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Locus-specific brackets for reliable typing of Y-chromosome short tandem repeat markers

Short tandem repeat (STR) loci, widely used as genetic markers in disease diagnostic studies and human identity applications, are traditionally genotyped through comparison of allele sizes to a sequenced allelic ladder. Allelic ladders permit a floating bin allele calling method to be utilized, which enables reliable allele calling across laboratories, instrument platforms, and electrophoretic conditions. Precise sizing methods for STR allele calling involving fixed bins can also be used when a high degree of precision has been demonstrated within an instrument platform and a set of electrophoretic conditions. An alternative method for reliable genotyping of STR markers, locus-specific brackets (LSBs), is introduced here. LSBs are artificial alleles created through molecular biology manipulations to be shorter or longer than alleles commonly seen in populations under investigation. The size and repeat number of measured alleles are interpolated between the two LSB products that are mixed with the polymerase chain reaction-amplified STR alleles. The advantages and limitations of the LSB approach are described along with a concordance study between the LSB typing approach and other STR typing methods. Complete agreement was observed with 162 samples studied at 5 Y-chromosome loci.

Keywords: DNA size standards / DNA typing / Locus-specific bracket / Short tandem repeat / Y-Short tandem repeat
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1 Introduction

STR loci, sometimes referred to as microsatellites or simple sequence repeats (SSRs), are popular markers for genetic linkage studies, disease diagnostics, plant and animal population studies, and human identity testing applications including parentage testing and forensic casework [1]. Multiple STRs can be abundantly amplified in multiplex reactions from low amounts of initial DNA template using the PCR [2–4].

Reliable typing of STR length variants depends on methods of calibrating measured DNA fragment electrophoretic migration distance to allele repeat number or "type" [5–7]. When STR markers are first discovered and are being evaluated in research laboratories, typing of samples is often performed using an internal size standard run with each sample to convert migration distance to number-of-bp size. In the first demonstration of the value of an internal size standard with CE systems, Guttman *et al.* [8] used the migration of a 40-mer oligonucleotide relative to that of a 50-mer to improve run-to-run migration distance precision from 4.8% RSD to a sizing precision of 0.7% RSD.

Given sufficient run-to-run sizing precision, a sequenced reference sample containing only one allele can be used to calibrate fragment size to allelic type. However, many experimental factors (*e.g.*, the chemical composition of the electrophorectic medium and buffer system, electric field strength, and temperature) can alter the relative electrophoretic mobility of sample alleles and the components of the internal size standard [9–12]. These changes in relative mobility will result in different apparent allelic size, and possibly different type, if analyzed under sufficiently different experimental conditions.

Allelic ladders composed of common alleles observed in population studies enable reliable comparison of typing results among laboratories [1]. Each allele in an allelic ladder supplied as part of a commercial kit is usually sequenced by the manufacturer and alleles present in the ladder typically span the expected range of previously observed alleles for that locus (*e.g.*, [2, 3]). The alleles present in the allelic ladder are usually sequenced to provide a characterized reference sample [13]. Com-

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Abbreviation: LSB, locus specific bracket

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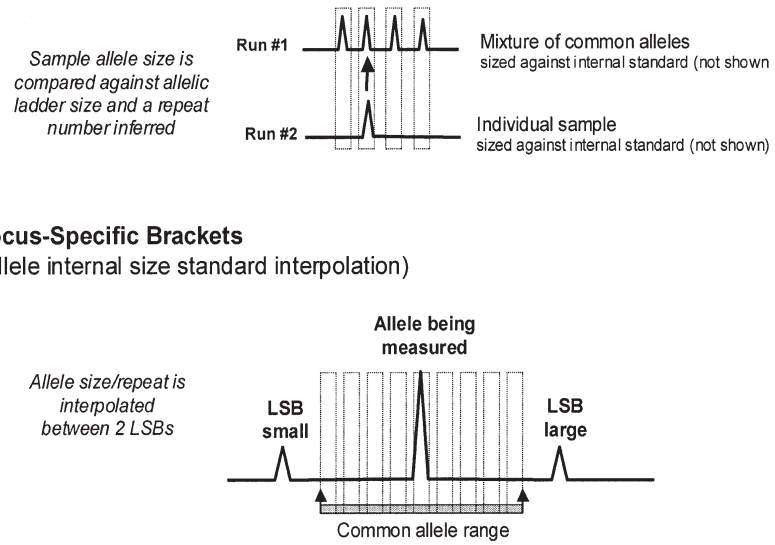

