Constructing STR Multiplex Assays

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Abstract

Multiplex PCR refers to the simultaneous amplification of multiple regions of DNA using the polymerase chain reaction (PCR). Commercial short tandem repeat (STR) assays that can co-amplify as many as 16 different loci have become widely used in forensic DNA typing. This chapter will focus on some of the aspects of constructing robust STR multiplex assays including careful design and quality control of PCR primers. Examples from the development of a cat STR 12plex and a human Y chromosome STR 20plex are used to illustrate the importance of various parts of the protocol. Primer design parameters and internet-accessible resources are discussed, as are solutions to problems with residual dye artifacts that result from impure primers.

Key Words: Multiplex PCR, short tandem repeat, STR, forensic DNA, quality control, PCR primer design, primer compatibility

1. Introduction

Short tandem repeat (STR) markers are abundant throughout most genomes and sufficiently polymorphic to serve as effective genetic markers (1). A number of fields utilize STRs including gene mapping, disease diagnostics, evolutionary biology, and human identification. The ability to study multiple STR markers in parallel with multi-color fluorescence detection technologies has revolutionized the amount of information that can be collected in a timely-fashion. The relatively small sizes for the tandem 2-6 bp repeat regions make them accessible to amplification using the polymerase chain reaction (PCR). Multiplex PCR, where multiple regions are simultaneously amplified in a single reaction, has greatly benefited forensic DNA typing because less DNA material is required to obtain results from multiple loci. In addition, the amount of labor required to obtain results at all of the markers is reduced since loci are being typed in parallel rather than sequentially.

Commercial STR assays that can co-amplify as many as 16 different loci (2-3) have become widely used in forensic DNA typing (**Fig. 1**). These kits have been embraced largely because they simplify sample processing, they promote uniformity across the community and enable database compatibility, and they remove the burden of reagent quality control from the individual laboratories. While almost all forensic DNA laboratories utilize commercial kits, it is beneficial to understand the challenges with multiplex PCR assay development. In addition, there may be some situations where it would be helpful to have in-house assays to assess the usefulness of various markers prior to finalizing a set for database or casework purposes (e.g., Y-chromosome STR loci). A

laboratory may have a set of markers that they are interested in examining that are of no interest or perceived commercial value to a company and thus would have little hope of being included in a commercial assay.

Several different strategies have been taken in the literature for PCR multiplex development (4,5). In many cases, extensive PCR optimization experiments are conducted with multiplex development that may seem daunting to some laboratories (5). Since compatible primers are the key to successful multiplex PCR, careful primer design and appropriate quality control measurements are essential to insure that the PCR primers will work under uniform PCR conditions and will not adversely interact with one another (6-8). Upfront informatics plays an important role, as does empirical experimentation.

An overview of the steps used in a careful primer design and testing approach is illustrated in **Fig. 2**. Primer design can be performed with a variety of computer programs that will be described in the materials and methods sections. Creation of a two dimensional plot that illustrates spatial and spectral aspects of STR allele ranges (**Fig. 3**) makes it easier to conceptualize desired PCR product sizes (*see* **Note 1**). Following the purchase of dye-labeled and unlabeled PCR primers, quality control of each individual primer is done prior to combining them for multiplex assay testing (*see* **Note 2**). Selection of fluorescent dye combinations can be important to ensure compatibility with detection instrumentation and to provide the smallest amount of spectral overlap and potential bleed through between dye colors (*see* **Note 3**).

The approach described here has been used to construct a Y-STR 10plex (8) and a cat STR 12plex (9) with 3 different dye labels, and a Y-chromosome STR 20plex using 4 dye labels (10,11). In addition, a number of multiplex single nucleotide polymorphism (SNP) detection assays have been performed with this approach (12). Examples from the cat STR 12plex and Y-STR 20plex will be illustrated.

