closest to the matching probe. This is illustrated by reference sample 9948, which is homozygous for locus D16S539 (11:11). The peak with the highest hybridization temperature corresponds to true allele 11, followed by closest neighboring probes 10 and 12. Similar patterns are observed for all other samples. These unique patterns can be exploited for genotyping. The peak corresponding to the highest hybridization temperature should always be called as present. If a neighboring peak is observed very close to the highest peak, it should also be called as present, e.g., sample 9947a. On the other hand, if a non-neighboring peak is observed at a temperature similar to the mismatch neighboring probes, e.g. probe 9 for sample 2800M, it should also be called as present. In some instances, no hybridization peak is observed at all for a mismatch probe. For sample 5 and sample 10, which are both homozygous for locus D16S539 (9:9), probe 13 does not show a hybridization peak. As the difference in repeat number is too high between the probe and the sample, the sensor region does not hybridize, leaving the probe intact. On the contrary, a quenching effect is observed instead of dequenching. This can be explained by the hybridization of the sample to the repeat region, where the fluorophore is positioned. As some nucleotides possess electron-donor properties, additional quenching of the fluorophore will occur.

3.3. Partial repeat genotyping

QueSTR probes rely on the observation of the destabilizing effect of a mismatch between sample and probe. This destabilizing effect is expected to be smaller when the mismatch is characterized by a partial repeat. To assess this, probes were designed for the TH01 locus. A commonly occurring allele for the TH01 locus is allele 9.3, which is characterized by 10 repeats, with a single-nucleotide deletion in the 4th repeat. Another complicating factor during probe design for the TH01 locus is the similarity between the repeat unit and the flanking region. The repeat unit of this locus is CATT. As the flanking sensor region starts with 'CA', followed by 'CAT', the sensor region was designed to be a little longer than the sensor region of other loci. Too much similarity between the sensor region and the repeat unit could cause hybridization of the sensor region of the probe to the repeat region of amplicons with a higher repeat number.

The obtained hybridization profiles are shown in Fig. 3 for all three reference samples, the results obtained for the buccal swabs and the blood samples are shown in Supplementary Fig. S3. Samples 8–10 were analyzed in triplicate. All reference samples contain a TH01 9.3 allele. The probes corresponding to the true alleles are observed at a higher temperature compared to the mismatch probes. As expected, mismatch probe 10, which only differs one nucleotide from true allele 9.3, also hybridizes at a relatively high temperature. Table 2 shows the hybridization temperature of probes 9.3 and 10 for all samples characterized by the presence of allele 9.3. The average difference in hybridization temperature between probe 9.3 and probe 10 is 1.53 °C, with a minimum of 1.34 °C and a standard deviation (SD) of 0.23 °C. These results show that differentiating match and mismatch probes is still possible, suggesting that accurate genotyping is not hampered by a partial repeat.

Table 2
Difference in hybridization temperature of matching probe 9.3 and mismatch probe 10.

Sample	Hybridization temperature Probe 9.3 (°C)	Hybridization temperature Probe 10 (°C)	Difference (°C)
9947a	73.55	72.20	1.35
9948	73.01	71.67	1.34
2800	71.70	69.96	1.74
Sample 2	72.24	70.85	1.39
Sample 5	72.59	71.19	1.40
Sample 7	72.05	70.31	1.74
Sample 9a	71.88	69.79	2.09
Sample 10a	71.53	70.13	1.40
Sample 9b	71.17	69.78	1.39
Sample 10b	70.82	69.43	1.39
Sample 9c	71.49	69.74	1.75
Sample 10c	71.49	70.09	1.40
Average	71.96	70.43	1.53
Standard deviation	0.75	0.83	0.23