Figure 1. Illustration of two different STR typing methods involving (A) allelic ladders and (B) two locus-specific brackets.

parison to allelic ladders also facilitates recognition of variant types, such as single nucleotide bp insertions or deletions [14]. Some early microchip CE demonstrations mixed an allelic ladder with the sample to enable direct calibration of electrophoretic migration time with allele type [15, 16]. This simultaneous analysis approach does not make efficient use of multicolor fluorescence detection systems.

Sequential analysis of allelic ladder and sample materials is more efficient for detection systems that resolve three or more dye colors. A two-step calibration strategy is required. First, migration times are converted to apparent DNA size by calibration to a set of internal size standard DNA fragments labeled in a different dye color from the dye-labeled PCR products being analyzed. Second, the apparent DNA sizes are converted to allelic types by calibration to the allelic ladders (also calibrated to the same internal size standard) analyzed as separate samples shortly before or after the sample. Allele sizes in the samples are compared to the sizes in the allelic ladder to make the allele calls. Figure 1A illustrates the floating bin method for allele designation that involves an allelic ladder (run #1) run separately from a sample (run #2).

Temperature variation and subsequent variation in the effective sieving polymer concentration can significantly degrade run-to-run sizing precision [17]. Frequent recalibration to an allelic ladder is a common fix for this problem: *e.g.*, every ten injections on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA)*. New instruments with better temperature control, such as the multicapillary ABI Prism 3100, have improved run-to-run sizing precision and eliminated the need for such frequent allelic ladder analysis [18].

We discuss here an alternative approach to sizing and typing of STR alleles with electrophoretic systems, using dual internal standards constructed of DNA sequences similar to the loci being studied. These locus-specific brackets (LSBs) are artificial alleles created to be smaller or larger than the naturally occurring alleles for an STR locus but possessing the same (or very similar) flanking sequences. These LSB fragments have similar electrophoretic behavior as naturally occurring alleles, enabling allele typing by simple interpolation. In principle, this approach maximizes analysis efficiency of multicolor systems since all colors can be used for sample detection. The LSB approach is illustrated in Fig. 1B. The study presented here compares traditional DNA typing techniques with this LSB technology and evaluates the reliability and usefulness of LSBs using five different STR loci present on the human Y-chromosome.

* Certain commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments, or equipment identified are necessarily the best available for the purpose.

2 Materials and methods

2.1 DNA samples

Liquid blood samples from anonymous males with self-declared ethnicities were purchased from Millennium Biotech, Inc. (Ft. Lauderdale, FL, USA) and Interstate Blood Bank (Memphis, TN, USA) in accordance with human subjects review board approval at the National Institute of Standards and Technology. The extraction, quantification, and typing of these samples with autosomal STR markers has been previously described [19]. A subset of 162 samples representing 61 US Caucasians, 61 African Americans, 37 Hispanics, and three Asians were examined in this study.

2.2 LSB materials

A small LSB (LSB_S,) and a large LSB (LSB_L,) were synthesized as described by Dau and Liu [12] for several Y-chromosome STR loci with specific numbers of repeats in order to bracket the naturally occurring common alleles for these markers (see Table 1). These LSB materials were tagged with specific fluorescent dyes using a dilution and reamplification strategy [20, 21], empirically mixed to yield balanced LSB products for each locus, and then combined to make a single balanced LSB mixture. A large batch of the mixed LSB material was created so that it could be consistently added to the PCR amplified sample products (amplicons).

