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ORIGINAL PAPER

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Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci

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Abstract The simultaneous amplification of multiple regions of a DNA template is routinely performed using the polymerase chain reaction (PCR) in a process termed multiplex PCR. A useful strategy involving the design, testing, and optimization of multiplex PCR primer mixtures will be presented. Other multiplex design protocols have focused on the testing and optimization of primers, or the use of chimeric primers. The design of primers, through the close examination of predicted DNA oligomer melting temperatures (T_m) and primer–dimer interactions, can reduce the amount of testing and optimization required to obtain a well-balanced set of amplicons. The testing and optimization of the multiplex PCR primer mixture constructed here revolves around varying the primer concentrations rather than testing multiple primer combinations. By solely adjusting primer concentrations, a well-balanced set of amplicons should result if the primers were designed properly. As a model system to illustrate this multiplex design protocol, a 10-loci multiplex (10plex) Y chromosome short tandem repeat (STR) assay is used.

Keywords Multiplex PCR design · Y chromosome · Short tandem repeats · Primer design

Introduction

Multiplex polymerase chain reaction (PCR) is defined as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture. Since first being described in 1988, PCR multiplexing has been applied in many areas of DNA testing including the analysis of deletions, mutations, and short tandem repeats (STRs) [1, 2, 3, 4]. Furthermore, the wide availability of genetic information due to the publishing of the sequence of the human genome makes the demand for multiplex PCR even greater [5, 6]. For example, more than 1.4 million single nucleotide polymorphisms (SNPs) have been identified in the human genome [5, 6]. Multiplex PCR primer sets have been used for linkage studies to track genetic diseases [7, 8].

Eukaryotic genomes are full of repeated DNA sequences. These repeated sequences come in all sizes, and are designated by the length of the core repeat unit and the number of contiguous repeat units or the overall length of the repeat region. These regions are often referred to as satellite DNA. DNA regions with repeat units that are 2–6 bp in length are called microsatellites or short tandem repeats (STRs) and can be readily amplified with PCR. STR markers are dispersed throughout the genome and occur on average every 10,000 nucleotides [9]. Y chromosome STRs have a number of applications in human identity testing including typing the perpetrator of sexual assault cases without differential extraction [10]. The inheritance of the non-combining region of the Y chromosome has also made it a popular tool for tracing human evolution through male lineages [11].

Use of the Y chromosome for forensic purposes was until recently restricted by a lack of polymorphic markers [12]. The Y chromosome is less variable than the other chromosomes. The majority of Y STRs including the ones presented here are located within its non-recombining region and are passed without recombination. Thus, results from individual markers cannot be combined using the product rule. Many markers are thus needed to obtain a high degree of discrimination between unrelated males. For Y STR systems to become more highly discriminatory and gain acceptance within the forensic DNA community robust multiplexes are required. Y STR multiplexes have been described previously [10, 13, 14]; however, the highest number of loci amplified simultaneously has been six or less.

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Multiplex PCR primer design and optimization is a greater challenge than designing singleplex PCR primer pairs because multiple primer annealing events need to occur under the same annealing conditions without interfering with one another. Extensive optimization is normally required to obtain a good balance between amplicons of the various loci being amplified [15, 16]. Previous efforts on multiplex PCR design and optimization have focused on chimeric primers [17] and on varying experimental conditions such as $MgCl_2$ concentration, reaction buffer concentrations, annealing temperature, and polymerase concentration [15].

In this study a multiplex PCR primer set design protocol has been developed that focuses primarily on the design of the oligonucleotide prior to actual testing in the laboratory. For a multiplex reaction to work properly, the primer pairs need to be compatible. Since multiplex assays are run under the same PCR conditions, the primers used need to have fairly similar characteristics such as melting temperatures (T_m) and should not exhibit significant interactions with each other, themselves, or unwanted regions of the template. Excessive regions of complementarity between primers must be avoided to prevent the formation of primer–dimers that may cause the primers to bind to one another instead of the template DNA. Through stringent initial primer selection the time-consuming and often costly process of optimization can be reduced. The primary experimental condition used to optimize the multiplex PCR system in this study is the primer pair concentrations; other variables, thermal cycling number, reaction components, annealing temperatures, etc, are fixed.

An increasingly popular method for the detection of STRs is the use of fluorescence-based detection assays [18, 19, 20]. In the application to DNA typing with STR markers, the fluorescent dye is attached to a PCR primer that is incorporated into the amplified target region of DNA. The amplified STR alleles are visualized as bands on a gel or represented by peaks on an electropherogram. One of the first methods developed for the automated analysis of fluorescent labeled PCR products has been polyacrylamide or agarose gel electrophoresis [4]. The development of capillary electrophoresis (CE) has led to a number of advantages in the detection of DNA-amplified products. Some of these advantages include high resolution, high throughput, automatic operation, and on-line detection with automated data acquisition [21, 22].

This work presents a strategy for rapidly preparing multiplex PCR primer mixtures that utilize fluorescent labeled primers for detection by CE. The strategy has been successfully used to develop multiplex PCR assays that amplify as many as 20 different regions of the human Y chromosome [23].

Materials and methods

Primer design and synthesis

The primer design programs were used including Gene Runner (Hastings Software, Inc., Hastings NY), and Primer 3 version 0.2 over the World Wide Web www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi [24]. Potential primer cross-reactions were examined via an algorithm that was run using a computer program written in Visual Basic 6.0 [16].