3.4. Influence of the length and sequence of the anchor region

QueSTR probes were designed for the D7S820 locus. One of both flanking regions of this commonly used forensic STR locus strongly resembles the repeat region, and should therefore preferably not be used as the sensor region. This flanking region also contains a homopolymeric tract of 8 adenosine units, immediately followed by a Single Nucleotide Polymorphism (SNP). To assess the influence of the presence of such a homopolymeric tract in the anchor region, two sets of probes were designed for this locus. The first set was characterized by a rather long anchor region, spanning the homopolymeric tract and the SNP. The second set was designed with a shorter anchor region, avoiding the homopolymeric tract. The hybridization curves obtained for reference sample 2800M are shown in Fig. 4. From this, it is clear that the first set of probes, carrying a long anchor region, are not capable of discriminating fully complementary probes from mismatch probes. However, this issue was resolved by designing a shorter probe, which proved capable of accurately genotyping the D7S820 locus. A potential cause of this discrepancy is the formation of secondary structures in the anchor region of the probe, which might alter hybridization dynamics. Moreover, the destabilizing effect of a mismatch is less pronounced for longer probes. Therefore, homopolymeric tracts in the probe sequence should be avoided during assay design for additional loci. As shown in

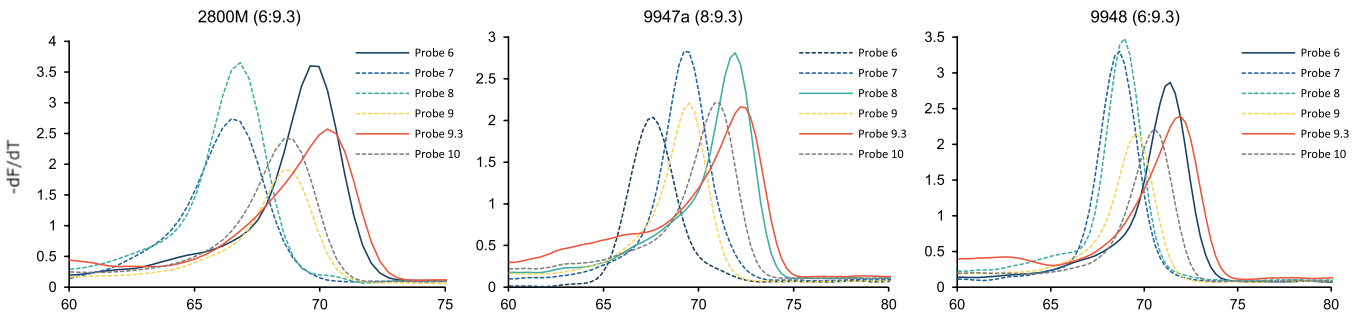

Fig. 3. TH01 hybridization profiles obtained for three reference DNA samples 2800M, 9947a, and 9948. Sample names and true genotypes are indicated above each graph. Hybridization curves of matching probes are represented as solid lines. Those of mismatch probes are represented as dotted lines.