2.3 PCR amplification

Unlabeled oligonucleotide PCR primers were purchased from Qiagen Operon (Germantown, MD, USA). Dye-labeled primers were obtained from Applied Biosystems with 6-FAM (blue), VIC (green), NED (yellow), and PET (red) dyes attached at the 5'-end. All oligonucleotides were quality-controlled upon receipt in terms of mass and concentration using a Bruker BIFLEX III TOF mass spectrometer (Billerica, MA, USA) and Cary 100 UV spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA, USA) using previously described methods [22]. Primer sequences used in this study with the concentrations employed for PCR amplification are listed in Table 2.

Table 1. Characteristics of Y-chromosome STR loci and LSBs used in this study

Locus	Dye	Known alleles[a)]	LSB_S		LSB_L		bp size[d)]
			Nom[b)]	Cal[c)]	Nom[b)]	Cal[c)]	
DYS389 I	6-FAM	9–17	7	7.21	19	18.16	144–189
DYS389 II	6-FAM	24–34	24	23.68	36	34.92	263–310
DYS390	VIC	17–28	15	14.91	30	29.87	204–264
DYS391	NED	6–14	5	4.98	16	15.94	104–149
DYS385 a/b	PET	7–28	5	5.13	21	20.99	302–362

a) Range of repeat sizes for known alleles [23]. Underlined alleles show potential overlap or extension beyond the corresponding LSB material used in this study.
b) Nominal number of repeat size for LSB, based upon synthetic design
c) Calibrated number of repeat size for LSB, based on calibration to allelic ladder
d) Bp size range of LSB_S to LSB_L relative to GS500 LIZ internal sizing standard

Table 2. PCR primer sequences used for amplification of Y-STR loci and reamplification of original LSB materials

Primer name	Dye label	PCR primer sequence (5'-3')	Concentration (μmol/L)
DYS389I/II-F	6-FAM	ACTTGAGGAACACAATTATCCCTGA	0.60
DYS389I-R		GACTCTCATCTGTATTATCTATGTATCTG	0.25
DYS389II-R		GACTCTCATCTGTATTATCTATGTGTGTG	0.51
DYS390-F		GCCGCCATGGTAGCATAATAGAAA	0.15
DYS390-R	VIC	CCTGCATTTTGGTACCCCATA	0.32
DYS391-F	NED	CTATTCATTCAATCATACACCCATATCTG	0.58
DYS391-R		GGTTGCAAGCAATTGCCATAGAG	0.29
DYS385a/b-F		ATTTGCTGACCAGATTTCTTTCTG	1.1
DYS385a/b-R	PET	TAGACACCATGCCAAACAACAAC	1.4

PCR reactions were performed in 20 μL volumes and consisted of a primer mixture with the concentrations listed in Table 2, 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1 × Gold Buffer, 1.5 mmol/L $MgCl_2$, 250 mmol/L dNTPs (Amersham, Piscataway, NJ, USA), 0.16 mg/mL BSA (Sigma, St. Louis, MO, USA), and approximately 1–2 ng of each DNA template. A GeneAmp 9700 thermal cycler (Applied Biosystems) was used under the following conditions with ramp speeds of 1°C/s (9600 emulation mode): 95.0°C for 11 min; four cycles of (94.0°C for 30 s, 63.0°C for 45 s, 72.0°C for 45 s); 32 cycles of (94.0°C for 30 s, 59.0°C for 30 s, 72.0°C for 45 s); 72.0°C for 60 min; and 25.0°C until samples were removed from the thermal cycler.