2. Materials

1. A number of internet-accessible computer programs and databases can be used for PCR primer design including Primer3 and GenBank. The programs utilized in the approach described here are listed in **Box 1**.
2. An in-house program written in Visual Basic 6.0 has been developed that can check potential primer-primer interactions in a pairwise and batch mode fashion (6; Vallone, in preparation).
3. Numerous commercial sources exist for oligonucleotide synthesis. For these studies, unlabeled oligonucleotides were purchased from Qiagen Operon (Alameda, CA). Fluorescently labeled PCR primers were obtained from Applied Biosystems (Foster City, CA) with 6FAM (blue), VIC (green), NED (yellow), or PET (red) dyes attached.
4. Primers are received lyophilized. The unlabeled primers are brought to 100 µmol/L or 200 µmol/L stocks with appropriate volumes of deionized water. The dye labeled primers are brought to 100 or 200 µmol/L concentrations with TE buffer (10 mmol/L Tris, 1 mmol/L EDTA; **Note 4**). The primer stocks are stored

in the dark at 4 ^{o}C. Frequent freeze-thaw cycles can accelerate break down of the dye attachment to the oligonucleotide.

5. The first quality control test performed is usually mass spectrometry to ensure that the primer was properly synthesized. Poor quality primers that contain numerous synthesis by-products (e.g., **Fig. 4**) are returned to the manufacturer for resynthesis prior to proceeding with primer testing.
6. Additional quality control tests that may be performed include UV spectrophometry at 260 nm to evaluate the quantity of the received oligonucleotide (*see* **Note 5**) and HPLC to assess purity of labeled primers (and remove impurities from incomplete dye attachment).
7. For initial testing purposes, the forward and reverse primers for a locus are mixed at a concentration of 1 µmol/L to create singleplex and multiplex working primer solutions. Empirical adjustment of primer concentrations is later performed in an effort to balance PCR product yields.
8. Other reagents typically included in PCR reactions (*see* **Note 6**) include a DNA polymerase (*see* **Note 7**), 250-300 µmol/L dNTPs, 1.5-5 mmol/L $MgCl_2$, 1X DNA polymerase buffer, and 1.6 mg/mL bovine serum albumin (BSA; Sigma).
9. Equipment required includes:
    a. Pipette with tips capable of accurately dispensing volumes as low as 1 µL
    b. Thermal cycler, such as GeneAmp 9700 (Applied Biosystems)
    c. Capillary electrophoresis instrument with multi-color detection capabilities, such as single capillary ABI 310 Genetic Analyzer or 16-capillary ABI 3100 Genetic Analyzer (Applied Biosystems)

10. Optional equipment for quality control of primers includes (*see* **Note 1**):
    a. MALDI-TOF instrument, such as BIFLEX III (Bruker Daltonics)
    b. UV spectrophotometer, such as Cary 100 (Varian Instruments)
    c. HPLC, such as Varian Helix (Varian Instruments)
11. Software for STR data analysis: GeneScan/Genotyper (Applied Biosystems)

3. Methods

This section will go through the steps in multiplex PCR assay development that are illustrated in Fig. 2.

A. Primer Design

1. Select loci to include in the multiplex. It is best to select all loci up front as the assay development is being initiated. While it is possible to add loci after earlier loci are put together, primer design options become less flexible as space on the 2-D assay design layout fills up.
2. Compile reference sequences and determine reference allele size (repeat number). For example, GenBank accession AC022486, which serves as the reference sequence for the DYS385 locus, contains 11 GAAA repeats (*see* **Note 8**). Doing an initial BLAST or BLAT search with a particular locus may uncover sequences entries from multiple clones in GenBank or in the human genome itself. If multiple entries are observed, sequence alignments can be helpful to create a consensus reference sequence (*see* **Note 9**).

3. Determine allele ranges for each STR locus. If extensive population studies have been performed for a particular STR locus, then full allele ranges are probably reasonably well known and PCR products from multiple loci can be placed closer together in the same dye color without fear of overlapping sizes. Typically it is wise to leave room for one or two possible undiscovered alleles on each end of the STR locus range. This strategy would mean that the smallest allele for one tetranucleotide locus could be 10-18 bp larger than the largest allele of the neighboring locus in the same dye color.
4. Layout multiplex schematic with candidate positions for each locus (see Fig. 3). Estimate spacing between loci and calculate required size for each PCR product from its reference sequence.
5. Design primers using Primer3 with fixed PCR product sizes and narrow annealing temperature range
    a. Select desired PCR annealing temp and design primers to be approximately 2-5 ^{o}C above PCR annealing temperature where possible (*see* **Note 10**). Try to keep the calculated primer annealing temperatures within ±5 ^{o}C. At this stage of the primer design process, do not worry about potential cross-reactions from primers used to amplify other loci.
    b. Some flanking regions are less desirable than others for primer design due to palindromic sequences or long polynucleotide stretches. Those loci with unstable flanking regions usually will become the larger loci in an assay, as more flexibility is required in terms of primer placement. Likewise,

