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Development of a new Human Identity Testing Kit for the Simultaneous Amplification of the 13 CODIS STR Loci plus Amelogenin Using Locus Specific Brackets (LSBs)

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Development of a new Human Identity Testing Kit for the Simultaneous Amplification of the 13 CODIS STR Loci plus Amelogenin Using Locus Specific Brackets (LSBs)

Lizmery Suarez Ferguson

Thesis

Submitted to the Eberly College of Arts and Sciences
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in
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ABSTRACT

Development of a new Human Identity Testing Kit for the Simultaneous Amplification of the 13 CODIS STR Loci plus Amelogenin Using Locus Specific Brackets (LSBs)

Lizmery Suarez Ferguson

STR loci consist of short, repetitive sequence elements 2–7 base pairs in length. An STR genotype is typically determined by PCR amplification followed by fragment size estimation with electrophoresis. Commercial multiplex STR amplification kits are available for the 13 CORE loci used in the USA for human identity testing. The genotyping rationale with these multiplex STR amplification kits involves a two-fold calibration analysis: 1) An internal size standard (ISS) labeled with a separate dye establishes the size of the unknown DNA fragment and 2) an allelic ladder which contains most of the common alleles for each locus is also sized by the internal sized standard and used as a reference for the allele call. Locus-Specific Brackets (LSBs) offer an alternative STR sizing method that may be less susceptible to the problems caused by variation in electrophoretic conditions. An LSB multiplex may also be cheaper to produce and use than a conventional kit. LSBs are internal size standards that flank each targeted locus. The brackets have the same repeat structure as the locus, are 1 or more repeat units shorter or longer than the common alleles, and are labeled with the same dye. Because LSBs have the same electrophoretic properties as the sample alleles, their sizing function should not be impaired by injection-to-injection variation in conditions. The aim of this project was to produce an LSB-based human STR multiplex reaction kit, free of allelic dropout and with appropriate LSB size standards for analysis by custom genotyping software, and to do a simple performance evaluation of the kit by testing the reproducibility of profiles and the sensitivity of the reaction. The result was a new human STR multiplex that can amplify all 13 STR loci plus amelogenin in a single reaction. The new LSB multiplex has a sensitivity range of 0.1-1ng of DNA template where full genetic profiles can be produced. Also developed were new locus specific brackets needed to accommodate necessary shifts allele size range. Existing software failed to successfully genotype all alleles; however manual analysis of the data showed the correct genotypes for 81% of all tested alleles, with few incorrect calls as fragment were incorrectly sized by the size standard. Although the further development of the kit is clearly required, the LSB kit remains promising as a new and alternative method for forensic DNA analysis.

DEDICATION

**This thesis is dedicated to my mother who taught me that education
is the way forward and hard work is the path to success**

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**Part One: Development of a new Multiplex for the Simultaneous
Amplification of the 13 CODIS STR Loci plus Amelogenin using
Locus Specific Brackets (LSBs)**

Introduction

General Background on Short Tandem Repeats

Selection of the commonly used STR loci and STR Kits

More than 99.7% of the human genome does not vary between individuals; however the 0.3% that remains has been used to generate profiles that can differentiate individuals at the genetic level [1]. Certain regions of the human genome contain repeated DNA sequences, and those with core repeat units 2-7 bp in length are commonly known as microsatellites, or short tandem repeats (STRs) [2]. Thousands of polymorphic microsatellites have been found throughout the human genome but despite the high number, only a few have been chosen for the purpose of human identity testing as not all of them have proven to have the necessary characteristics that make them especially suitable for human identification [3]. Such characteristics include being easily amplified by polymerase chain reaction without the problem of differential amplification; that is, given the small size of STR alleles, their PCR products are generally similar in amount, making analysis easier. STR loci have also been chosen due to their high level of polymorphism which is necessary in order to discriminate among individuals. The size of STR alleles is another advantage that makes them desirable in forensic applications. Many range from ~100 to 400bp in length allowing in many cases the recovery of full genetic profiles even when the DNA recovered from a crime scene is highly degraded. Their size also allows the capability of multiplexing; that is, the simultaneous PCR amplification of multiple locations of the human genome [4]

Loci with a tetranucleotide repeat are less prone to artifact alleles than shorter repeats and allow for better resolution of heterozygous alleles one repeat apart during electrophoretic analysis [5].

STR nomenclature is based on location. If the STR locus is within a gene, then the gene name is used for designation, e.g. TH01 within intron 01 of the human tyrosine hydroxylase gene. The name of an STR not within a gene indicates its chromosomal position, e.g. D5S818 where D: DNA; 5: chromosome 5; S: single copy sequence; and 818: is the 818th locus being described on chromosome 5 [6].

STR alleles have been further categorized by repeat structure. Simple repeats contain units of identical length and sequence, compound repeats contain two or more adjacent simple repeats and complex repeats several repeat blocks of variable unit length as well as variable intervening sequences. Some tetranucleotide STR loci useful in forensics contain non-integer alleles which differ in size by only 1 or 2 nucleotides; therefore, a forensic DNA analysis instrument must permit genotyping accuracy to within 0.5 nucleotides [7].

In 1996 the Federal Bureau of Investigation solicited the advice of a wide range of forensic analysts to establish core STR loci for inclusion within the national database known as the Combined DNA Index system or CODIS (also known as the National DNA Index System, NDIS). By October of 1998 the FBI had selected 13 STR loci and it was officially launched throughout the United States. The loci chosen were D13S317, TH01, TPOX, D5S818, D7S820, D3S1358, FGA, CSF1PO, D8S1179, vWA, D16S539, D18S51, and D21S11, with amelogenin as the sex determining marker [8]. Currently NDIS contains over 6.5 million STR profiles and links all the 50 states in the United States making it possible to search criminal DNA profiles [9]. STRs are also now widely used in other areas where genotyping comparison is needed, e.g. disease research and diagnostics, parentage assessment as well as investigation of human diversity [10].

Current approach for STR typing

The process for STR genotyping begins by amplifying all loci through PCR. Once the DNA has been amplified the amplicon size is determined by gel or (much more commonly) capillary electrophoresis [11]. The use of fluorescent technology allows two loci with overlapping allele size ranges to be co-electrophoresed, because each can be labeled a different color. One of the PCR primers for a given locus is labeled on the 5' end with a fluorescent dye. As the labeled DNA molecule passes a capillary window a laser hits the fluorophore or dye attached to the DNA fragment. [12]. The dye absorbs the energy of the laser and then emits light at a lower energy level. This energy is then converted to an electronic signal measured in relative fluorescent units (RFUs) and makes up the peaks observed on the electropherogram just like bands are observed on a gel **Error! Reference source not found.** [13].

The current typing method first uses an internal size standard (ISS) added to the amplified alleles prior to electrophoresis to generate a function for estimating sample allele size. The internal size standard DNA fragments span a size range larger than the sample alleles and it must be labeled with a different dye from the sample alleles, Figure 3. The sizing function relates the electrophoresis time of the known fragments, i.e. those of the internal standard and the allelic ladder.

Once the fragments have been sized each fragment gets assigned a repeat number (i.e. an allele call). For this step it is necessary to use an allelic ladder. The ladder contains most of the common alleles for each STR locus present in the population each labeled by its specific dye and it is used to calibrate the PCR product sizes to STR repeat numbers [14]. Alleles found in the ladder are PCR-amplified with the same primers provided by the STR kit to test unknown

samples; therefore, every sample amplified by the kit will yield alleles that are identical in size as alleles present in the allelic ladder [15]. Each Allele in the ladder has been characterized by the number of repeats it contains and the range of each allele bin is $\pm 0.5\text{bp}$ around each allele; therefore, when a sample allele matches the allele from the ladder an allele call is assigned. Contrary to the internal size standard, the allelic ladder is injected separately from the samples. It is normally run within a short time period from the other sequentially processed samples alleles, whether is as part of the same batch as the samples being typed, or shortly before or after the sample set, therefore, it is assumed that it runs under the same electrophoretic conditions as all other samples being processed [16]. For analysis, the ladder is sized by the internal size standard and then the software allows the conversion from size (bp) in to a repeat number **Figure 2**. The most common algorithm used to determine the size of a DNA fragment is called Local Southern Method [17]. It uses two known peaks on either size of the unknown fragment being measured in order to calculate its size **Figure 3**. Local southern works very well for calculating sizes that range from 100 to 400 base pairs because DNA molecules in this size range have typically a linear relationship between size and time; however, because the samples and the allelic ladder have separate injections, run-to-run fluctuations in electrophoresis conditions (e.g. temperature, buffer pH, etc.) may cause inconsistencies in sizing reproducibility that can potentially lead to an inaccurate allele call.

STR allele calls are made in an automated fashion. However due to the presence of artifact peaks, an expert scientist is needed to double check the data. Artifacts are divided into two categories: biology related (stutter products, incomplete adenylation,) and technology related (matrix failure, dye blobs, air, and sample contaminants).

Biological related artifacts

Stutter product: is an incorrect STR allele produced by strand slippage during PCR. According to this model the newly formed strand of DNA skips one repeat unit starting the complementary base pairing with the next repeat (misalignment), pushing out a non-base pair loop from the template strand of the DNA and normally causing a deletion of one repeat unit [18]. In tetranucleotides they commonly appear as one repeat shorter than the main allele (n-4) but they can also be observed as one repeat larger (non-base pair loop inserted) than the main allele (n+4)

Figure 4a. The acceptable upper limit for stutter is 3SD above the highest stutter percentage normally observed at that locus or a general value of 15% [19]

Incomplete adenylation: incomplete adenylation is the result of PCR amplification with too much DNA template. During the PCR reaction the *Taq* polymerase catalyzes the addition of an extra nucleotide (adenine) at the 3' end of the PCR product. However when this fails to happen split peaks are produced known as -A and +A. Because one of the alleles will be off ladder, manual edition by the expert is needed **Figure 4b** [20].

Technology related artifacts

Matrix failure: known as pull up, matrix failure results when the instrument fails to resolve the dye colors. The spectral overlaps occur for examples when a sample is overloaded and it exceeds the linear range of detection. The result is peaks of >1 color for a single fragment, or a highly elevated baseline for any given color [1].

Dye blobs: dye blobs occur when the fluorescent molecule detaches itself from the primer and migrates through the capillary independently. Dye blob peaks are typically broader than an allele [21].

Sample contaminants: any extra material that becomes visible within the analysis spectrum tends to interfere with the results as they appear as peaks. Many contaminants appear as broad peaks that can be distinguished from the true alleles [22].