2.4 Sample preparation and electrophoresis

The separation and detection of Y-STR alleles and LSBs were accomplished using both an ABI Prism 310 single-capillary and an ABI Prism 3100 Genetic Analyzer 16-capillary array system following manufacturer protocols, using the G5 matrix filter set to detect the five dyes 6-FAM, VIC, NED, PET, and LIZ (orange). Prior to sample analysis, a spectral matrix was established using matrix standard set DS-33 (Applied Biosystems). Samples were prepared with 20 μL Hi-Di formamide (Applied Biosystems), 0.5 μL GS500 LIZ size standard (Applied Biosystems), 1 μL amplicon, and 3 μL LSB mixture (see Section 2.2). On the ABI 310, samples were injected one at a time for 5 s at 15 kV and separated at 15 kV for 28 min at a run temperature of 60°C. Separations were performed using 3100 POP-4 sieving polymer matrix (Applied Biosystems), 1 × Genetic Analyzer buffer (Applied Biosystems), and a 47 cm × 50 μm capillary (Applied Biosystems). On the ABI 3100, samples were injected 16 at a time for 10 s at 3 kV and separated at 15 kV for 44 min at a run temperature of 60°C. Separations were performed using 3700 POP-6 sieving polymer matrix (Applied Biosystems), 1 × A.C.E. buffer (Amresco, Solon, OH, USA), and a 36 cm × 50 μm capillary array (Applied Biosystems).

2.5 Data analysis for LSB typing

Following data collection, samples were analyzed with GeneScan 3.7 (Applied Biosystems) and Genotyper 3.7 (Applied Biosystems) software programs to determine scan number (data points), relative DNA size compared to the GS500 LIZ size standard, and peak height for each LSB and sample allele tested.

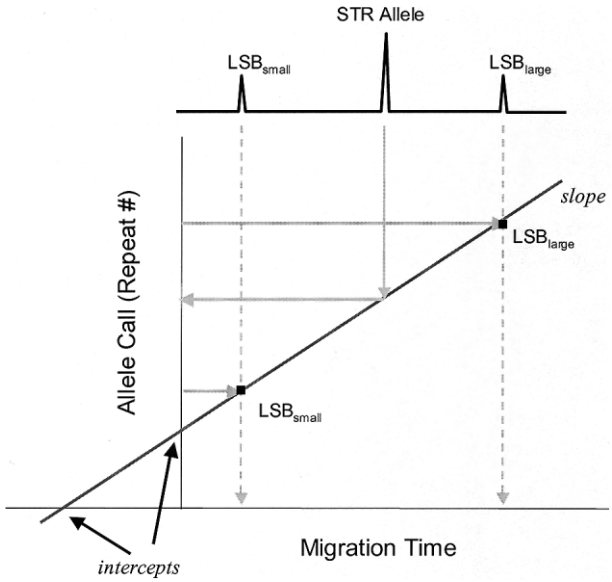

Figure 2. Illustration of method for allele size determination based on two LSBs created to be smaller and larger than common alleles at a particular STR locus.

The repeat number of each allele was calculated using the following formula [6, 12]:

$$\text{Repeat\# Allele} = \text{CalRep\#LSB}_s + \frac{(\text{Scan\#Allele} - \text{Scan\#LSB}_S)(\text{CalRep\#LSB}_L - \text{CalRep\#LSB}_S)}{(\text{Scan\#LSB}_L - \text{Scan\#LSB}_S)}$$

where Scan# is the time scale data point for the highest signal in the pertinent peak detected in the GeneScan software and CalRep# is the calibrated repeat number for the LSBs. Calibration of the apparent DNA size of the LSBs to that of sizing ladder alleles of the same nominal repeat number corrects for any small differences in electrophorectic migration behavior between the artificial LSB and native alleles. Figure 2 illustrates this process. This two-point calibration is possible since the electrophoretic migration of DNA is fairly linear over the size range of approximately 100–400 bp of the PCR products of forensic interest. Within this Ogsten regime, movement through the separation medium is primarily on the basis of fragment size; *i.e.*, the number of bps [9, 11].