STR loci with long repeat stretches will by necessity have larger PCR product sizes than loci with a smaller number of repeats.

6. Check for potential primer interactions across the various STR loci that will be included in the multiplex assay (*see* **Note 11**).
7. BLAST all newly designed primers to search for potential mispriming sites that may occur in other parts of the human genome beyond the intended target region.
8. Select a primer in each pair to be fluorescently labeled. It is often convenient to select the forward primer for each locus to maintain consistency when purchasing the primers.
9. Add a 5'tail to unlabeled primers to promote full adenylation (i.e., non-template addition) during PCR (*see* **Note 12**).
10. Place order for PCR primers. Until the primer combinations have been demonstrated to work well in a multiplex format, it is advisable to purchase the smallest possible quantity of each primer in order to decrease development costs.

B. Primer Quality Control and Testing

1. Check primer quality with MALDI-TOF mass spectrometry.
2. Check primer quantity with UV spec (*see* **Note 5**).
3. Mix primers in locus-specific pairs at 1 µmol/L concentration.
4. Test primer pairs in singleplex reactions with standard PCR conditions to ensure that the correct size product(s) is generated.
5. Combine equimolar amounts (e.g., 1 µmol/L) of primers in multiplex set.
6. Test initial equimolar primer mix with standard PCR conditions (8,10).

7. Balance primer mix empirically based on PCR product yields.
8. Test adjusted primer mix on the same quantity of multiple DNA templates to ensure consistency across samples (*see* **Notes 13** and **14**).
9. Perform sensitivity studies with serial dilutions of DNA templates.
10. Create allelic ladders with common alleles. Alternatively typing may be performed without allelic ladders using precise sizing and sequence information from a single sample (11).
11. Write Genotyper macro for allele calling.

4. Notes

1. The creation of spatial and spectral two-dimensional layouts (**Fig. 3**) is beneficial in examining potential PCR product sizes for the loci intended to be present in a multiplex PCR assay. Most multiplex STR assays will involve PCR products in the size range of 75-400 bp and utilize three different dye labels. With polymorphic STR markers it is important to have a good idea of overall allele ranges prior to designing the assay in order to avoid putting loci too close together that are next to one another in the same dye color. Typically leaving room for one or two new alleles at each end of the expected allele range is prudent. It is also important to remember that the reference sequence allele (represented by the vertical arrows along the horizontal locus allele range bars in Fig. 3), which is used for primer design, only represents one of the possible alleles and could be anywhere along the expected allele range.

2. Full characterization and quality control of PCR primers involves a UV spectrophometry measurement to determine concentration, an HPLC run to evaluate purity, a matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis to confirm correct sequence and purity, and a capillary electrophoresis (CE) run to determine the level of residual dye artifacts ("dye blobs"). Dye artifacts are only a problem with CE systems as they are co-injected during electrokinetic injection. Dye artifacts originating from impure primers usually have minimal impact on DNA separations in gel systems.

3. A number of fluorescent dye combination choices exist. Selection of dye labels used in a multiplex PCR assay impacts vendor options. Publicly available dyes such as fluorescein and tetramethyl rhodamine are less expensive and more widely available. However, they may not work as well as proprietary fluorescent dye labels in terms of brightness and spectral resolution. In our laboratory, we have chosen to use Applied Biosystems dyes as they work best with the ABI instruments we have in terms of color separation and sensitivity. Thus, our multiplexes contain 6FAM (blue), VIC (green), NED (yellow), and PET (red) for dye labels. Regardless of which dye combinations are selected for a multiplex assay, it is essential to use an appropriate color separation matrix to avoid pull-up between dye colors.