Additional peaks present in STR Profiles

Triallelic pattern: rare individuals show three alleles at a single locus instead of two. One explanation is a somatic mutation of an allele, resulting in a chimera with some cells containing the original alleles and some cells containing the mutant allele. This produces uneven peak heights for two of the affected alleles that when added together are equal in height as the normal allele. The second type occurs as a localized duplication or chromosomal aneuploidy producing peaks that are all similar in heights **Figure 4c** [23].

Mixed sample results: a mixture of >1 individual is apparent when multiple loci have more than two alleles of equal intensities at a particular locus or severe heterozygous imbalance. Determining the individual genotypes in a mixture is particularly difficult if one of the contributors is, 1/10th of the major contributor, or if there are multiple individuals [24].

Advantages of Multiplex PCR

Multiplex PCR, the amplification of several loci within the same PCR reaction allows for the efficient use of sample DNA and reduces the possibility of contamination or data mix-up compared to many separate reactions [25]. After the 13 core loci for CODIS were adopted in the United States, Life technologies (Applied Biosystems) and Promega Corporation marketed multiplex STR kits for typing some or all CODIS loci plus amelogenin [26]. Common problems

in PCR-based STR analyses are the production of false negatives due to reaction failure or false positives due to contamination of the PCR reaction. In multiplex assays each amplicon provides an internal positive control for the other loci. A failure to amplify one but not all loci, points to incomplete template degradation rather than complete degradation or a problem with the reaction mix. An external (separate tube) positive control is still needed to monitor conditions such as thermal cycler function [27].

Factors affecting DNA mobility during electrophoresis

Whether the separation occurs on gel or capillary the basic principle is the same. Molecules are separated on the basis of their rate of movement through a gel/capillary under the influence of an electrical field. Electrophoretic mobility is a function of DNA fragment size and shape, the density of the gel/polymer, temperature, buffer, and the strength of the electrical field [13]. During electrophoresis DNA interacts efficiently with the sieving polymer, and its size is proportional to its length, a relationship that is the key to accurate genotyping by CE. [28]. Denaturing conditions are maintained by chemicals such as urea, formamide, and 2-pyrrolidinone, but even under strong denaturing conditions DNA can assume its secondary structure, e.g. a loop/hairpin. For this reason high temperature is also used to promote denaturation [29].

Commercial Control of Life Science Products and Automated Platforms

The current technology surrounded STR technology has been for decades dominated by two major companies in the United States, Applied Biosystems (AB) and Promega Corporation. These two companies hold the vast majority of the market from kits, reagents and software to the

integrated platforms that are currently in almost every forensic lab in the United States [30]. As the demands of generating, entering and maintaining the DNA profiles in the national database increases, so does the need for new automation that can increase sample throughput, reduce cost and allow for full resolution of STR base composition. A new approach to allow this type of resolution has been developed by Roche laboratories. The Roche genome sequencer FLX platform has been used to simultaneously sequence multiple core STR loci and directly compare these sequences to conventional CE-based STR technology. This new method is advantageous as it reveals DNA base substitutions and repeat sequence variations that would not have been identified with the current STR typing method [31]. Another new significant development has been the combination of PCR with CE on a microchip to produce a fully integrated and automated STR analysis system. The advantage of such a system would increase throughput separation of forensic samples increase workflow and lower the costs by reducing the electrophoretic analysis from submicroliter to nanoliter sample volumes [32].

STR Typing with Locus-Specific Brackets (LSBs)

Locus Specific Brackets were developed as an alternative sizing method for detecting STR fragment length. LSBs are internal size standards that flank each targeted locus. They are artificial alleles created to be 1 or more repeat units shorter or longer than the common alleles of an STR locus but having identical (or very similar) flanking regions **Figure 5** [33]. As the sample allele and LSB sequences are nearly identical, the electrophoretic behavior is expected to be nearly identical. Therefore the sizing function should be much more robust between-injection variation in electrophoretic conditions compared to sizing using an ISS. [34]. Just as in any other STR kit, alleles can fall outside the desired range. If shorter or longer than its respective brackets

it will migrate between sets of LSBs from adjacent loci (equivalent to an off-ladder allele in the conventional method); however, if an allele were to be exactly the same size and sequence as the bracket, the true allele would be masked by the LSB and the genotype would be incorrect e.g. a heterozygous locus would appear as homozygous. Just like the conventional approach Locus Specific Brackets utilizes the local southern method to determine the size of the unknown DNA fragments by using the reciprocal relationship between fragment length and mobility.

Other potential advantages of Locus Specific Brackets over conventional human STR kits are that the same number of loci requires one fewer dye per kit; that used for the ISS, and the lack of an allelic ladder means every injection can yield a sample profile. Therefore an LSB kit could reduce costs and analysis time compared to the kits currently in use.

Y-STR Typing with Locus Specific Brackets

Locus-Specific Brackets were found to be reliable for Y-chromosome STR typing. Potential problems compared to a conventional kit discussed by the authors include masking of a sample allele by an LSB and the fact that new sample fragment sizes cannot be developed for the LSB size ranges [35].

Preliminary Validation of the LSB Multiplex

The now defunct company known as Oligotrail, LCC, developed an LSB multiplex for the 13 CODIS STR loci plus amelogenin [36]. Prior to the current study, members of our research lab attempted to validate this kit by measuring concordance with profiles from the same human

subjects produced by the commercial kit identifier (AB). The reagents, manual and genotyping software were supplied by a former Oligotrail research scientist.

99 human subjects were profiled using both kits for a total of 1330 allele comparisons (unpublished). 1315 alleles were concordant 14 of the samples amplified with the LSB kit had allele dropout at loci D13S317 and vWA. DNA sequencing of these individuals revealed a primer annealing site polymorphism for D13S317, a likely explanation for the allelic dropout. One LSB vWA primer site overlapped the repeat region, also a likely explanation for the dropout. Oligotrail had not encountered these problems, perhaps because they relied on a geographically limited selection of research subjects (East Asian), while our study was a mixture of ethnicities.

I took over the project to make a new reaction (LSB mixes reagents were getting scarce), and to design new primers to solve the dropout problem and include them in the new mix. During the course of my preliminary work I encountered unexpected challenges that changed the original course of the project. Almost all fragment sizes generated by the a reaction mix I made with primer sequences from the Oligotrail genotyping software (called Oligocode) manual were in disagreement with fragment sizes from the previous validation study (same sample). Further investigation revealed that primer sequences given in the LSB manual were not the same as the primers present in the LSB multiplex used during the previous validation study (**current and original LSB sequences are given in appendix Table 1**). Due to the observed differences in primer annealing sites, the new mix faced a potential overlap between adjacent loci of the same color, and a [new] reverse primer site included a polymorphism that could potentially create locus imbalance or allele dropout. Shifts in fragment size also meant that profiles would not be correctly genotyped with Oligocode.

Because at this point the optimization of the new LSB mix was very advanced, and primers designed to solve dropout issues had already been included in the new mix, I opted to keep the current LSB multiplex, design new primers for affected loci and synthesize new brackets.

Goal

The goal of this project was to produce an LSB-based human STR multiplex reaction kit, free of allelic dropout and with appropriate LSB size standards for analysis by the Oligotrail genotyping software, and to do a simple performance evaluation of the kit by testing the reproducibility of profiles and the sensitivity of the reaction.

Materials & Methods

DNA Sample preparation

DNA samples were already extracted and quantified as recommended by SWGDAM revised validation guidelines during the previous validation of the LSB kit [37]. Liquid blood samples (100ul) and buccal swabs were collected from 100 anonymous (WVU IRB protocol 16279). DNA extraction from the liquid blood samples was done using Qiagen (Valencia, CA) DNeasy kit according to protocol for whole nucleated blood, with a final elution of 100uL. Buccal swab samples were extracted using the Qiagen DNeasy kit according to manufacturers' directions for DNA extraction from tissues, with a final elution of 100uL. All samples were quantified for nucleic acid content using the nanodrop spectrophotometer and further diluted to a working concentration of 1ng/ μ l.

LSB-Multiplex Primer design & synthesis

All primes were designed using the Oligoanalyzer tool from Integrated DNA Technology, and only primers that fit the best criteria (e.g. specificity, melting temperature, GC content, hairpin structure, self-dimer-structures etc.) for ultimate performance were chosen [38]. All primer lengths were between 18 – 30 bases to maintain high sequence specificity provided that the annealing temperature was optimal. The GC content was kept between 40-60 percent, allowing for a melting temperature between 54°C - 58°C. HPLC purified 5' fluorescently labeled primers were purchased from Operon Biotechnologies, Inc (Huntsville, AL) containing either FAM (carboxyfluorescein), JOE (β -carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein) TAMRA (N,N,N',N'-tetramethyl- β -carboxyrhodamine), or ROX (β -carboxy-X-rhodamine). Non-fluorescently labeled primers were purchased from Integrated DNA Technologies (Coralville,

IA) and the method of purification was standard desalted (**primer sequences are given in table 1 of appendix**).

D13S317: A new unlabeled forward primer was designed containing the nucleotide present in the sample with the mutation (**Table1**). The original and new primers were combined at equal ratios to act as a degenerate primer that will bind whether a T or a C is present and were tested on a sample for which dropout was previously observed and the 5' label was transferred from the forward to the reverse primer (**Figure 6**).

vWA: A new unlabeled reverse primer (vWA-RLS) was designed to avoid the overlap between the primer and the repeat region by shifting the primer binding site 9 base pairs away from the repeat region (**Figure 7**). Again, the primer was tested on a sample for which dropout was observed (**Table 1**)

D18S51: A new unlabeled reverse primer was designed for this locus because the smallest allele generated by the Oligotrail primer falls on the wrong side the long Bracket of D21S11. The primer was shifted 46 bp away from the repeat region. The primer sequence is given in **Table 1**.

CSF1PO: A new unlabeled reverse primer was designed for this locus because the Oligotrail primers yield an allele size range overlapping the long bracket of D16S539. The primer was shifted 29 bp away from the repeat region. The primer sequence is given in **Table 1**.

Test for Signal Strength in Monoplex PCR reactions

For initial testing purposes all primers were diluted to a final concentration of 5pmol/uL. Each locus was amplified individually under the PCR reaction conditions and Thermal Cycler parameters described below (see section on PCR amplification), to ensure that the correct size product was generated.