2.6 Y-STR typing with Y-PLEX 6 and Y-PLEX 5 kits

Y-PLEX 6 and Y-PLEX 5 kits from ReliaGene Technologies (New Orleans, LA, USA) were used to amplify a subset of the 162 samples for the Y STR loci DYS19, DYS385a/b, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS438, and DYS439. PCR amplification was performed with 25 μL volumes and 1–2 ng DNA template according to the manufacturer's instructions. The separation and

detection of the Y-PLEX 6 and Y-PLEX 5 kit generated PCR products that were accomplished with an ABI Prism® 310 Genetic Analyzer using filter set A. The matrix was established with matrix standards for the four dyes FAM (blue), HEX (green), TAMRA (yellow), and ROX (red) (Applied Biosystems). Each sample was prepared by adding 1 μL amplicon to 20 μL of deionized formamide containing 0.75 μL GS500 ROX size standard (Applied Biosystems). Samples were injected for 5 s at 15 kV and separated at 15 kV for 26 min with a run temperature of 60°C using POP-4 sieving polymer matrix (Applied Biosystems), 1 × Genetic Analyzer Buffer with EDTA (Applied Biosystems), and a 47 cm × 50 μm capillary (Applied Biosystems). Following data collection, samples were analyzed with GeneScan 3.7 and allele calls were determined by comparison to allelic ladders using Genotyper 3.7 and the Y-PLEX 6 310 v3.0 and Y-PLEX 5 310 v1.0 genotyping templates provided by ReliaGene.

2.7 DNA sequencing

As *per* the manufacturer protocol, half reactions of the BigDye Version 3 kit (Applied Biosystems) were used with approximately 7 ng of amplicon treated with ExoSAP (USB, Cleveland, OH, USA) to remove original PCR primers and remaining dNTPs from the PCR reaction. Individual, unlabeled PCR primers were used for sequencing. Sequencing products were cleaned up on Performa DTR columns or 96-well plates (Edge Biosciences, Gaithersburg, MD, USA) and separated on an ABI 3100 Genetic Analyzer using POP-6, a 36-cm array, and an electrokinetic injection of 22 s at 1 kV. Forward and reverse sequences were aligned and analyzed with Sequencer (GeneCodes, Ann Arbor, MI, USA).

3 Results

3.1 Locus-specific brackets used in this study

Table 1 lists the nominal and calibrated repeat numbers for the LSB_S and LSB_L materials examined in this study. Figure 3 contains electropherograms showing PCR products from the 5 Y-STR loci examined along with the corresponding LSB_S and LSB_L products. Repeat numbers for each LSB are indicated above their respective peaks. Note that the DYS389I and DYS389II alleles contain split peaks due to incomplete or poor adenylation [1]. Since LSBs are also PCR products, they contain stutter products that become more prevalent with longer alleles (*e.g.*, LSB_L for DYS385). The 5 Y-STR markers examined in this study were run simultaneously with the following locus/dye combinations: DYS389I/6-FAM, DYS389II/6-FAM, DYS390/VIC, DYS391/NED, DYS385a/PET, and DY385b/PET.

