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Short communication

STR sequence analysis for characterizing normal, variant, and null alleles

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ABSTRACT

DNA sequence variation is known to exist in and around the repeat region of short tandem repeat (STR) loci used in human identity testing. While the vast majority of STR alleles measured in forensic DNA laboratories worldwide type as "normal" alleles compared with STR kit allelic ladders, a number of variant alleles have been reported. In addition, a sequence difference at a polymerase chain reaction (PCR) primer binding site in the DNA template can cause allele drop-out (i.e., a "null" or "silent" allele) with one set of primers and not with another. Our group at the National Institute of Standards and Technology (NIST) has been sequencing variant and null alleles supplied by forensic labs and cataloging this information on the NIST STRBase website for the past decade. The PCR primer sequences and strategy used for our STR allele sequencing work involving 23 autosomal STRs and 17 Y-chromosome STRs are described along with the results from 111 variant and 17 null alleles.

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1. Introduction

Short tandem repeat (STR) loci are the primary genetic markers used for human identity testing and are typically examined with multiplex polymerase chain reaction (PCR) assays that measure PCR product length relative to sequenced allelic ladders [1]. A common set of about 20 autosomal STRs is routinely examined in various combinations as part of commercially available kits [2]. While the vast majority of the millions of STR alleles examined worldwide each year fall within the sizing bins used to correlate allelic ladder repeat number to the tested sample's STR repeat length, occasionally variant or "off-ladder" alleles are observed [3–9]. Our group at NIST has provided variant and null allele sequence analysis as a service to the forensic DNA community for the past decade in addition to cataloging this information on the STRBase website [59]; http://www.cstl.nist.gov/biotech/strbase/. As of February 2010, there were 528 variants cataloged. In addition, DNA sequence variation is known to exist in the repeat region and the flanking regions of most of the widely-used STRs [10,11]. A mismatch due to sequence variation in the DNA template can cause allele dropout when it falls within PCR primer binding sites. The resulting "null alleles" can be detected when concordance studies are performed using sets of PCR primers with different annealing positions [12–37].

In order to understand at the molecular level the reason for a variant or null allele, the DNA sample needs to be sequenced at the STR locus. If the locus in question is heterozygous and contains two distinguishable alleles, these alleles must be separated from one another prior to sequencing. Sets of new PCR primers have been developed that anneal outside all known STR kit primer positions to enable examination of the nucleotide sequences underlying these primer binding sites [Supplemental Table S1]. While there have been other reports with DNA sequence information from a few STR loci [38–58], this article describes comprehensive coverage for all the common human identity testing STR markers.

2. Materials and methods

2.1. DNA samples

DNA samples used in this study came from our in-house set of more than 650 U.S. population samples described previously [60] or were variant alleles provided by outside DNA testing laboratories. Samples with previously confirmed or suspected null alleles [26] were typically provided as DNA extracts or blood stains. All the samples were completely stripped of any personal identifiers and made anonymous prior to testing at NIST in accordance with our laboratory's institutional review board policy. DNA extractions from the bloodstains on paper were performed with Chelex as previously described [61] or by DNA IQ extraction (Promega Corp., Madison, WI) following manufacturer's protocol. If limited in size, bloodstains supplied as FTA punches were purified by manufacturer's protocol (Whatman Inc., Piscataway, NJ) and the purified punch used in the initial PCR.

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2.2. Primer design for PCR and sequencing primers

PCR primers were designed against each reference sequence using web-based Primer3 [62]. Typically the default parameters were used, including melting temperature (T_m) values in the range of 57–63 °C. The region encompassing known kit primer sequences [63] was highlighted to ensure that it would be included within the PCR product upon the sequencing primer design.

Unlabeled oligonucleotides were purchased from Qiagen Operon (Alameda, CA). Table S1 contains a list of the DNA sequencing primers used in these experiments. These primers were quality controlled prior to use via UV absorbance at 260 nm using a Cary 100 UV-Vis spectrophotometer (Varian, Palo Alto, CA). All the primers were used in 1.0 μmol/L concentrations and stored at 4 °C.