Optimization of each single dye multiplex

After all primer pairs were individually tested, all loci labeled with the same dye were combined. At this point the entire multiplex was still divided into four reactions. These were the FAM-labeled triplex of D13S317, TH01, and TPOX; JOE-labeled tetraplex of Amelogenin, D5S818, D7S820 and FGA; TAMRA-labeled triplex of vWA, D21S11 and D18S51, and ROX-labeled tetraplex of D3S1358, D8S1179, D16S539, and CSF1PO.

Peak height was balanced by increasing or decreasing primer concentration for an individual locus depending on whether the peaks were too low or too high (**Primer concentrations are given in Table 2 of appendix**).

Multiplex optimization (all colors)

Once optimization was done by color, the process was repeated with all colors combined. This involved extensive trial and error, because the more primers combined, the less predictable the optimization process becomes as increased competition between the primers produces nonlinear relationships between primer concentration and peak height (**Primer concentrations are given in Table 3 of appendix**).

PCR amplification

For all single locus amplifications each PCR reaction included 3 μ L of 5X LSB buffer (Tris HCl 67Mm, Ph 8.3, KCl 333Mm, MgCl₂ 10Mm, gelatin .007%) and 3 μ L of 5X dNTPs mix (25mM each from Promega Corporation, Madison, WI). The 5U Immolase Taq polymerase from Bioline USA Inc, was diluted to 1U and one microliter was used per reaction. 2 μ L of each 5pmol primer was used together with 3 μ L of water and 1ng DNA template for a total of 15 μ L. For single dye

and full multiplex reactions 1.5 uL of primer mix were used, 1.5ul of 10X dNTPs and 1uL of 5U of Taq polymerase were used instead. All samples were amplified on a Perkin-Elmer Thermal Cycler Model 9700 with the following cycling parameters 95°C for 10 minutes, then 10 cycles of 94°C for 30 seconds, (touchdown)71-60°C for 30 seconds (30%rpm), 72°C for 60 seconds (20%rpm), then 26 cycles of 94°C for 30 seconds, 59°C for 30 seconds (30%rpm), 72°C for 60 seconds (20%rpm), and a final extension of 60 minutes at 70°C.

Capillary Electrophoresis

1uL of amplified PCR product and 0.5uL of ROX-500 [39] size standard were combined with 15uL of HiDi foramide (Applied Biosystems). This mixture was denatured at 95°C for 3 minutes followed by snap cooling on ice for 3 minutes. Samples were then loaded onto the ABI Prism 310 Genetic analyzer (AB) using a 60cm capillary and POP-4 polymer (Applied Biosystems). Injection was done at 15.0 kV for 5sec, with a run temperature of 60°C and a run time of 30 minutes per sample.

Peak Height Ratio (PHR)

Intra-locus peak height ratios (PHR) were calculated for all 13 loci by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the value as percentage. The ratio was calculated using 3 samples, at DNA concentrations of 1, 0.5, 0.1, and 0.01 nanograms [40].

Stutter

Stutter percentages were calculated by dividing the height of the stutter peak by the height of the allele [19]. Stutter was not calculated in some loci as the level of noise in the electropherograms obscured any stutter, and in others stutter was simply not observed.

Results

Allele dropout from previous LSB work

The new primers successfully amplified alleles at locus D13S317 (genotype 9, 11) and vWA (14, 18) that displayed dropout in the earlier multiplex (**Figure 8 & 9 respectively**).

Size overlap

Primers designed to shift the size ranges of D18S51 and CSF1PO away from the LSB of an adjacent locus failed to amplify. Therefore the multiplex was prepared with the original primers for these loci.

Test for Signal Strength in Single Locus PCR reactions

All 13 STR loci individually amplified were detected and separated through the ABIPrism -310 Genetic analyzer, and sized with ROX-500. Every peak was above 1000 RFUs, and differences among heterozygous allele sizes were concordant with identifier profile from the same sample confirming the specificity of the LSB primers.

Multiplex optimization (single dye only)

After a series of PCR reactions where primer titrations were constantly being adjusted a relatively balanced multiplex was produced for each color **Figure 10-13**. In general the increase/decrease of peak height was unpredictable. However, in many cases the balance was improved by simply increasing the primer concentration of the larger fragments while keeping the concentration of smaller fragment the same or by reducing the primer concentrations of the smaller fragments and increasing the primer concentrations of the larger ones.

Multiplex optimization

At this step titration of 29 primers became a lot more complicated as more primers competed for reagents. The titration process was the same as the previous step and after a thorough optimization a relatively balanced multiplex was obtained **Figure14**.

LSB Multiplex PHR

The peak height ratio for the multiplex was calculated based on the data generated by GeneScan software (AB), for all heterozygous loci present in all three samples. At 1ng all 12 heterozygous loci present in all three samples presented peak height ratios above 50% except locus TPOX. With a lower concentration of 0.5ng Locus D5 showed a PHR of 45% in two samples for all injections and TPOX imbalanced increased with a PHR of 17% in one sample and 8% in another. With 0.1ng of DNA template more loci presented a PHR of less than 50% such as amelogenin, D7S820, D8S1179 D3S1358, and D5S818 with ratios between 40 and 50 percent, and D16S539 and D21S11 with ratios between 35 and 40 percent. At this concentration TPOX experienced allele dropout. At 0.001ng, the entire multiplex experienced severe allele imbalanced in all loci with the majority of loci with ratios below 50%. Many loci also experienced allele dropout **Figure 15**. Although the allele imbalance at both 0.5 and 0.1 nanograms of template is less than 50%, all allele heights were above 400 relative fluorescent units (RFU).

Stutter percentage

Stutter peaks were not observed in D13S317, TH01, TPOX, amelogenin, CSF1PO and for D5S818, D8S1179 and D16S539 the high background noised made it impossible to accurate

measure stutter peaks. Stutter percentage was calculated for loci D7S820, FGA, vWA, D21S11, D18S51 and D3S1358. High percent stutter was found for allele 10 in locus D7S820 (22%), allele 17 in locus D3S1358 (32%) and alleles 33.2 and 29 in locus D21S11 (26% %21%) respectively. All other alleles were below the 20% range (**Table 2**).

Table 1. New primers for D13S317, vWA, D18S51 & CSFIPO STR loci. These primers were designed to resolve the allelic dropout, and fragment overlap issues

Locus	Sequence (5' → 3')	Length (bp)
D13S317		
Forward	ACT CTG ACC CAT CTA ATG CCT A	22
vWA-RLS		
Forward	ACAGATGATAAATACATAGGATGGATGG	28
D18S51.R		
Forward	TCG GCG GTT AAT GAA GTA TTG GAT AAG	27
CSFIPO.R		
Forward	TCT TCC TCA TCC CTG CAT CT	

Table 2. Stutter percentage on various STR loci with LSB multiplex

Loci	Allele	Sample	Average	SD
D7S820	10	B483	22%	3.16%
FGA	23	B483	13%	2.43%
FGA	18	B910	11%	0.66%
FGA	21	B910	12%	2.35%
FGA	19	BKA	7%	2.33%
VWA	17	B483	11%	1.17%
VWA	18	B910	12%	0.04%
D21S11	29	B483	21%	11.05%
D21S11	33.2	B910	26%	0.32%
D21S11	30	BKA	14%	2.39%
D18S51	16	B483	16%	1.00%
D18S51	14	B910	19%	5.63%
D3S1358	17	B483	32%	7.67%
D3S1358	15	B910	11%	1.88%

Figure 1: Schematic of Capillary electrophoresis (a) and fluorescence process (b). Samples are loaded into a tray and injected into polymer-filled capillary by applying high voltage. As the fluorescently labeled samples pass through the detection a laser beam hits the dye labeled molecule (a). The laser beam excites the fluorophore from its ground state to an excited transitions state to eventually release a photon at a lower energy level hence (b) [1].

Figure 2: Allelic ladder used in STR typing to convert sizes into an allele call. Just like the alleles of unknown samples, all alleles present in the allelic ladder for each locus are sized by the internal size standard for comparison.) [1].

Figure 3: Internal Size Standard GS500-ROX (a) and its data points used during calibration. The internal size standard contains DNA fragments of various lengths (a) and is used to size both the allelic ladder and unknown samples (b) [1]

Figure 4: Artifacts commonly observed in STR profiles. Their identification is necessary as they can interfere with the analysis, especially mixture [41].

Figure 5: Locus-Specific brackets. A short (S) and a long (L) bracket is used to size and genotype each locus by making the brackets one or more repeats shorter/longer than the smallest/larger common allele for each locus [33].

Figure 6: Illustration of D13S317 primer modifications to solve the issue of allele dropout. Arrows indicate the directions of the primer. Labeled has been switched from the forward primer to the reverse primer.

Figure 7: Illustration of vWA primer modifications to solve the issue of allele dropout. The arrows indicate the position of the primer. The new reverse primer is shown shifted away from the repeat region in order avoid overlap.

Figure 8. Smaller allele at locus D13S317 recovered with the new primer

Figure 9. Smaller allele at locus vWA recovered with new designed primer.

Figure 10. Optimized 5'-FAM labeled multiplex.

Figure 11. Optimized JOE labeled multiplex.

Figure 12. Optimized TAMRA labeled multiplex.

Figure 13. Optimized ROX labeled multiplex.

Figure 14. Optimized LSB multiplex.

Figure 15: Average heterozygote peak height ratio for the LSB multiplex. The X axis indicates the input DNA concentration and the y axis indicates the heterozygote peak height ratio (PHR) at each individual loci (injections were not averaged). The results illustrate 1 amplification of three separate genomic DNA extracts with the LSB multiplex for concentrations ranging from 1-0.01ng.

Part Two: Design and synthesis of Locus Specific Brackets

Introduction

STR allele sizing by Locus Specific Brackets requires pairs of sequence compatible DNA standards that can closely bracket a locus without overlapping with its alleles upon electrophoresis [33]. Because they are nearly identical in sequence and fragment length to alleles of the genetic loci they calibrate, all locus specific brackets can be co-electrophoresed with sample alleles in the same lane allowing for precise calibration standards [35]. Locus-Specific Brackets can be created by using an existing allele as a starting template. With the appropriate primers, the existing allele can be PCR-amplified to be shorter than the smallest common allele, and larger than the largest common allele for a particular locus. The number of repeats present in the template must be the smallest and largest available alleles for each locus. Because in order to create a bracket only a single allele needs to be present, the desired allele must be isolated when present in a heterozygous form. This can be achieved by amplifying the locus through PCR and extracting alleles from non-denaturing polyacrylamide gels (PAGE), DNA cloning. Once isolated, the allele must be amplified by PCR, separated and detected by electrophoresis. Another way of obtaining a bracket without the rigorous lab process is simply by commercially synthesizing the desired allele sequence.