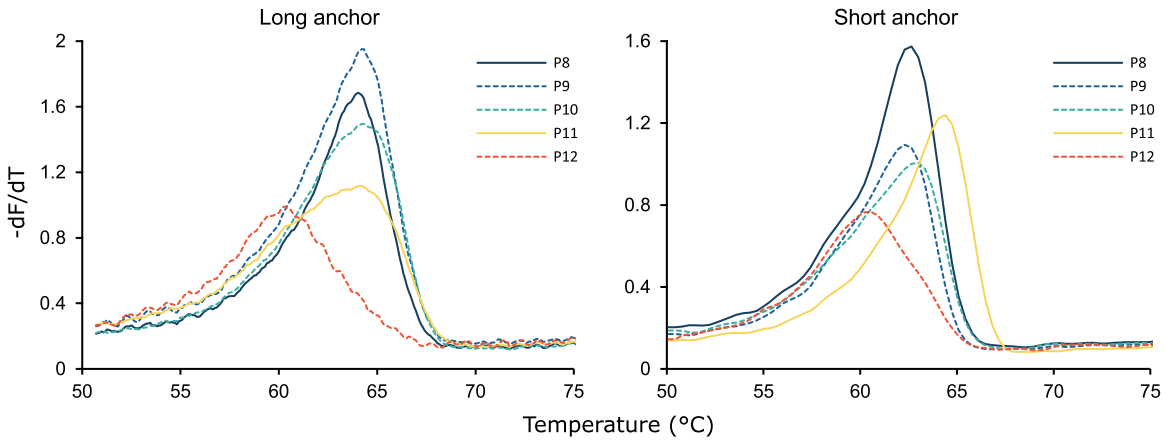

Fig. 4. Locus D7S820 hybridization profiles obtained for reference DNA sample 2800M (true genotype 8:11), using two different sets of probes. Left: hybridization profile obtained using probes with a long anchor region, containing a homopolymeric tract. Right: hybridization profile obtained using probes with a short anchor region, avoiding the homopolymeric tract. Hybridization curves of matching probes are represented as solid lines, those of mismatch probes are represented as dotted lines.

Supplementary Fig. S4, all samples could be genotyped correctly using the second set of probes, which harbor the shorter anchor region.

3.5. Sensitivity and repeatability assessment

A crucial characteristic of a forensic assay is its sensitivity, as often only a limited amount of input material is available. To assess the sensitivity of the QueSTR probe assay, the TH01 locus was analyzed in a dilution series of reference sample 9947a. The input for asymmetric PCR ranged from 31 pg to 500 pg DNA. The obtained melting profiles are shown in Supplementary Fig. S5. Genotyping was successful, even when an input of 31 pg was used, which is comparable with the sensitivity of commercially available STR kits for CE analysis. However, the hybridization peaks for the lowest input showed some allelic imbalance.

Besides the sensitivity, the repeatability of QueSTR genotyping was assessed, by analyzing samples 8–10 in triplicate, for all three loci, on three different days. These results are shown in Supplementary Figs. S2-S4. As discussed previously, the results for the TH01 locus, which contains a partial repeat, show reproducible melting profiles. The genotyping results for loci D16S539 and D7S820 also show a high reproducibility for these loci. The minor variations observed in the hybridization profiles result from differences in asymmetric PCR efficiency, variations in the pipetting steps, and imperfect temperature measurements.

3.6. Comparison of QueSTR probes versus STRide probes

The herein presented QueSTR probes are a valuable alternative for the previously described STRide probes. While the STRide probes require the presence of a guanine moiety close to the repeat region, the QueSTR probes do not suffer from this design constraint. This allows for a more universal probe design, by keeping the sensor region as short as possible. For the loci D16S539 and D7S820, probes were designed with a sensor region of three nucleotides. For locus TH01, the sensor region was extended to 7 nucleotides, due to some sequence homology between the repeat region and the sensor region. Still, accurate genotyping was obtained, despite the presence of a partial repeat.

Some drawbacks of the QueSTR probes compared to the STRide probes should be noted. Firstly, the assay relies on hybridization curve analysis, whereas STRide probes are assessed by a melting curve analysis. As a consequence, the obtained hybridization peaks are broader compared to the melting peaks obtained using STRide probes, due to the slower kinetics of DNA hybridization. Second, hybridization of a sample and a mismatch probe can be initiated at the sensor region, even in case of a mismatch, which causes a signal to occur immediately when performing QueSTR genotyping. Using the STRide probes, the duplex can still re-align to its thermodynamically most favored conformation. Lastly, the QueSTR probes are more expensive compared to the STRide probes due to the inclusion of a ribonucleotide and a quencher. Therefore, QueSTR probes could be implemented in a microarray alongside the STRide probes. This will make assay design much more flexible, and will enable analysis of a broader panel of STR loci. The presence of the RNase H2 enzyme should not interfere with the STRide probe assay, as it only recognizes RNA:DNA duplexes. Only for those loci that are not suited for STRide probe analysis, QueSTR probes will be employed. One such locus might be STR locus D7S820, which yields a less pronounced signal when using STRide probes than the other loci included in our proof-of-concept study [9]. As discussed in that study, data-analysis is envisioned to be performed automated. Therefore, combining two assays in one microarray should not complicate data analysis and interpretation for the end-user.