2.3. PCR amplification

PCR amplifications were performed as described previously [36] with approximately 5–7 ng extracted DNA amplified with locus-specific annealing temperature (see Table S1) in the range of 50–60 °C.

2.4. Post-amplification quantification

PCR products were evaluated for specificity and quantity following amplification using 1.2% or 2.2% agarose FlashGel System, with FlashGel QuantLadder, (Lonza Rockland Inc., Rockland, ME). For each sample, 3 μL of product was examined.

2.5. Gel electrophoresis for STR allele isolation

Allele separation for heterozygote allele samples was performed on acrylamide based gels prepared to be in the range of 9–12% total acrylamide with 3% cross link of bis-acrylamide in a 50 mmol/L Tris-formate buffer pH 9.0, (50 mmol/L of formate obtained using formic acid and titrating the solution to pH9.0 using Tris Base). The gel matrix used was dependent on the size of the PCR product to be separated. Large PCR amplicons (>500 bp) were separated on the 9% gels while smaller amplicons <300 bp were separated on 12% gels. Homozygous allele samples do not require gel separation. Details of the gel casting, electrophoresis and silver staining can be found in Supplemental Protocols 1 and 2.

Using a scalpel or razor blade, the desired silver stained allele bands were excised from the gel and placed overnight in 50–100 μL of TE^{-4} buffer (10 mmol/L Tris–HCl, 0.1 mmol/L EDTA, pH 8.0). The volume of TE^{-4} buffer was dependent on the color/concentration of the DNA obtained from the gel cut.

2.6. Reamplification of separated heterozygous alleles

After the gel-separated alleles from heterozygote samples sat overnight in TE^{-4} buffer, a 5 μL aliquot was reamplified using the same PCR conditions as described [36].

2.7. DNA sequencing reactions

All the STR alleles from homozygous individuals and reamplified gel-separated alleles from heterozygous individuals were processed using Big Dye v 3.1 as described previously [36] to the analysis of the sequencing product.

2.8. Analysis on the ABI PRISM 3130xl Genetic Analyzer

Purified cycle sequencing samples were prepared for analysis on the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) by adding 1 μL of sample to 12 μL of Hi-Di formamide in a 96 well reaction tray. Although a variety of instrument configurations have been used over the years, we have found the 80 cm array and POP7 polymer (Applied Biosystems) to work optimally. Samples were injected onto the capillaries with 22 s of 1 kV and electrophoretically separated at 15 kV.

2.9. Data analysis

DNA sequences were analyzed using Sequence Analysis version 5.2 (Applied Biosystems) and LaserGene SeqMan Version 7.1.0 (DNASTAR inc., Madison WI). Sequence information for each STR locus was aligned against a GenBank reference sequence with the number of repeat units listed in Table S1. Reference sequences for most of the STR loci used are available at http://www.cstl.nist.gov/biotech/strbase/seq_ref.htm.

3. Results and discussion

The steps involved in our STR allele sequencing strategy are noted in Fig. 1. First, a sample is identified that is in need of sequencing to understand the molecular basis for allele dropout or to characterize the repeat region or flanking region surrounding an STR marker. The null allele samples are typically identified as part of a concordance study [28,33] where a heterozygote is observed with one primer set while a homozygote is seen with a different primer set. Variant alleles are often "off-ladder" alleles observed by a DNA testing laboratory and provided to NIST as DNA extracts or bloodstain punches for further evaluation.

The DNA samples of interest are PCR-amplified with forward and reverse sequencing primers. These sequencing primers have been designed to anneal far enough away from the STR repeat in order to generate PCR products that encompass the primer binding regions for all the known kit primers surrounding commonly used STR loci. The DNA sequencing PCR primers listed in Table S1 were all tested with homozygous samples to verify that clean sequence information could be obtained for each of the 23 autosomal and 17 Y-chromosome STR loci listed. Locus-specific annealing temperatures were used to obtain optimal results.