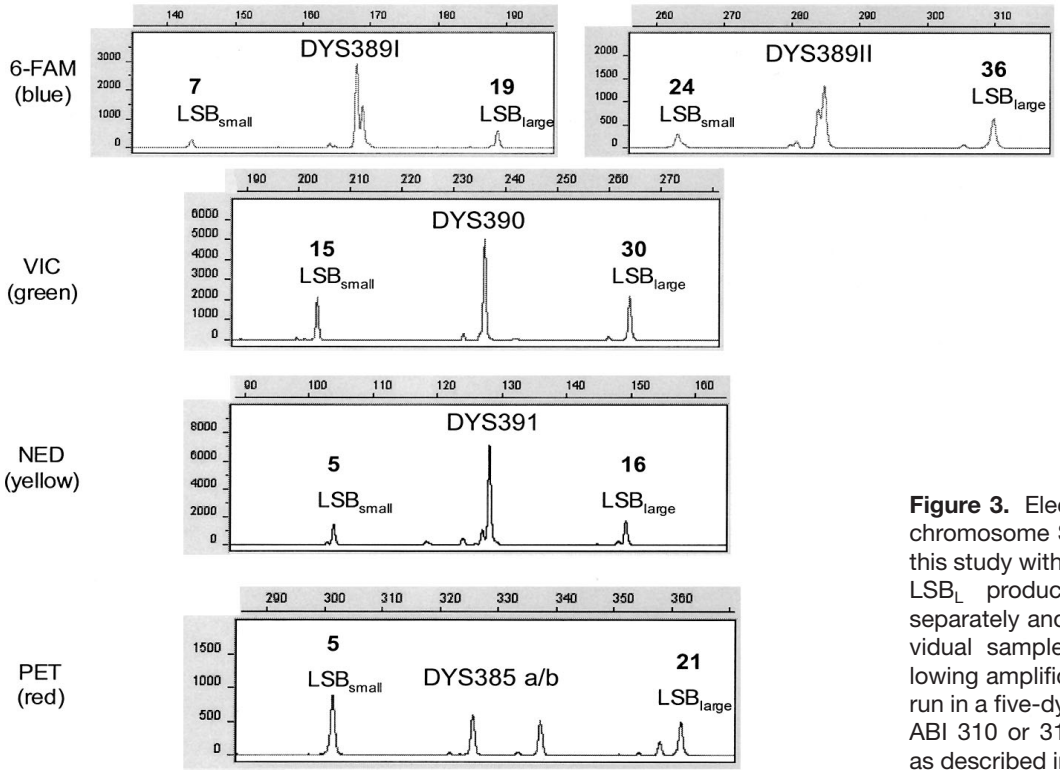

Figure 3. Electropherograms of Y-chromosome STR loci examined in this study with LSBs. The LSB_S and LSB_L products were generated separately and mixed with the individual sample PCR products following amplification. Samples were run in a five-dye format on either an ABI 310 or 3100 Genetic Analyzer as described in the Section 2.

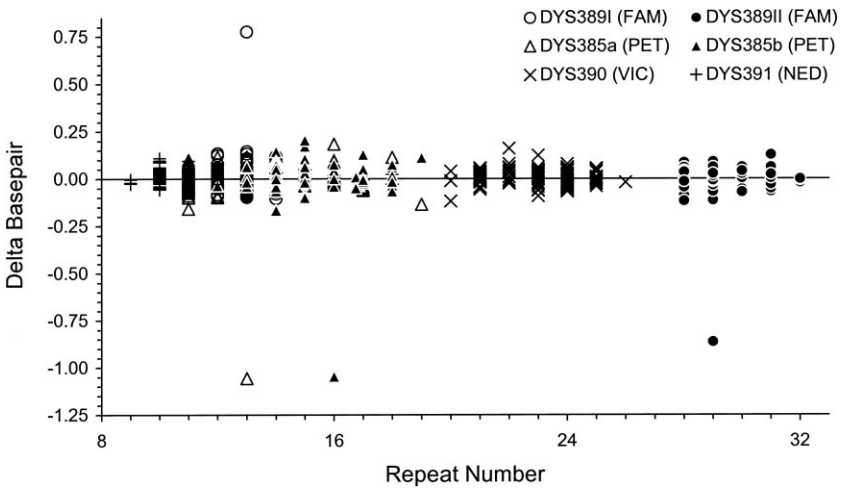

Figure 4. Plot of repeat number determined by the LSB method *versus* difference from the nearest integer repeat "delta basepair" for five different loci across 162 samples.