Isolating STR alleles through *Polyacrylamide Gel Electrophoresis* (PAGE)

PAGE is a great electrophoretic technique developed to separate and purify DNA and proteins on the basis of their size, conformation, and net charge. Depending on the composition of the gel the technique can resolve DNA fragments that are short as well as in close proximity [42]. PAGE gels have much smaller size pores ($\sim 100\text{-}200\text{ \AA}$) than agarose gels ($\sim 1500\text{-}2000\text{ \AA}$) allowing for higher resolution [9]. With native polyacrylamide gels, DNA fragments are separated but remain

in their double stranded form while under denaturing conditions DNA fragments remain single stranded [43]. Generally a higher level of resolution is obtained with denaturing gels given that single stranded DNA is more flexible than double stranded and interacts better with the medium, allowing closely spaced DNA fragments to be separated [44]. The disadvantage of using this method is that the process is very time consuming; the materials used for creating the gel are highly neurotoxic, back flow of DNA while loading the gels can easily cause contamination with adjacent wells, and the thin and delicate gel needs to be handled with extreme care to avoid breakage [45]

Isolating STR alleles with TA DNA cloning

DNA cloning is simply a recombinant technique that allows the transfer of a DNA fragment of interest from one organism to a self-replicating genetic element such as a bacterial plasmid [46]. Generally DNA cloning involves four basic steps. First the source and vector DNA are isolated. Second restriction enzymes cut the two DNA strands, creating ends that can connect the DNA template with the vector. The third step involves bonding the DNA template to the vector using a DNA ligase enzyme and finally the DNA is transformed into a host cell [47]. Because STR multiplex PCR products are amplified with Taq DNA polymerase TA cloning is one of the simplest methods that can be used for the purpose of allele isolation. The name TA comes from the ability of the *Taq* polymerase to add a single A (deoxyadenosine) to the 3' end of the PCR products, which in turn is used to directly clone the target to a linearized vector with a 3' T overhang [48]. A topoisomerase enzyme binds to Duplex DNA and cleaves the backbone of the DNA strand forming a covalent bond between the 3' phosphate of the cleaved strand and the topoisomerase (**Figure 16**). The ligation process of the PCR product causes a disruption in the

fusion of the vector's lethal *E-coli* gene *ccdB* and the *lacZα* gene permitting growth of only positive recombinants upon transformation on the competent cells [49]. Once bacterial colonies have grown the plasmid DNA can be isolated and used for sequencing or direct PCR amplification.

Generating STR alleles through artificial gene synthesis

Gene synthesis is rapidly becoming the preferred method for applications requiring the assembly of DNA sequences, both natural and engineered. These genes are normally generated by the synthesis of long oligomers, with overlapping nucleotides (~20bp) that are later ligated and assembled into full length genes. In the same manner short tandem repeats can be synthesized and used as template for a PCR reaction. The advantage of this option is that gene synthesis services are commercially available through any major biotechnology company [50]. However a major disadvantage is that the costs are quite high.

Materials and Methods

DNA Sample preparation

DNA samples were already extracted and quantified during the previous validation of the LSB kit as recommended by SWGDAM revised validation guidelines. Liquid blood samples (100ul) and buccal swabs were collected from 100 anonymous (WVU IRB protocol 16279). DNA extraction from the liquid blood samples was done using Qiagen (Valencia, CA) DNeasy kit according to protocol for whole nucleated blood, with a final elution of 100uL. Buccal swab samples were extracted using the Qiagen DNeasy kit according to manufacturers' directions for DNA extraction from tissues, with a final elution of 100uL. All samples were quantified for nucleic acid content using the nanodrop spectrophotometer and further diluted to a working concentration of 1ng/μl. Samples were chosen depending on the number of repeats needed to make the brackets (**refer to appendix section Table 4**).

LSB New Primers

New reverse primers were designed to amplify the targeted regions except D13S317 for which a new forward primer was designed. Primers for the short brackets were designed by deleting nucleotides of the 5'end of the existing primer for a particular locus or by shifting the entire primer annealing site, and the primers for long brackets were design by adding nucleotides to the 5'end of the existing primer for a particular locus or by shifting the entire primer annealing site. Primers were ordered from Integrated DNA Technologies (Coralville, IA. Because all bracket specific primers would yield PCR products less than 400bp, a second set of primers was designed to increase the overall size of each PCR product to be cloned (**primer sequences are**

given in Table 5 of appendix section). These primers were also designed in a way that if the primer pair failed to work together, a combination of both bracket and cloning specific primers would yield a fragment that is larger than 500bp and increase the success of the cloning reaction (**Figure 17**). The cloning specific primer pairs did not work together in all cases, but one or both primer combinations worked during the PCR reaction and only the pair that produced the larger fragment size was used to clone the brackets.

PCR amplification (starting material)

All samples were amplified with 3 μ L of 5X LSB buffer (Tris HCl 67Mm, Ph 8.3, KCl 333Mm, MgCl₂ 10Mm, gelatin .007%) and 3 μ L of 5X dNTPs mix (25mM each from Promega Corporation, Madison, WI). The 5U Immolase Taq polymerase from Bioline USA Inc, was diluted to 1U and one microliter was used per reaction. 2 μ L of each 5pmol primer was used together with 3 μ L of water and 1 μ L of 1ng DNA template for a total of 15 μ L. All samples were amplified on a Perkin-Elmer Thermal Cycler Model 9700 model with the following cycling parameters 95°C for 10 minutes, then 10 cycles of 94°C for 30 seconds, (touchdown) 71-62 °C for 30 seconds (30% rmp), 72°C for 60 seconds (20% rmp), then 26 cycles of 94°C for 30 seconds, 59°C for 30 seconds (30% rmp), 72°C for 60 seconds (20% rmp), and a final extension of 60 minutes at 70°C.

PCR purification

All PCR products were purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol.

Direct PCR amplification of bacterial colonies

All samples were amplified with 10µl of Promega master mix, 1µl of both M13 forward and reverse primer 7µl of water and 1µl (plasmid) or 3µl (direct colony) template. All samples were amplified on a Perkin-Elmer Thermal Cycler Model 9700 model with the following cycling parameters 94°C for 10 minutes, 94°C for 30 seconds, 47°C for 30 seconds, 72°C for 30 seconds, and a final extension of 10 minutes at 72°C.

Polyacrylamide Gel Electrophoresis

A 12% nondenaturing gel was prepared with 21ml of 40% acrylamide/bis- acrylamide (37:1 ratio), 500µl of 10% ammonium persulfate (APS) and 14 ml of 5X Tris-Buffered Saline (TBS), and water for a total of 70ml. The solution was degassed and then divided in half. For each 35ml half, 12.3µl of tetramethylethylenediamine (TEMED) was added. 15µl of PCR product plus 7µl of PAGE 6X loading dye was loaded into the well. The gel was run for 16 hours, washed and stained with ethidium bromide (EtBr) and then visualized under UV light. A 200 µl tip was used to make, 3 punctures in the center of the desire band and place in 20 µl of water to soak overnight allowing the DNA to slowly diffuse into the water. The water was used the following day for PCR as a template, and amplification was confirmed using a 2% agarose yield gel.

TOPO TA Cloning

All cloning reactions were carried out using the TOPO TA cloning kit for sequencing from Invitrogen.

LB-Agar plates

All plates were prepared by adding 250ml of dH₂O, 20g of LB-Agar powder (VWR), mix thoroughly on a heat plate for 1 min, autoclave the solution for 20 min, let agar cool to ~55°C, add 50 µl of 20mg/ml of ampicillin and pour before solidification begun. Each plate was labeled and sealed appropriately. *X-gal* was dissolved in dimethoxy formamide (40mg/ml) and 40 µl were added to each at the appropriate time.

Analyzing transformants through Direct PCR

Each colony was collected with a toothpick; transferred to a reference plate for regrowth and the remaining cells were placed in 10 µl of water. This was then placed in the thermal cycler for 10 minutes at 95°C, 8 µl were used as template for a PCR reaction (see section above). Each sample was loaded onto a 2% agarose gel for identification of positive colonies.

Plasmid Purification

After having grown the bacterial colonies on LB-Broth overnight, all plasmids were purified according to the instruction from Qiagen Plasmid Purification kit.

Bracket mixture

Once amplified each labeled bracket was quantified and diluted to a 10ng concentration. They were then combined to specific ratios into one mixture **Table 3**.

Capillary electrophoresis

1uL of bracket PCR mixture and 0.5uL of ROX-500 size standard were combined with 15uL of HiDi foramide (Applied Biosystems). This mixture was denatured at 95°C for 3 minutes followed by snap cooling on ice for 3 minutes. Samples were then loaded onto the ABI Prism 310 Genetic analyzer (Applied Biosystems) using a 60cm capillary and POP-4 polymer (Applied Biosystems). Injection was done at 15.0 kV for 5sec, with a run temperature of 60°C and a run time of 30 minutes per sample.

Artificially synthesized Brackets

Brackets to be commercially synthesized were modified (repeat numbers were added/decreased) to meet the expected size of the desired bracket.

Bracket precision study

All JOE/FAM labeled brackets data was exported from GeneScan software into Microsoft Excel and multiple injections were averaged prior to subsequent calculations. The observed sizes were subtracted from the expected and plotted against the size deviation. Both average and standard deviation were calculated for each bracket and the standard deviation was multiplied by 3 for a 99.7% confidence interval.

Results

Allele Isolation with polyacrylamide gel

Bracket isolation from a 12% non-denaturing polyacrylamide gel failed. Samples were contaminated as indicated by the extra peaks observed in the electrophoregram, and therefore were not included in the LSB mixture.

Cloned Brackets

TA-cloning successfully generated all FAM-labeled loci brackets: D13S317, TH01, and TPOX as well as JOE-labeled brackets Amelogenin short, D5S818 short, D7S820 short and long, and FGA long (**Figure 18 & 19**). ROX-500 confirmed the expected sizes for each bracket. Both TAMRA & ROX-labeled brackets were not produced due to the lack of reagents. The artificially synthesized brackets (D5S818 long and FGA short) were successfully amplified and used for genotyping in combination with the TA-cloned brackets.