4. Storage of dye labeled primers in a slightly basic solution, such as TE at pH 8.0, rather than deionized water, which is typically pH 5.0, can reduce degradation of

the primer. If resuspended at pH <7.0, the fluorescent dye molecular can begin to degrade more rapidly and give rise to more residual dye artifacts ("blobs").

5. A concentration determination of PCR primers using a UV spec in one's own laboratory may reveal that oligonucleotides are not always quantified uniformly within and between manufacturers. If primers are being purchased from multiple vendors, then conducting a UV spec quantification check in one's own laboratory is useful in order to properly match primer pair concentrations. A UV spec check will also help maintain consistency if multiple batches of a primer are used over time. Maintaining consistent relative primer concentrations is especially important with multiplex PCR assays where a dozen or more primers are expected to work together in a single tube at empirically defined concentrations.

6. PCR volumes tested with multiplexes constructed in our laboratory have ranged from 5-25 µL. Tubes or trays used in thermal cycling must be well sealed for low volume reactions to prevent any evaporation. The rubber gasket supplied with GeneAmp 9700 cyclers for 96well plates works well.

7. A hot start enzyme like AmpliTaq Gold DNA Polymerase (Applied Biosystems) that activates at a high temperature is beneficial with multiplex PCR as it minimizes extension from primer-primer interactions that can form at lower temperatures than the assay annealing temperature and produce competing primer dimers.

8. Sequence entries in GenBank are often from the complementary strand. Thus, these reference sequences need to be made reverse and complement (r&c) in order to conform to familiar STR repeat and primer position nomenclatures. For example, the GenBank accession AC022486 for DYS385 contains 11 TTTC repeats that can be converted to the more familiar GAAA repeat structure reported by Schneider *et al.* (13).

9. A consensus reference sequence can be created by aligning multiple GenBank entries in an effort to identify possible primer binding site polymorphisms between the various STR alleles reported in GenBank. Alignments may be performed via the BCM Search Launcher program (**Box 1**) once candidate reference sequences have been put into a FASTA format. Sequence differences in flanking regions around the STR repeat can be flagged as possible polymorphisms and these regions should be avoided during primer design.

10. Although it is usually best to have primers with calculated annealing temperatures above the PCR annealing temperature, this is not always necessary. Sometimes it is not possible to design a primer with a higher Tm due to lack of available sequence information or sequence issues (e.g., palindromes or polynucleotide stretches). For example, in the cat STR 12plex, one of the primers has a calculated Tm of 50 ^{o}C because of limited sequence information yet it works fine with a PCR annealing temperature of 59 ^{o}C (data not shown). Thus, a lower than desired

primer Tm does not mean that the primer pair will not work well in a multiplex PCR environment and empirical testing is always required to prove the value of each primer.

11. A computer program has been written within our group at NIST that enables automated screening of potential primer-primer interactions via batch analysis. This Autodimer program has been written in Visual Basic and described in previous publications (6,8). It will be made publicly available in the near future through the STRBase website (http://www.cstl.nist.gov/biotech/strbase).

12. Use of a single G or a 7-base tail GTGTCTT on the 5'end of the unlabeled primer within a locus-specific primer pair can promote full adenylation of PCR products amplified from that locus. **Fig. 5** demonstrates how the partially adenylated doublet peaks for a STR locus from a heterozygous individual are converted to fully adenylated ones using the 7-base tail with identical PCR conditions.

13. Residual dye molecules exist for most fluorescent dye labeled primers that have not been extensively purified or stored properly (see Note 4). These dye blobs can interfere with allele calls in some size ranges but can be removed with Edge Bioscience spin columns following PCR amplification (14) as shown in **Fig. 6**.

14. The addition of bovine serum albumin (BSA) improves PCR amplification in multiplex reactions as shown in **Fig. 7**, most likely because BSA helps reduce

inhibition of the polymerase by the residual dye molecules present from the multiple primers in the PCR reaction.