3.2 Precision and accuracy in sizing PCR products

Since two alleles are possible with the duplicated locus DYS385 [23], the ability to reliably size STR alleles from multiple Y-chromosome loci using the LSB approach was effectively explored with six PCR products across four dye colors. In this particular study, we included an additional internal size standard in the fifth dye channel to permit conversion of migration time (scan number or data point) to effective DNA size with the GS500 LIZ size standard and GeneScan software. The collected data were imported into the Genotyper software after the alleles and LSBs were sized in GeneScan. This permitted an evaluation of precision of LSB materials and STR alleles with and without the traditional sizing calculations commonly used in genotyping applications. Generally, the SDs of the calculated fragment sizes were in the order of 0.1 bp in agreement with the run-to-run precision noted by Lazaruk *et al.* [5].

Figure 4 displays the allele repeat numbers for each of the five loci as determined with the LSBs against the difference between the calculated bp size and the bp size of the closest integer number of repeats for all 162 samples. For example, if the equation described in Section 2.5 calculated an allele to contain 12.99 repeats, then the "true" allele repeat count was assumed to be 13.00. Since all alleles examined in this study are tetranucleotide repeats, the bp size difference is (12.99–13.00 repeats) $\times$ 4 (bp/repeat) = -0.04 bp. This "delta basepair" is a measure of typing accuracy under the assumption that all sample alleles are exact integer repeats.

With the exception of four data points, all LSB-determined allele sizes fell within ± 0.2 bp. Note that the four samples in question fall approximately 0.8–1.2 bp or approximately a single nucleotide off from a complete tetranucleotide repeat. These four samples were sequenced and found to be variant alleles possessing single base insertions or deletions in the flanking region near the STR repeat [14]. For example, alleles 12.3 and 15.3 at DYS385 were found to possess the same T deletion in a polyT stretch that is 73 bases downstream of the repeat region as noted previously by Schoske *et al.* [24]. After correcting the true allele type assignment of these four variant alleles, the average delta basepair for the population of 937 typed alleles was 0.01 bp with an SD 0.05 bp.

3.3 Concordance of LSB typing to traditional methods

The concordance of allele calls made with the LSB typing approach was evaluated relative to either commercial kits or a previous study performed at NIST involving precise sizing and sequencing of representative alleles [24]. With the exception of one DYS385 allele noted below, all $6 \times 162 = 972$ allele designations were fully concordant across the various methodologies employed.

A sample possessing alleles 13 and 21 at DYS385 based on allelic ladder measurements (Fig. 5) appeared to contain only allele 13 with the LSB method (Fig. 6C). Upon finding this discrepancy the sample was retested on the ABI 310 with and without adding the LSB markers (Fig. 6). Running the sample amplicon without the LSB_S and LSB_L products (Fig. 6A) clearly shows the presence of both alleles. In this case, with the LSB data alone, the DYS385 allele 21 would have been missed in this particular sample because it was masked by the LSB_L allele. Thus, a disadvantage of using LSB markers of exactly the same

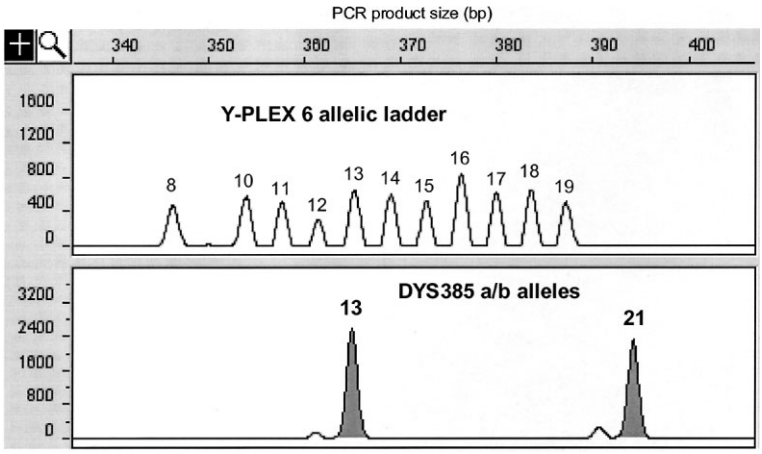

Figure 5. Electropherograms of a DYS385 allelic ladder from the Y-PLEX 6 kit (top panel) and a sample containing alleles 13 and 21 (bottom panel).

size and sequence as a true allele is the possibility of masking a true allele of the same repeat number. Of the 162 samples analyzed, only one sample exhibited this problem where an allele was masked by an LSB. Other synthetic designs, *e.g.*, including a 2 bp insertion or deletion variant, could minimize such masking.