Brackets Precision

The graphs in **figures 20 & 21** depict the size deviation of the newly designed brackets. All FAM labeled brackets were within the 0.5bp limit as expected; however, when the average and 3SD were calculated TPOX long bracket was over the 0.5bp limit with a 3SD equal to 0.57bp (**Table 4**). Most of the green brackets were within the 0.5bp limit; however with 3SD the values were greater than the 0.5bp for all green brackets except locus D5S818 with 3SD of 0.25bp and 0.23bp (**Table 5**).

Table 3. FAM/JOE-Bracket Mixtures concentrations

Bracket	PCR product (ng)	Volume (µl)
D13S317		
Short	10	1.0
Long	10	1.0
TH01		
Short	10	1.1
Long	10	1.4
TH01		
Short	10	2.0
Long	10	1.6
Water		1.9
Amelogenin		
Short	10	1.0
D5S818		
Short	10	1.0
Long	10	1.0
D7S820		
Short	10	1.0
Long	10	1.0
FGA		
Short	10	1.0
Long	10	1.2
water		2.3

Table 4 FAM-labeled Locus Specific Brackets precision study (no sample present)

Brackets	D13S317-S	D13S317-L	TH01-S	TH01-L	TPOX-S	TPOX-L
Bracket exp. (bp)	99	149	167	207	212	251
Mean	99.08	148.94	167.31	206.71	211.79	250.76
SD	0.08	0.06	0.08	0.06	0.07	0.19
3*SD	0.24	0.18	0.24	0.18	0.21	0.57
n	10	10	10	10	10	10

Table 5 JOE-labeled Locus Specific Brackets precision Study

Brackets	Amelo-S	D5S818-S	D5S818-L	D7S820-S	D7S820-L	FGA-S	FGA-L
Size (bp)	98	138	181	196	235	251	317
Mean	98.30	138.24	180.74	195.66	235.43	251.17	316.74
SD	0.28	0.08	0.08	0.20	0.18	0.19	0.17
3*SD	0.85	0.25	0.23	0.61	0.55	0.58	0.51
n	13	13	13	13	13	13	13

Figure 16. TOPO[®] TA Cloning[®] of *Taq*-amplified DNA (Available at: <http://www.invitrogen.com>)

Figure 17. Illustration of primer design strategy to clone-locus specific brackets

Figure 18 New FAM-labeled locus specific brackets.

Figure 19. New JOE-labeled locus specific brackets

Figure 20. Size deviation for all FAM-labeled brackets

Figure 21. Size deviation for all JOE-labeled brackets

**Part Three: Preliminary evaluation of the Performance of Locus
Specific Brackets as a new Human Identification Testing Kit**

Introduction

In the United States the DNA advisory Board (DAB) and the Technical Working Group on DNA Analysis Methods (TWGDAM) were established to aid the forensic community with the revision of the *Quality Assurance Standard for Forensic Caseworking Laboratories* and the *Quality assurance Standards for DNA Databasing Laboratories* [51]. Under these standards the process of validation is an important part of forensic DNA typing as it establishes the limitations and characteristics of the method and whether or not it fits the purpose for which it was developed [52]. Validation is also necessary because it demonstrates the robustness reliability and reproducibility of the method. A robust method is one in which successful results are obtained a high percentage of the time and few, if any, samples need to be repeated. A reliable method refers to one in which the obtained results are accurate and correctly reflect the sample being tested, while a reproducible method means that the same or very similar results are obtained each time a sample is tested [53]. In order to test the performance of the LSB kit two experiments were chosen to test the sensitivity and reproducibility of the kit.

Reproducibility Study

A high degree of precision is needed between multiple runs in order for the procedure to be considered reliable. Precision for the separation must be $\pm 0.5\text{bp}$ to accurately distinguish between microvariant (partial repeat) alleles and complete repeats that differ by one nucleotide. Imprecision is normally expressed as the standard deviation of observed fragment size. Precision gives reproducible sizing data, precise enough to produce the expected genotype under a given set of experimental conditions [54].

Sensitivity Study

For an STR multiplex sensitivity refers to the lowest sample DNA concentration that can be measured with confidence; also known as Limit of Detection (LOD), and the lowest concentration of DNA that can be determined in a precise and accurate manner given a set of experimental conditions [37]. Sensitivity is expressed as the lower and upper limit of DNA template concentrations that can reproducibly produce a full profile with peak heights above the analysis threshold [55].

Materials and Methods

DNA Sample preparation

DNA samples were already extracted and quantified during the previous validation of the LSB kit as recommended by SWGDAM revised validation guidelines. Liquid blood samples (100ul) and buccal swabs were collected from 100 anonymous (WVU IRB protocol 16279). DNA extraction from the liquid blood samples was done using Qiagen (Valencia, CA) DNeasy kit according to protocol for whole nucleated blood, with a final elution of 100uL. Buccal swab samples were extracted using the Qiagen DNeasy kit according to manufacturers' directions for DNA extraction from tissues, with a final elution of 100uL. All samples were quantified for nucleic acid content using the nanodrop spectrophotometer.

PCR amplification

All samples were amplified with 3μL of 5X LSB buffer (Tris HCl 67Mm, Ph 8.3, KCl 333Mm, MgCl₂ 10Mm, gelatin .007%) and 3μL of 5X dNTPs mix (25mM each from Promega Corporation, Madison, WI). The 5U Immolase Taq polymerase from Bioline USA Inc, was diluted to 1U and one microliter was used per reaction. 2uL of each 5pmol primer was used together with 3uL of water and 1uL of 1ng DNA template for a total of 15uL. All samples were amplified on a Perkin-Elmer Thermal Cycler Model 9700 model with the following cycling parameters 95°C for 10 minutes, then 10 cycles of 94°C for 30 seconds, (touchdown) 71-62 °C for 30 seconds (30% rpm), 72°C for 60 seconds (20% rpm), then 26 cycles of 94°C for 30 seconds, 59°C for 30 seconds (30% rpm), 72°C for 60 seconds (20% rpm), and a final extension of 60 minutes at 70°C.

Capillary Electrophoresis

1 μ L of PCR product, 0.5 μ L of ROX-500, and 1 μ L of JOE/FAM LSB brackets mixture were combined with a 15 μ L of HiDi foramide (Applied Biosystems). This mixture was denatured at 95°C for 3 minutes followed by snap cooling on ice for 3 minutes. Samples were then loaded onto the ABI Prism 310 Genetic analyzer (Applied Biosystems) using a 60cm capillary and POP4 polymer (Applied Biosystems). Injection was done at 15.0 kV for 5sec, with a run temperature of 60°C and a run time of 30 minutes per sample.

Data Analysis

Sensitivity

Three samples were tested; each at DNA template concentrations of 1ng, 0.5ng, 0.1ng, and 0.01ng for a total of 12 samples and all 12 samples were run on the genetic analyzer on three separate days. The data were exported from GeneScan software into Microsoft Excel and peak height values from all 3 injections were averaged prior to subsequent calculations. Intralocus balance or heterozygous peak height ratio was calculated by dividing the height of the smallest allele by the height of the largest allele at each locus and then expressed as a percentage. Intracolor was evaluated by first averaging all heterozygote peak heights and then dividing homozygous peak height in half. Once normalized for diploidy, the lowest allele height was divided by the largest allele height labeled with a given dye and then expressed as a percentage. Interlocus balance was calculated in a similar manner to intralocus, except the comparison was made across all loci regardless of the dye color [56]. The average of all peak heights were also calculated per locus, based on all three samples and three separate injections.

Precision

Data from GeneScan software were imported into Microsoft Excel. The observed allele sizes were subtracted from their expected values. The size deviation was plotted against their expected size. Their mean and standard deviations were calculated. TAMRA & ROX-labeled loci were not analyzed for this study because their brackets are yet to be developed.

Genotype concordance

Nine samples amplified with the FAM labeled multiplex and 3 samples for the JOE labeled multiplex were used for genotyping and their allele calls were compared with the genotypes obtained from the Identifiler kit. Using Microsoft Excel simple genotyping software was written to automatically generate allele calls based on comparison to a ROX internal size standard. The software simply subtracts the conserved region length (primer binding site to the edge of repeat) from the total fragment length, the remainder is divided by 4 and the results are assigned as the allele call. For partial repeats the software was simply modified to convert repeats with decimal point of .25, .5 .75 to .1, .2, and .3 allele calls (e.g. an allele call of 9.75 repeat is converted into 9.3).

Results

LSB Multiplex Sensitivity

For three separate genomic DNA sources with input ranges from 0.01ng – 1ng all but one sample (BKA, 0.5ng) were successfully amplified (**Figure 22**) with the highest analyzed peak at 7945 RFUs for 1ng amplification at locus TH01 and the lowest analyzed peak was observed in locus D8S1179 at 362 RFUs also at 1ng of DNA concentration. Peak heights decreased with decreasing DNA concentration. At 0.5ng of DNA the highest RFU was observed at locus D7S820 with a score of 7098 RFUs and the lowest at locus D16S539 with a score of 258RFUs. At 0.1ng, the highest score was 5417 RFUs at locus TH01 and the lowest was D8S1179 with a score of 380RFUs, while one sample experienced allele dropout in locus TPOX. All alleles average heights were calculated based on 3 separate injections for concentrations of 1ng and 0.1ng and based on two injections for 0.5ng (**Figure23**).

At 1ng the peak height ratio for most alleles were above 60% except 2 outliers, one at 26.1% (TPOX sample B910) and the other with 50.3% (D8S1179 sample BKA). At 0.5ng 4 outliers were observed with high peak imbalance, TPOX sample B910 (13%), D5S818 sample B910 with 44%, D8S1179 for both samples B910 and B483 with 54%. Greater locus imbalance was observed with 0.1ng with one allele dropout at TPOX for sample B910 while most loci experienced allele dropout at 0.01ng (**Figure 24**).

With 1ng of DNA input and 3SD the average heterozygous peak ratio for TH01, D7S820, FGA, vWA, D21S11, D18S51, D3S1358, and D16S539 were observed above 60% as they should be. However; D5S818 and D13S313 were observed slightly higher than 46% , while D8S1179 and

CSF1PO were at 37% and 39% respectively **Table 6**. Due to the high allele imbalance at locus TPOX the average PHR was difficult to accurately be calculated and Intracolor balance (expected at around 50%) was not obtained at the expected level. The closest to this balance was 49% by TAMRA-labeled at 1ng, 46% and 0.5ng. Only a 7% intercolor balance was observed at 1ng, 10% at 0.5ng and was not accurately calculated at 0.1ng due the presence of allele dropout in locus TPOX **Table 7**. The same applies for 0.01ng were most alleles failed to be successfully amplified.