Acknowledgments

This multiplex PCR assay design process has been made easier with the development of an autodimer program to screen for potential primer-primer interactions written by Dr. Peter Vallone in our laboratory. The work of Richard Schoske on the Y-STR multiplexes and Margaret Kline for the PowerPlex 16 and Identifiler data is also gratefully acknowledged. 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. This work was funded in part by the National Institute of Justice through an interagency agreement with the NIST Office of Law Enforcement Standards.

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Box 1. Internet sites useful for PCR primer design process

GenBank
http://www.ncbi.nlm.nih.gov/Genbank/
GenBank contains DNA sequence entries that may serve as reference sequences for primer design. A particular sequence may be located within GenBank by performing a BLAST search with a portion of the sequence for a locus, such as a published PCR primer.

Primer3
http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
Primer3 permits rapid and flexible PCR primer design for one reference sequence at a time. A reference sequence is pasted into an Internet browser window and the user indicates primer design parameters. A set of possible primer pairs is returned over the World Wide Web in a matter of seconds. The default primer Tm values of 57-63 ^{o}C generally select primers that work quite well with a PCR annealing temperature of 55 ^{o}C.

BLAST
http://www.ncbi.nlm.nih.gov/BLAST/
BLAST (Basic Local Alignment Search Tool) enables a rapid search of GenBank or other DNA sequence databases to determine if similar sequences to the query are present. If the query is a PCR primer, then similar sequences could indicate possible mispriming sites. If a high amount of similarity is seen with an undesired sequence or sequences, then the PCR primer should be redesigned to avoid the potential mispriming sites that would reduce the efficiency of PCR amplification.

BLAT
http://genome.ucsc.edu/cgi-bin/hgBlat
BLAT (BLAST Like Alignment Tool) performs homology searches. BLAT on DNA sequences is designed to quickly find sequences of 95% and greater similarity of length 40 bases or more. BLAT does not work well for querying with PCR primers rather it is useful for mapping large portions of a locus into the human genome reference sequence.

BCM Search Launcher: Multiple Sequence Alignments
http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html
A user can input multiple DNA sequences in FASTA format and obtain back an alignment of those sequences. This information can be useful in evaluating multiple sequence entries from GenBank in a search for possible single nucleotide polymorphisms that may disrupt PCR primer annealing.

FIGURE CAPTIONS

Fig. 1. Example of results from two commercial multiplex STR kits each capable of simultaneous amplification of 16 different loci. The top panel depicts Identifiler (Applied Biosystems) kit results on a DNA sample while the bottom panel contains PowerPlex 16 (Promega Corporation) kit results on the same sample. The loci names are listed above the corresponding peaks.

Fig. 2. Steps for development of STR multiplexes described in this chapter.

Fig. 3. Multiplex STR design layout using spatial (PCR product size) and spectral (dye label color) dimensions. PCR product size ranges for the various loci can be easily seen with this approach along with the size of the reference allele used for PCR primer design.

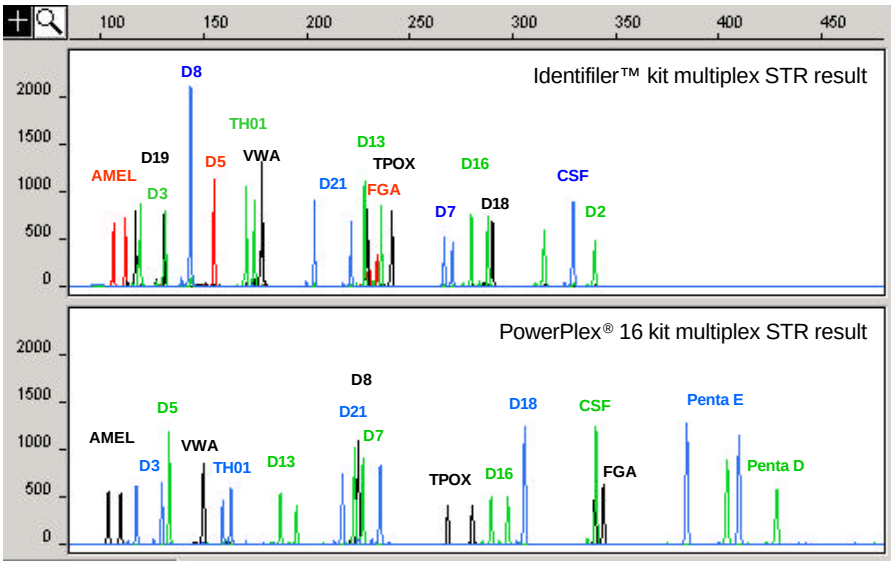
Fig. 4. Mass spectrum of a poor quality primer that contains numerous failure sequences and is therefore undesirable in a multiplex PCR reaction. This result was generated with ~10 picomoles of unpurified oligonucleotide using conditions described previously (7).