4 Concluding remarks

As demonstrated in this work, LSBs can provide reliable STR typing. These artificial alleles provide an internal calibration unique to each marker that can be used to adjust for electrophoretic run-to-run differences. No allelic ladder or separate internal size standard is required. Sequenced LSB alleles can provide the calibrants necessary to accurately convert electrophoretic mobility of a PCR product into the number of repeats present. All colors evaluated by a given detection system may be used for labeling the PCR products because a separate dye channel is not needed for the internal size standard.

However, the LSB approach to STR typing has several potential limitations: (1) LSBs may mask a true allele as illustrated in Fig. 6. (2) It may not be possible to pack loci together as tightly in a multiplex because of the space needed on either side of the common set of alleles to fit the LSB_S and LSB_L markers. (3) Negative controls do not appear "clean" when data files are reviewed because the LSB peaks are present in each dye color. (4) Current genotyping software, such as GeneScan and Genotyper, are designed to work with an internal size standard labeled with a fluorescent dye that is readily distinguished from the colors used to label the alleles. For automatic LSB typing, experimental and data analysis procedures must be designed that enable the LSB peaks to be reliably located.

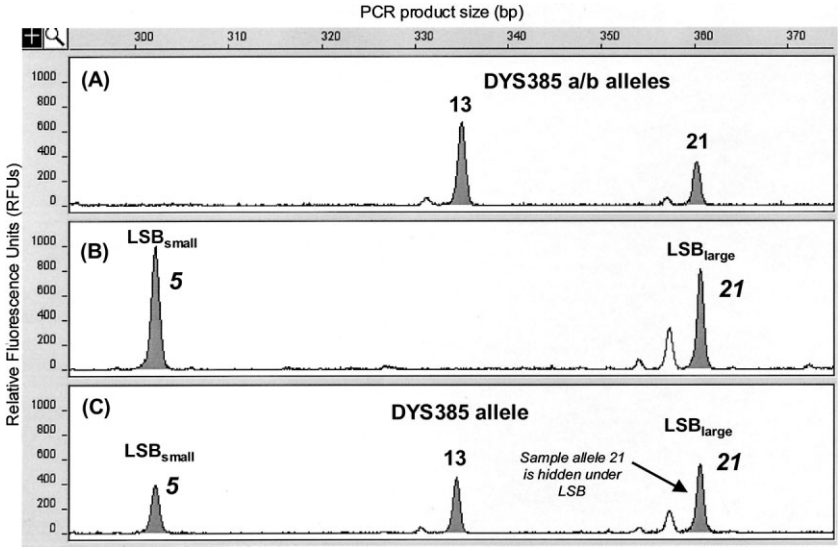

Figure 6. Electropherograms from the same DNA sample shown in Fig. 5 but amplified with the primers listed for DYS385 shown in Table 2: (A) sample without the DYS385 LSB_S and LSB_L products, (B) the DYS385 LSB_S and LSB_L products by themselves, (C) combination of the sample and the DYS385 LSB_S and LSB_L products.

The LSBs method described here was originally developed to eliminate the need to construct allelic ladders or use an internal size standard labeled with a different fluorescent dye. While neither LSBs nor automated genotyping software to process samples are commercially available, this approach does permit reliable typing of STR markers with single or multicolor fluorescence detection. However, a complete validation of the LSB approach would require the study of temperature effects on the accuracy of STR allele typing, but this should await first the implementation of redesigned LSBs that do not mask true alleles.

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