Precision test

Based on nine samples and three injections, a total of 150 measurements were obtained for all FAM-Labeled alleles. At locus D13S317 53/54 alleles were within the 0.5bp limit, as well as all 39/39 for locus TH01 and 35/39 for TPOX. All 78 JOE-labeled allele were also measured. For locus D5S818 23/25 fell within the expected range, 28/29 for locus D7S820 and 22/24 for locus FGA **Figures 25 & 26**. For amelogenin marker 13/16 measured fragments were also within the expected range. Based on 3SDs the majority of FAM-labeled alleles were well within the expected 0.5bp limit except TH01 allele 6, and TPOX allele 11 see **Table 8**. For JOE labeled loci FGA allele 19 was over 0.5bp limit with 3SD of 0.72bp **Table 9**.

Genotype concordance

A total of 228 comparisons were made for loci D13S317, TH01, TPOX, D5S818, D7S820, FGA, and amelogenin between the LSB kit and the commercial kit identifier. Full concordance was observed at locus D13S317 and TH01 were all 58/58 genotype comparisons were correct. Locus TPOX was the only one affected at alleles 8 and 11 were systematically called 8.1 & 11.1

(these sizes were also 1bp higher than expected) for all three injections in 3 samples; however in two occasion two samples were genotyped correctly during one injection but incorrectly in the others for a total of 36/58 correct genotypes (sizes match the incorrect genotype in both samples). For JOE-labeled loci, a total of 77 genotype comparisons were made. 50/72 genotypes were correct while 22 were incorrect. The discrepancies were observed at locus amelogenin where 2 out of the three samples had both X & Y fragments “off ladder” on two out of the three injections. Locus D5S818 had 2 samples were allele 12, was consistently called 12.1 in all three injections. Locus D7S820 alleles 11 and 12 were genotyped as 11.1 & 12.1 in one sample and locus FGA, two samples repeatedly called alleles 18 & 19 as 18.1 and 19.1 respectively; In all of these cases, the fragment sizes were also off by 1bp. See **Table 10 & 11**.

Table 6 Average peak Height Ratio after all three sample injections were averaged

STR Locus	Average PHR	SD	3SD
D13S317	76%	10%	46%
TH01	96%	2%	91%
TPOX	N/A	N/A	N/A
Amel	93%	7%	70%
D5S818	82%	11%	50%
D7S820	96%	3%	87%
FGA	82%	4%	69%
vWA	88%	8%	65%
D21S11	89%	4%	78%
D18S51	80%	2%	75%
D3S1358	93%	6%	75%
D8S1179	58%	7%	37%
D16S539	83%	6%	64%
CSF1PO	77%	13%	39%

Table 7. Intra locus & intracolor balance for all STR loci at different DNA concentrations (all three injections were averaged per sample)

Loci	Template 1ng	Template 0.5ng	Template 0.1ng
D13S317	31%	66%	51%
TH01	94%	77%	55%
TPOX	26%	13%	dropped
Amel	48%	49%	15%
D5S818	42%	82%	30%
D7S820	77%	79%	31%
FGA	77%	87%	47%
vWA	62%	70%	41%
D21S11	58%	68%	23%
D18S51	65%	51%	50%
D3S1358	47%	70%	46%
D8S1179	46%	54%	28%
D16S539	68%	58%	45%
CSF1PO	65%	85%	21%
Intracolor			
FAM	20%	10%	11%
JOE	30%	22%	20%
TAMRA	49%	46%	23%
ROX	7%	12%	10%

Table 8 Average sizes for FAM-labeled loci. All allele sizes are based on ROX-500 size standard and sized by GeneScan Software.

D13S317	8	9	11	12	13
Mean	111.99	116.00	123.95	128.09	132.17
SD	0.08	0.05	0.09	0.08	0.06
3*SD	0.23	0.15	0.26	0.23	0.19
Exp	112	116	124	128	132
n	15	3	20	9	6
TH01	6	7	8	9	9.3
Mean	179.16	183.15	187.03	190.97	193.83
SD	0.30	0.11	0.07	0.06	0.07
3*SD	0.90	0.34	0.20	0.18	0.22
Exp	179	183	187	191	194
n	18	12	9	12	6
TPOX	8	9	10	11	
Mean	226.26	230.60	234.86	239.29	
SD	0.07	0.10	0.11	0.22	
3*SD	0.22	0.30	0.32	0.67	
Exp	226	231	235	239	
n	12	9	3	15	

Table 9. Average sizes for JOE-labeled loci. All allele sizes are based on ROX-500 size standard and sized by GeneScan software.

Amelogenin	X	Y				
Mean	103.12	108.44				
SD	0.58	0.05				
3*SD	1.75	0.16				
exp	103	108				
n	9	6				
D5S818	11	12	13			
Mean	156.13	160.42	164.44			
SD	0.07	0.08	0.04			
3*SD	0.22	0.23	0.13			
Exp	156	160	164			
n	11	9	5			
D7S820	8	10	11			
Mean	208.21	216.92	221.37			
SD	0.06	0.09	0.21			
3*SD	0.18	0.27	0.62			
Exp	208	217	221			
n	7	12	5			
FGA	18	19	21	22	23	24
Mean	261.83	265.79	273.24	277.12	280.86	284.62
SD	0.11	0.24	0.09	0.19	0.12	0.08
3*SD	0.32	0.72	0.27	0.57	0.35	0.24
Exp	262	266	273	277	281	285
n	3	4	4	5	5	3

Table 10. Genotype concordance test between the LSB kit and Indentifiler kit for all FAM-labeled loci.

Sample	D13S317 M1	D13S317 M2	D13S317 M3	Identifiler
B551	11,13	11,13	11,13	11, 13
S972	8,12	8,12	8,12	8,12
B276	11,12	11,12	11,12	11,12
B985	8,11	8,11	8,11	8,11
S999	8,11	8,11	8,11	8,11
B581	8,11	8,11	8,11	8,11
B188	12,12	12,12	12,12	12,12
S800	8,11	8,11	8,11	8,11
B142	9,13	9,13	9,13	9,13
	TH01M1	TH01 M2	TH01 M3	
B551	6,7	6,7	6,7	6,7
S972	9,9	9,9	9,9	9,9
B276	6,8	6,8	6,8	6,8
B985	6,9.3	6,9.3	6,9.3	6,9.3
S999	6,9.3	6,9.3	6,9.3	6,9.3
B581	7,8	7,8	7,8	7,8
B188	6,7	6,7	6,7	6,7
S800	6,9	6,9	6,9	6,9
B142	7,9	7,9	7,9	7,9
	TPOX M1	TPOX M2	TPOX M3	
B551	11,11	11.1,11.1	10.3,10.3	11,11
S972	11,11	11,11	11,11	11,11
B276	7.3,9	7.3,9	7.3,9	8,7
B985	10,11	10,11	10,11	10,11
S999	9,11.1	9,11	9,11	9,11
B581	7.3,7.3	7.3,7.3	7.3,7.3	8,8
B188	9,9	9,9	9,9	9,9
S800	11,11	11,11	11,11	11,11
B142	8,8	7.3,7.3	7.3,7.3	8,8

Table 11. Genotype concordance test between the LSB kit and Identifiler kit for all JOE-labeled loci.

Sample	Amelogenin M1	Amelogenin M2	Amelogenin M3	Identifiler
B483	X,Y	X,Y	X,Y	X,Y
B910	OL,OL	X,X	X,X	X,X
BKA	X,Y	X,Y	X,OL	X,Y
	D5S818	D5S818	D5S818	
B483	11,12	11,12	11,12.1	11,12
B910	11,13	11,13	11,13	11,13
BKA	11,12	11,12	11,12	11,12
	D7S820	D7S820	D7S820	
B483	8,10	8,10	8,10.1	8,10
B910	10.1,11.1	10.1,11.1	10.1,11.1	10,11
BKA	8,10.1	8,10.1	8,10.1	8,10
	FGA	FGA	FGA	
B483	23,24	23,24	23,24	23,24
B910	18.1,21	18.1,21	18.1,21	18,21
BKA	19.1,22	19.1,22	19.1,22	19,22

Figure 22. LSBs allele average peak heights. Based on 3 separate injections from three distinct samples sources at DNA concentrations of 1, 0.5, and 0.1(0.01ng not included due to failure to obtain full profiles).

Figure 173. Heterozygous peak height ratios with various inputs of DNA concentrations. The x axis indicates the input DNA concentration and the y axis indicates the heterozygote peak height ratio (PHR) at each individual loci (multiple injections were averaged). The results illustrate 1 amplification of three separate genomic DNA with the LSB multiplex for concentrations of 1, 0.5, and 0.1, and 0.01ng.

Figure 24. Size deviation for all FAM-labeled alleles based on sizes generated by ROX internal size standard. All data points are based on 9 samples with three separate injections.

Figure 25. Size deviation for all JOE-labeled alleles based on sizes generated by ROX internal size standard. All data points are based on 3 samples with three separate injections and three samples that were only injected once.

Discussion & Future Research

The LSB STR multiplex kit correctly typed 81% of all alleles using 1ng of DNA concentration from a single source, and in all three samples tested, full profiles were recovered, with allele heights well above the analysis threshold. Although full profiles were obtained, the low heterozygous PHR observed together with the high level of stutter may cause problems during future mixture studies. The LSB kit also showed that 87% of all sample alleles were accurately sized; by the ABI Prism 310. Although 13% represents a very high level of variation for the kit, a larger sample size and number of measurements should be increased to fully test the reliability of the system. A larger sample size will also reveal with more certainty the level of allele dropout that can be encountered with the new LSB multiplex. Currently there is only one locus that seems to have problems with primer binding site mutations, but given that the majority of the primers are different from the original multiplex, it is possible that with a larger sample size dropout may increase.

Sensitivity of the LSB multiplex system

Of particular significance to the development of the multiplex is its performance evaluation. Performance criteria included peak heights, intercolor, intralocus, and intracolor balance. With 1ng of DNA template peak heights were evaluated to verify that homozygous peaks were not off scale and the overall peak amplitude was sufficient to allow for detection of 1ng of template or less. Intercolor balance was also evaluated in relation to the ability to produce full profiles within the recommended range. Although intralocus balance greater than 70% was not achieved with the tested samples, the multiplex can produce full profiles under the recommended concentration range of 0.1 to 1 nanograms as the overall peak height ratio was greater than 50% for all loci

except TPOX and their peak height were above 200RFUs. It is known that the heights of the peaks will vary from sample-to-sample, even for the same DNA sample amplified in parallel. Because the three single source samples were not amplified at least in duplicates for any given concentration it becomes more difficult to explain if the low intralocus balance was influenced perhaps by inappropriate dilution, inhibition or pipetting error.