Fig. 5. Full adenylation improves with the addition of a 5'tail to the unlabeled primer. In the top panel, both alleles at this heterozygous locus exhibit doublets from –A and +A peaks that are ~1 bp apart. The bottom panel shows the same DNA sample amplified at the same locus with 5'tailed primers that promote full adenlyation of both alleles. The PCR product sizes for this STR locus are ~7 bp larger with the 7-base GTGTCTT 5'tail in the bottom panel.

Fig. 6. Residual dye removal with Edge spin columns (see ref. 14). Arrows in the left panel indicate positions of dye blobs that exist for each primer dye label used in the NIST Y-STR 20plex assay (10). The right panel shows the same PCR product after purification with an Edge column.

Fig. 7. Benefits of BSA addition to cat STR 12plex assay (9). DNA sample concentration and PCR components are identical between the two panels except that the top panel contains 0.16 mg/mL BSA.

Fig. 1


100
150
200
250
300
350
400
450
2000
1500
1000
500
0
Identifiler™ kit multiplex STR result
D8
TH01
D13
D19
D5
VWA
TPOX
D16
AMEL
D21
CSF
D3
FGA
D7
D18
D2
PowerPlex® 16 kit multiplex STR result
D8
D5
D21
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TPOX
D16
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Penta D

Fig. 2

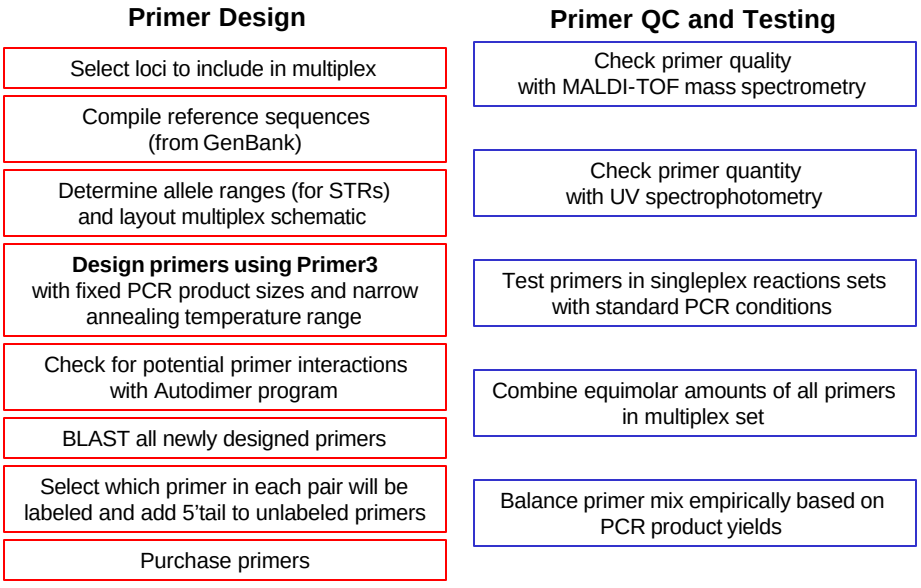

Primer Design
Select loci to include in multiplex
Compile reference sequences (from GenBank)
Determine allele ranges (for STRs) and layout multiplex schematic
Design primers using Primer3 with fixed PCR product sizes and narrow annealing temperature range
Check for potential primer interactions with Autodimer program
BLAST all newly designed primers
Select which primer in each pair will be labeled and add 5'tail to unlabeled primers
Purchase primers
Primer QC and Testing
Check primer quality with MALDI-TOF mass spectrometry
Check primer quantity with UV spectrophotometry
Test primers in singleplex reactions sets with standard PCR conditions
Combine equimolar amounts of all primers in multiplex set
Balance primer mix empirically based on PCR product yields