3.7. Comparison to state-of-the-art CE analysis

The envisioned main purpose of the QueSTR probe assay is to analyze reference samples with a disposable chip in a portable device. High-resolution CE is hard to implement in a lab-on-a-chip. The QueSTR probes could be spotted in a small microarray. Another advantage of QueSTR probe genotyping over CE is the multiplex capability. The number of targets included is virtually only limited by the spatial restrictions of the designed microarray. Using CE, however, the number of targets is limited, as only four to six fluorescent markers are employed. Higher-order multiplexing might affect the sensitivity of the amplification step, yet extremely high-order multiplexed forensic kits are available [4].

Some limitations inherent to hybridization-based genotyping of STRs should be pointed out. First, some commonly assessed STR loci are characterized by high repeat numbers. Longer probes should be developed for these loci, which is disadvantageous for hybridization-based assays. Longer probes result in smaller differences in hybridization temperature between matching and mismatch probes. Therefore, this assay is not ideally suited for analysis of loci with extremely high repeat numbers. Second, complex repeat patterns, characterized by sequence variants within the repeat region, and partial repeats, will make accurate analysis of these long probes even more challenging. Third, variations outside the probe region, such as length variations, will not be detected by hybridization-based technologies. This might cause discrepancies between CE analysis and QueSTR probe genotyping. Lastly,

the analysis of unbalanced mixture samples is challenging for the QueSTR probes. The obtained signal is dominated by the most abundant amplicons present in the PCR product. This explains why no signal for stutter PCR products is observed in the data obtained in this study. Typically, these stutter products represent 5–10% of the amplicons. Most probe(s) corresponding to the stutter allele(s) hybridize to (one of) the true allele amplicon(s). Therefore, the main application of this assay is the analysis of reference samples, such as buccal swabs. Enabling fast analysis of these reference samples in a booking station would realize a major benefit for most investigations [5]. Moreover, innocent suspects could be exonerated much faster by using rapid DNA typing technology.

4. Conclusion

QueSTR probes, which are DNA oligonucleotide probes containing one RNA moiety, were developed for STR genotyping. The probes are labeled with a fluorophore and a quencher, separated from each other by the RNA moiety. Their working mechanism relies on the recognition and cleavage of an RNA:DNA duplex by RNase H2. Probes were developed for three CODIS core loci. After asymmetric amplification, genotyping was performed by hybridization curve analysis. Completely matching alleles form the most stable duplex with their corresponding probe, and thus the probe with the highest hybridization temperature indicates the allele present in the sample. Successful genotyping by performing hybridization curve analysis was obtained for all three investigated STR loci. The TH01 9.3 allele could also accurately be genotyped, although it only differs one nucleotide from allele 10. QueSTR probes provide a valuable alternative to the previously developed STRide probes, as they do not suffer from some design constraints, and allow more uniform probe design. Taken together, these different types of hybridization-based STR identification probes represent an important step towards miniaturized and portable STR genotyping.

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CRediT authorship contribution statement

Olivier Tytgat: Writing – original draft; **Sonja Škevin**: Writing – review & editing; **Maarten Fauvart**: Writing – review & editing; Supervision; **Tim Stakenborg**: Writing – review & editing; Supervision; **Dieter Deforce**: Conceptualization; Writing – review & editing; Supervision; **Filip Van Nieuwerburgh**: Conceptualization; Writing – review & editing; Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: O.T., D.D., and F.V.N. are inventors on a pending patent application disclosing the STR-probes described in this publication (WO 2021/175762 A1).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.131714.

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