The range of sensitivity of this assay indicates that the LSB can produce a profile under a wide range of DNA template concentration, however is not suitable for low copy number. The current average peak heights showed no indication of saturation as the peaks were within the detection range for the ABI Prism 310, however using more than 1ng with the current multiplex will cause higher allele imbalance, incorrect allele sizing and incomplete adenylation.

Sizing Precision

During this study both brackets and alleles were co-electrophoresed to test their migration consistency and reproducibility on the ABI Prism 310 genetic analyzer system. The principle is to expose both known brackets and questioned alleles to the same electrophoretic conditions, assuming that each LSB bracket pair will size its respective alleles each time a sample was tested. The sample reproducibility (based on sizes generated by ROX-500) was assessed through the calculation of SDs around mean values for each bracket and its discrete alleles. The study demonstrated that on average most brackets and alleles would fall within the 0.5bp limit; however, for a confidence interval of 99.7%, this might not be the case. JOE-labeled brackets and alleles showed more variation than the FAM-labeled ones but it is worth noticing that these samples were only run on three separate occasions, and that perhaps the number of replications would have to be larger in order to get a more accurate sizing reproducibility.

Genotype consistencies

One key aspect of any genotyping system is the ability to provide reproducible results. The LSB multiplex was able to demonstrate in all three injections that the sample sizes and genotypes were reproducible, although in various loci the results were not accurate. The genotype inconsistencies however can be justified as the sizes for these alleles were also inaccurately sized by R0X-500.

LSB kit limitations

Although the LSB multiplex can be used to amplify non-degraded samples and produce full genetic profiles the kit still remains with limitations that can potentially challenge the reliability of the system. These limitations are described below:

Allele dropout

The issue of allele dropout in loci vWA and D13S317 that contributed to the failure of the previous validation study, was successfully overcome in the new LSB multiplex by selecting primers free of point mutations as well as primers with reduced potential competition for building blocks, and undesired complementarity both between primers (primer dimer) and primers and genomic DNA template (mispriming). However, the newly discovered point mutation at locus TPOX during rigorous preliminary research creates future problems of heterozygous allele imbalance as observed during the peak height ratio and sensitivity study, as well as unreliable results given that heterozygous alleles may appear as homozygous specially in templates with low DNA concentrations.

Size overlap

A multiplex with same-dye labeled alleles with potential for overlap with neighboring loci and in this case overlap with a locus specific bracket, creates problems for data analysis and interpretation increasing the potential for improper genotyping of a sample alleles. Although the overlap at loci CSF1PO & D18S1358 was not observed in the sample included in this project, it is expected to happen with samples that carry the smallest alleles for both of these loci.

Need for new genotyping software

The Oligocode genotyping software is absolutely necessary to genotype large data sets with the LSB method. The software has been specifically developed to analyze data that does not contain the internal size standard or an allelic ladder, but simply the sample alleles and the brackets.

Although the Oligocode manual claims to use the local southern method for genotyping unknown alleles, it is unclear how the software manages to use the local southern method with only two data points (both short and long brackets) present in the data set when local southern requires at least 4 data points to create a standard curve. The original LSB work [36] does mention that analysis can be done with only the LSBs as internal lane standards or the LSBs plus two known alleles for each locus as the external lane standard; however, it does not explain how this apply to every new data set.

Currently the software only works with brackets that are equivalent in size to a full repeat but not a partial one. This makes the new LSB impossible to genotype with the software as few of the brackets synthesized to genotype the current LSB multiplex, contain partial repeats.

Incomplete bracket set.

In order to genotype all 13 loci using the LSB method, all brackets need to be present. At this point there are only 14 brackets that can calibrate 7 loci.

Future research

The new human LSB multiplex remains with issues that make it unsuitable for forensic DNA analysis. In order to overcome this problem a new primer must be designed to solve for TPOX allele dropout. New primers must also be designed for loci D18S51 and CSF1PO to overcome the size overlap problems. If good primer candidates cannot be found for these loci, mobility modifiers or spacers can be implemented in order to keep the current primers. New TAMRA & ROX-labeled brackets must be designed to complete the set needed to calibrate all 13 loci. Oligocode software must be improved so that it can accommodate these new allele and LSB sizes. Once these issues have been solved, a new validation study must be done with a larger samples size.

Currently new STR technology and instruments are constantly being developed. Part of the technology includes kits with additional markers. Given that the current kit only amplifies all 13 loci, perhaps new markers can be included within the future-improved version of the LSB multiplex, making the kit more marketable. A new improved version of the kit may also encourage the development of commercial software for genotyping STR data without the use of an internal size standard or an allelic ladder and instead recognizes the presence of brackets. Given the current standards and regulations for validating a kit to be use in casework samples, the LSB kit might not be the system of choice; however, everyday many non-criminal paternity cases are being processed in different labs and a kit like the LSBs may become useful as the kit

in itself might be cheaper to process, reagents costs may decrease, and sample throughput may increase.

Having a kit like locus-specific bracket may benefit the forensic community as kits available from Applied Biosystems, or Promega are highly expensive and in many ways limited to their respective software and genetic analyzers. If an improved version of the LSB kit can be in the future created, one free of allelic dropout and duly validated, this kit may become valuable to many labs searching for cheaper and better kits.

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Appendix

Table 3. Original LSB primer sequences used to amplify the multiplex used during the validation study and current primer sequences according to the LSB manual.

Name	Current primer sequences 5'- 3'	Original primer sequences 5'- 3'
D13S317.F	ACT CTGACCCATCTA ACGCCT A	TCTGACCCATCTAACGCCTAT
D13S317R	CAG CCC AAA AAG ACA GAC AGA AAG	GCCCCAAAAGACAGACAGAAAGA
TH01.F	CAA AGG GTA TCT GGG CTC T	GGCAAATAGGGGGCAAATTCAAAG
TH01.R	TGG GCT GAA AAG CTC CCG ATT A	GAAAAGCTCCCGATTATCCAG
TPOX.F	TGG CAC AGA ACA GGC ACT TAG	CTTCCTCTGCTTCACTTTTCACC
TPOX.R	GAA CTG GGA ACC ACA CAG G	CCTTCTGTCCTTGTCAGCGTTTA
Amel.F	CCT GGG CTC TGT AAA GAA TAG TG	CCTGGGCTCTGTAAAGAATAGTG
Amel.R	ATC AGA GCT TAA ACT GGG AAG CTG	ATCAGAGCTTAAACTGGGAAGCTG
D5S818.F	GAC AAG GGT GAT TTT CCT CTT TGG T	GACAAGGGTGATTTTCCTCTTTGGT
D5S818.R	CAA GTG ATT CCA ATC ATA GCC ACA G	GTGATTCCAATCATAGCCACAG
D7S820.F	TCA GGC TGA CTA TGG AGT TAT TTT AAG G	AGGCTGACTATGGAGTTATTTTAAGG
D7S820.R	CAT TTA TCC TCA TTG ACA GAA TTG CAC C	TTATCCTCATTGACAGAATTGCAC
FGA.F	ATG CCC CAT AGG TTT TGA ACT CAC	GCAGGGCATAACATTATCCAAAAG
FGA.R	TCT CAG ATC CTC TGA CAC TCG	GATCCTCTGACACTCGGTTGTA
vWA.F	GAG ATG TGA AAG CCC TAG TGG A	GATGTGAAAGCCCTAGTGGA
vWA.R	AAA TAC ATA GGA TGG ATG GAT AGA TGG A	CATAGGATGGATGGATAGATGGA
D21S11.F	AAT TCC CCA AGT GAA TTG CCT TCT A	AATTCCCCAAGTGAATTGCCTTCTA
D21S11.R	TAT TAG TCA ATG TTC TCC AGA GAC AGA C	TCAATGTTCTCCAGAGACAGAC
D18S51.F	TTC ATG CCA CTG CAC TTC ACT CT	TTCATGCCACTGCACTTCACTCT
D18S51.R	CCG ACT ACC AGC AAC AAC AC	CCGACTACCAGCAACAACAC

D3S1358.F	TGC AGT CCA ATC TGG GTG ACA GA	ACTGCAGTCCAATCTGGGT
D3S1358.R	TGA AAT CAA CAG AGG CTT GCA TGT	ATGAAATCAACAGAGGCTTG
D8S1179.F	ATG TAT TTT TGT ATT TCA TGT GTA CAT TCG	ATGTATTTTTGTATTTTCATGTGTACATTCTG
D8S1179.R	CAC GTA GCT ATA ATT AGT TCA TTT TCA TCA	CACGTAGCTATAATTAGTTCATTTTCATCA
D16S539.F	TGT ACA AGT GCC AGA TGC TCG TT	TGTACAAGTGCCAGATGCTCGTT
D16S539.R	CCA TTT ACG TTT GTG TGT GCA TCT GTA A	CCATTTAGCGTTTGTGTGTGCATCTGT
CSF1PO.F	TGA GTC TGC CAA GGA CTA GC	CTGTGTCTCAGTTTTCTACCTGT
CSF1PO.R	CAC ACA CCA CTG GCC ATC TTC	TGGAGGTCATCCTTATCTCCTTTC

Table 4 Primer concentrations for multiplex optimization (same dye only). Initial concentration were provided by previous researcher and the optimize concentrations were needed to obtained an improved version.