Fig. 3

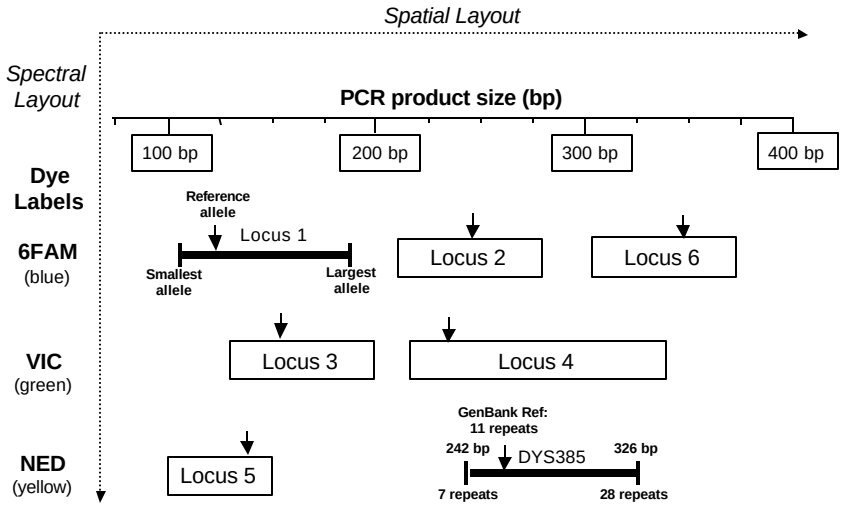
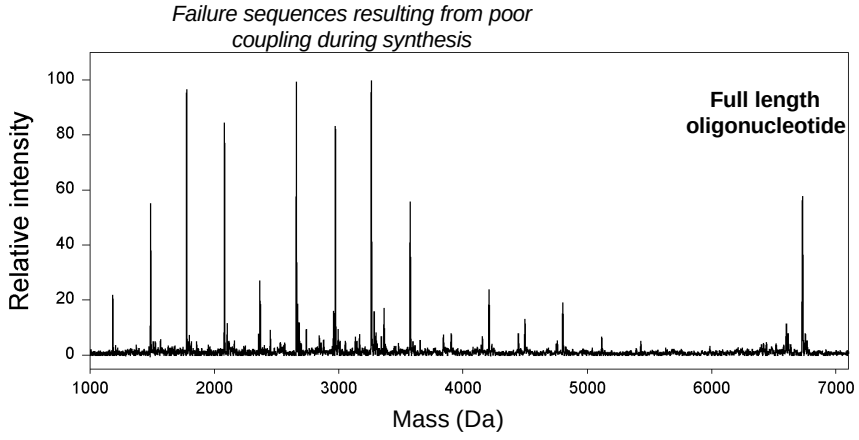

Spatial Layout
Spectral Layout
PCR product size (bp)
100 bp
200 bp
300 bp
400 bp
Dye Labels
6FAM (blue)
VIC (green)
NED (yellow)
Reference allele
Locus 1
Smallest allele
Largest allele
Locus 2
Locus 6
Locus 3
Locus 4
Locus 5
GenBank Ref: 11 repeats
242 bp
DYS385
326 bp
7 repeats
28 repeats

Fig. 4


Failure sequences resulting from poor coupling during synthesis
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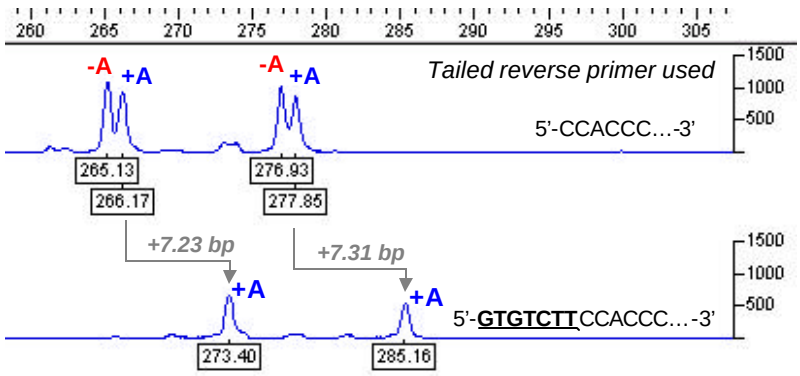
4000
5000
6000
7000
Mass (Da)