Following the generation of DNA sequence information using the steps outlined in Fig. 1, the forward (F) and reverse (R)

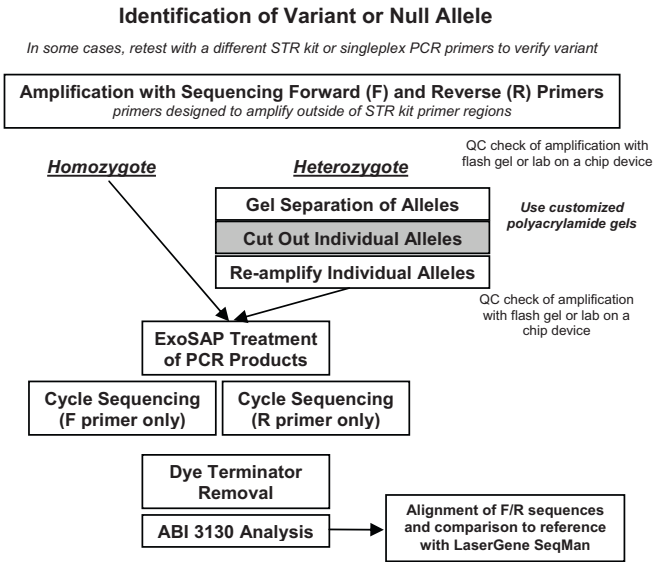

Fig. 1. Summary of the steps used in sequencing STR alleles.

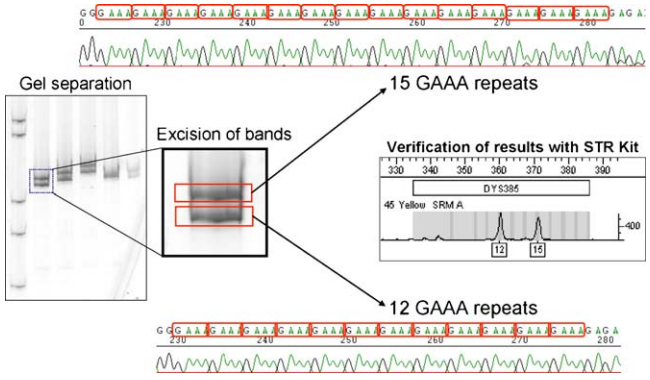

Fig. 2. Gel separation of several DYS385 PCR amplified samples with sequencing and typing results for a 12,15 sample.

sequences are aligned with one another and then compared to a reference sequence. Differences between the generated sequences from a putative variant allele were carefully examined and then compared to other sequenced alleles as well as the reference sequence, which originates from the GenBank accession numbers noted on STRBase for each locus: http://www.cstl.nist.gov/biotech/strbase/str_fact.htm. Fig. 2 illustrates a gel separation of the alleles present in several DYS385 PCR-amplified samples with the sequence information for each allele in a 12,15 type.

From the hundreds of normal and variant alleles sequenced over the past decade, 111 alleles from 19 autosomal and 4 Y-STR loci are included in Table S2. This information represents only a subset of more than 500 variant alleles cataloged on STRBase: http://www.cstl.nist.gov/biotech/strbase/var_tab.htm. In addition, 17 null alleles characterized following detection through concordance studies are reported in Table S3. Further information on null alleles is also available on STRBase at http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm.

In Tables S2 and S3, the nomenclature for reporting variation in the flanking region follows that recommended by the International Society for Forensic Genetics (ISFG) DNA Commission [64]. For example, a deletion of a "T" 14 bp downstream of the repeat region is listed as "D14Tdel".

4. Conclusions

DNA sequence analysis is helpful to understand the molecular basis for an allele that sizes "off-ladder" or one that fails to be amplified with a specific set of PCR primers. Through funding from the National Institute of Justice, NIST has provided STR allele sequence analysis free-of-charge to the human identity testing community. The supplemental files with this article provide the DNA sequencing primers used and a selection of results obtained with normal, variant, and null alleles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2010.09.005.

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