Locus	Initial concentration	Optimized Concentration
D13S317		
Forward	3.8 pmol/μl	3.9 pmol/μl
Reverse	3.8 pmol/μl	3.9 pmol/μl
TH01		
Forward	6.8 pmol/μl	6.0 pmol/μl
Reverse	6.8 pmol/μl	6.0 pmol/μl
TPOX		
Forward	6.7 pmol/μl	6.7 pmol/μl
Reverse	6.7 pmol/μl	6.7 pmol/μl
Amelogenin		
Forward	1.6 pmol/μl	1.0 pmol/μl
Reverse	1.6 pmol/μl	1.0 pmol/μl
D5S818		
Forward	1.2 pmol	1.0 pmol/μl
Reverse	1.2 pmol	1.0 pmol/μl
D7S820		
Forward	12.9 pmol/μl	11.5 pmol/μl
Reverse	12.9 pmol/μl	11.5 pmol/μl
FGA		
Forward	6.7 pmol/μl	6.3pmol/μl
Reverse	6.7 pmol/μl	6.3 pmol/μl
vWA		
Forward	9.2 pmol/μl	8.5 pmol/μl
Reverse	9.2 pmol/μl	8.5 pmol/μl
D21S11		
Forward	14.2 pmol/μl	15.3 pmol/μl
Reverse	14.2 pmol/μl	15.3 pmol/μl
D18S51		
Forward	15.7 pmol/μl	13.0 pmol/μl
Reverse	15.7 pmol/μl	13.0 pmol/μl
D3S1358		
Forward	5.8 pmol/μl	5.5 pmol/μl
Reverse	5.8 pmol/μl	5.5 pmol/μl
D8S1179		
Forward	14.5 pmol/μl	15.1 pmol/μl
Reverse	14.5 pmol/μl	15.1 pmol/μl
D16S539		
Forward	2.6 pmol/μl	2.9 pmol/μl
Reverse	2.6 pmol/μl	2.9 pmol/μl
CSF1PO		
Forward	10.3 pmol/μl	9.5 pmol/μl
Reverse	10.3 pmol/μl	9.5 pmol/μl

Table 5. Optimized primer concentration for LSB multiplex (all colors)

Name	Optimized Concentration
D13S317	
Forward	1.0 pmol/μl
Forward. FDT	1.0 pmol/μl
Reverse	2.0 pmol/μl
TH01	
Forward	26.0 pmol/μl
Reverse	26.0 pmol/μl
TPOX	
Forward	27.6 pmol/μl
Reverse	26.6 pmol/μl
Amelogenin	
Forward	1.4 pmol/μl
Reverse	1.4 pmol/μl
D5S818	
Forward	5.0 pmol/μl
Reverse	5.6 pmol/μl
D7S820	
Forward	22.7 pmol/μl
Reverse	23.8 pmol/μl
FGA	
Forward	25.0 pmol/μl
Reverse	25.0 pmol/μl
vWA	
Forward	5.0 pmol/μl
Reverse	5.0 pmol/μl
D21S11	
Forward	6.0 pmol/μl
Reverse	6.0 pmol/μl
D18S51	
Forward	25.0 pmol/μl
Reverse	25.0 pmol/μl
D3S1358	
Forward	8.6 pmol/μl
Reverse	7.7 pmol/μl
D8S1179	
Forward	50.0 pmol/μl
Reverse	50.0 pmol/μl
D16S539	
Forward	8.5 pmol/μl
Reverse	8.5 pmol/μl
CSF1PO	
Forward	24.7 pmol/μl
Reverse	24.1 pmol/μl

Table 6 Template source used for cloning locus-specific brackets

Locus	Allele Range	Sample	Bracket	Locus Type and Number of Repeats
D13S317	6-13	BANS	Short	Homozygous locus with 8R
		B511 & S511	Long	Heterozygous locus with 10R & 14R
TH01	4-11	BANS	Short	Homozygous locus with 6R
		B378 & S378	Long	Homozygous locus with 9R
TPOX	6-13	LMSF	Short & Long	Heterozygous locus with 6R & 12R
D5S818	7-16	B999 & S 999	Short	Homozygous locus with 9R
		B188 & S 188	Long	Heterozygous locus with 9R & 14R
D7S820	6-15	B 581 & S 581	Short	Heterozygous locus with 7R & 12R
		B551 & S 551	Long	Heterozygous locus with 10R & 14R
FGA	16-52	S 311	Short	Heterozygous locus with 16R & 19R
		LMSF	Long	Heterozygous locus with 27R & 30R
vWA	11-20	B114 & S 114	Long	Heterozygous locus with 17R & 20R
		B540 & S 540	Short	Homozygous locus with 14R
D21S11	24-38	B401 & S 401	Short	Heterozygous locus with 25R & 29R
		B909 & S 909	Long	Heterozygous locus with 30R & 34.2R
D18S51	9-27	B669 & S 669	Short	Heterozygous locus with 10R & 15R
		LMSF	Long	Heterozygous locus with 15R & 21R
D3S1358	12-19	B007 & S007	Long	Heterozygous locus with 17R & 18R
		DNA Technologies	Short	Homozygous locus with 10R
		B126 & S 126	Long	Heterozygous locus with 14R & 18R
D8S1179	7-19	BANS	Long	Heterozygous locus with 18R & 19R
		B999 & S999	Short	Heterozygous locus with 8R & 15R
		B276 & S 276	Short	Heterozygous locus with 8R & 14R
D16S539	5-15	B142 & S 142	Short	Heterozygous locus with 8R & 11R
		S483-C	Long	Heterozygous locus with 12R & 14R
CSF1PO	6-15	B311 & S 311	Short	Heterozygous locus with 7R & 11R
		B972 & S 972	Long	Heterozygous locus with 11R & 14R

Table 7. Primers designed for cloning locus-specific brackets. SB stands for short bracket, LB for long bracket and CSP stands for cloning specific primers.

Locus	Primer sequence 5' - 3'	Length (nt)	Tm (°C)
D13S317			
Forward-SB	TCG CCG CCT ATC TGT ATT TAC AAA T	25	56.4
Forward-LB	GTG ACT CTC TGG ACT CTG ACC C	22	58.5
Forward-CSP	TCC AAG CTC ACA GTG CCT AA	20	59.6
Reverse-CSP	TCC GAG GTT CTC TTC CTG TG	20	60.4
TH01			
Reverse-SB	CTC CCG ATT ATC CAG CCT GG	20	57.3
Reverse-LB	CTC CCG ATT ATC CAG CCT GG	22	59.4
Forward-CSP	CCT GGT GTT TGA GTC CCT GT	20	60.0
Reverse-CSP	CAG CTT CAT CCT GAG CTT CC	20	61.1
TPOX			
Reverse-SB	TGG GAA CCC CAC AGG TTA A	19	56.1
Reverse-LB	CAG GGG AGG AAC TGG GAA C	19	57.6
Forward-CSP	ATG CCC AGC ACA TGC CTA	18	61.2
Reverse-CSP	GTG TGC TCA CAC CAA GCA CT	20	59.9
¹Amelogenin			
Reverse-SB	CCT GGG CTC TGT AAA GAA TAG TG	23	55.0
Forward-CSP	TGT GTG CTG GTT TCT GCT TC	20	55.9
Reverse-CSP	CGG CCA TAT TTA GGA GGA CA	20	54.1
D5S818			
Reverse-SB	TAG CCA CAG TTT ACA ACA TTT GTA TC	26	53.7
Reverse-LB	GTG CTT TTT AGC CAA GTG ATT CCA	24	56.1
Forward-CSP	ACG CCT TTC CTC TGA AGT GA	20	59.9
Reverse-CSP	ACC TGA AAG GGA CAG GGT TT	20	59.8
D7S820			
Reverse-SB	G CCA TTG ACA GAA TTG CAC CAA ATA T	26	56.6
Reverse-LB	CGA TTC CAC ATT TAT CCT CAT TGA CAG A	28	56.8
Forward-CSP	CCT GAC CCC CTA TGG AAT TT	20	60.1
Reverse-CSP	TTA CAC GAT GGA AGG CAT CA	20	60.7
FGA			
Reverse-SB	AGA TCC TCT GAC ACT CGG TTG	21	56.2
Reverse-LB	TTC AGG ACT TCA ATT CTG CT	20	51.6
Forward-CSP	AGC TGG CAA GTG AGT GAT CC	20	60.4
Reverse-CSP	GGG ACC ACA GCC ACA TAC TT	20	59.9

vWA			
Reverse-SB	TAG GAT GGA TAG ATG GAT AGA TAG ATA G	28	52.3
Reverse-LB	TAG GAC AGA TGA TAA ATA CAT AGG ATG G	28	53.8
Forward-CSP	TCC CAC CTT CCA GAA GAA GA	20	55.2
Reverse-CSP	GGT GAA GTT GGG TCT GCA CT	20	57.6
D21S11			
Reverse-SB	AAT GTT CTC CAG AGA CAG ACT	21	53.0
Reverse-LB	CGG GGC GTG ATA TAT TAA AGA TGT TG	26	55.9
Forward-CSP	GGT GGG CTG AGT GGA GAA TA	20	59.8
Reverse-CSP	AGA GGC CTC ACA GGT TTT CA	20	59.6
D18S51			
Reverse-SB	AAT GTT CTC CAG AGA CAG ACT	21	53.0
Reverse-LB	CGG GGC GTG ATA TAT TAA AGA TGT TG	26	55.9
Forward-CSP	AAC AAT AGG CCA AGC GTG AT	20	59.60
Reverse-CSP	TAG CCG ACA AAA GGC TAT GG	20	60.2
²D3S1358			
Reverse-LB	GGC TTA TAC TCA TGA AAT CAA CAG AGG C	28	57.5
Forward-CSP	AGC ACT TTG GAA GGC TGA GA	20	60.1
Reverse-CSP	CCC ATA CCC TGA GCC ATA GA	20	59.9
D8S1179			
Reverse-SB	GTA TTA GTT CAT TTT CAT CAC TGT ATC GTA	30	53.5
Reverse-LB	AGT ATA GTT TCA CGT AGC TAT AAT TAG TTC	30	52.8
Forward-CSP	CCT TTG CCT GAG TTT TGC TC	20	59.9
Reverse-CSP	TGA GAC CGT GTC TTG CTC TG	20	60.2
D16S539			
Reverse-SB	CAT CTG TAA GCA TGT ATC TAT CAT CCA TCT	30	56.0
Reverse-LB	GGC GAG AGT GAT TCC ATT TTT ATA CCA TTT A	31	57.2
Forward-CSP	ATC TGC CCT CAT CAG TGG AC	20	60.1
Reverse-CSP	CAG CTA TTT GGG AGG CTG AG	20	59.9
CSF1PO			
Reverse-SB	ATC CCT GCA TCT CAG ACT CT	20	55.1
Reverse-LB	GGT CAT CCT TAT CTC CTT TCT C	22	52.2
Forward-CSP	TTC GAG AAG GTG GCC TCT AA	20	55.4
Reverse-CSP	TCA CAA TCT GCT GCA AGT CC	20	55.5

¹There is no reverse primer for the long bracket of amelogenin as the short bracket of locus D5S818 is used to size the amelogenin DNA fragments

²Due to space limitation, a reverse primer for the short bracket of D3S1358 was not designed.