Fig. 5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265
270
275
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285
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295
300
305
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+A
-A
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Tailed reverse primer used
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1500
1000
500
265.13
266.17
276.93
277.85
+7.23 bp
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+A
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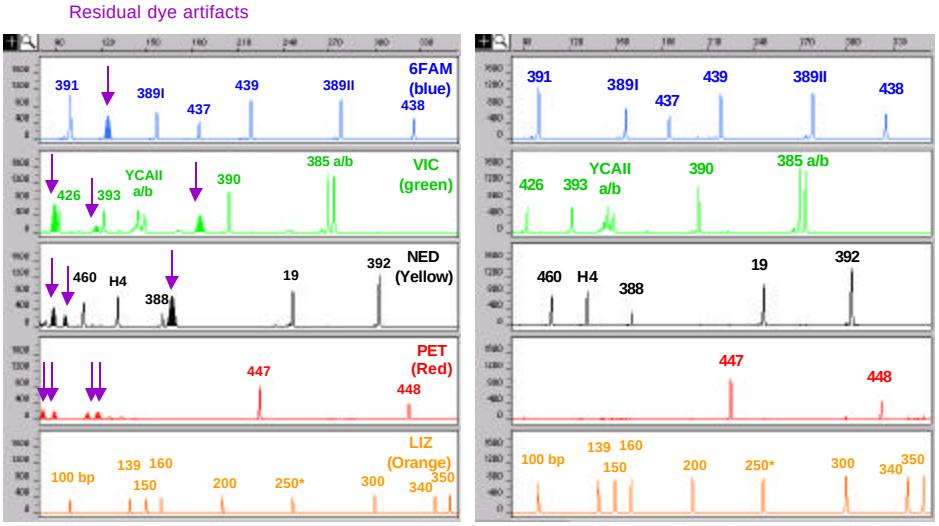
1000
500
273.40
285.16

Fig. 6


Residual dye artifacts
6FAM (blue)
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389I
437
439
389II
438
VIC (green)
426
393
YCAII a/b
390
385 a/b
NED (Yellow)
460
H4
388
19
392
PET (Red)
447
448
LIZ (Orange)
100 bp
139
160
150
200
250*
300
340
350
391
389I
437
439
389II
438
426
393
YCAII a/b
390
385 a/b
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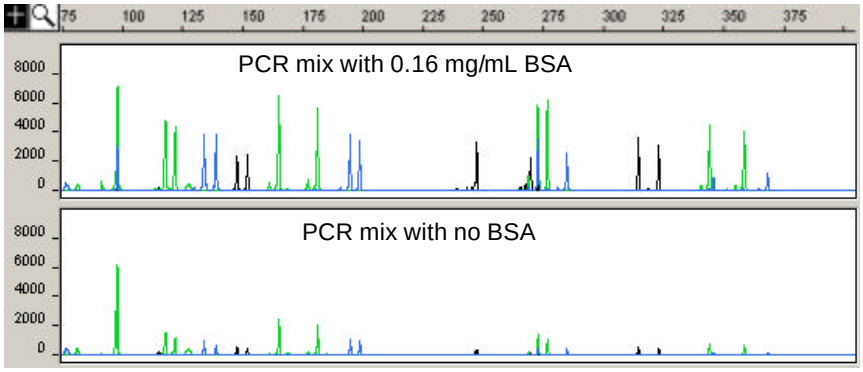
150
200
250*
300
340
350

Fig. 7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125
150
175
200
225
250
275
300
325
350
375
PCR mix with 0.16 mg/mL BSA
8000
6000
4000
2000
0
PCR mix with no BSA
8000
6000
4000
2000
0