PCR primer oligonucleotides were purchased from MWG Biotech (High Point, NC), Invitrogen (Carlsbad, CA), or Applied Biosystems (Foster City, CA). Primers were quality control tested using MALDI time-of-flight mass spectrometry prior to further testing to confirm proper synthesis using conditions described previously [25].

Sequence searches and alignments

Sequence searches were accomplished by a standard nucleotide BLAST search using GenBank(www.ncbi.nlm.nih.gov). Subsequent sequence alignments were also accomplished through the Baylor College of Medicine (BCM) search launcher located at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html.

Table 1 Y STR loci used in the Y STR 10plex[a]

STR locus	GenBankaccession	Reference allele	Repeat motif	New size range (bp)	Previous size range (bp)	Reference (previous size)
Y19	AC017019 (R&C)	15	$[TAGA]_3N_4[TAGA]_{12}$	176–212	176–212	[37]
Y391	AC011302	11	TCTA	156–184	272–300	[12]
Y392	AC011745	13	TAT	93–123	234–264	[12]
Y435	AC002992	9	TGGA	143–159	212–228	[33]
Y436	AC005820	12	GTT	87–101	129–144	[33]
Y437	AC002992	16	$[TCTA]_{10}[TCTG]_2[TCTA]_4$	185–197	185–197	[33]
Y438	AC002531	10	TTTTC	134–169	202–237	[33]
Y439	AC002992	20	$[GATA]_2N_4[GATA]_3N_{14}$ $[GATA]_1N_3[GATA]_1N_7[GATA]_{13}$	119–139	237–257	[33]
A7.1	AC009235	10	ATAG	100–120	162–182	[31]
H4	AC0011751	12	TAGA	121–141	353–373	[31]

[a]Size ranges calculated using the GenBank sequence as the reference allele. The GenBank accession numbers given are BAC clones containing STR sequences. Size ranges given are based on sequence information given in GenBank, and take into account the adenylation of PCR products through non-template addition. All primers were redesigned except DYS19 and DYS437 in order to fit into the multiplex design

PCR reaction and thermal cycling conditions

The Y STR 10plex amplifications were performed in reaction volumes of 20.0 µL with 2 units of AmpliTaq GoldDNA polymerase (Applied Biosystems, Foster City, CA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 µm deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), 4.4 µL of the Y-STR primer mix (see Table 1 for concentrations), and 2 µL DNA template at a concentration of approximately 2.5 ng μL^{-1}. Thermal cycling conditions were as follows: (1) 95 °C for 10 min, (2) 28 cycles: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, (3) 60 °C for 45 min, and (4) 25 °C hold.

Anonymous DNA samples from healthy blood donors were purchased as buffy coats from QC products Inc. (Pompano Beach, FL) and extracted using an organic extraction procedure as described elsewhere [26].

Capillary electrophoresis of PCR products

The separation and detection of PCR products were accomplished with the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using filter set C with 6-carboxy fluorescein (6-FAM), 4,7,2′,7′-tetrachloro-6-carboxyfluorescein (TET), 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX), 6-carboxy-X-rhodamine (ROX) matrix standards part #401546 (Applied Biosystems, Foster City CA) POP-4 sieving polymer matrix, 1X Genetic Analyzer Buffer with EDTA, and a 47 cm×50 µm uncoated capillary. Samples were injected for 5 s at 15,000 V and separated at 15,000 V for 24 min with a run 1 µL PCR product to 20 µL deionized formamide containing 0.75 µL ROX–GS500 temperature of 60 °C. DNA sizing was performed with ROX-labeled GS500 (Applied Biosystems, Foster City, CA) as the internal standard. Samples were prepared by adding standard. The samples were then loaded into the autosampler tray. Following data collection, samples were analyzed with the Genescan 3.1 software program.

Some DNA separations were also accomplished with the ABI Prism 3100 Genetic Analyzer 16-capillary array system following manufacturer's protocols (Applied Biosystems, Foster City, CA) using Matrix Z set-up with the 6-FAM, HEX, NED (Proprietary to Applied Biosystems), and ROX matrix standards part #4316100. HEX can be replaced with VIC (Proprietary to Applied Biosystems) matrix standard part #4323022 (Applied Biosystems, Foster City, CA). Additionally, proper set-up includes the GeneScan POP4 module, POP-4 sieving polymer matrix, 1X Genetic Analyzer Buffer with EDTA, and a 36 cm×50 µm capillary array part #4315931 (Applied Biosystems, Foster City, CA). Sixteen samples were injected at a time for 3 s at 3,000 V and separated at 15,000 V for 44 min with a run temperature of 60 °C. DNA sizing was performed with ROX-labeled GS500 (Applied Biosystems, Foster City, CA) as the internal standard. Samples were prepared by adding 1 µL PCR product to 20 µL deionized formamide containing 0.75 µL ROX–GS500 standard. Following data collection, samples were analyzed with the Genescan 3.1 software program.

Multiplex PCR primer design

Due to the complexity involved in the design of multiplex PCR primer sets it is helpful to view the strategy for primer selection in a systematic fashion. Figure 1 illustrates the process of multiplex PCR primer design, testing, and optimization used in this study. It starts with the selection of the loci to be examined and ends with the empirical test of the primer mix. Figure 1 is divided into two parts: part A focuses on the multiplex PCR primer mixture design, while part B describes the process of multiplex PCR primer mixture testing and optimization. Part A is the most time consuming and when done successfully limits the amount of actual primer testing and optimization. A successfully designed and optimized multiplex should result in the amplification of all desired loci and have similar yields (balance) between respective amplicons. The resulting multiplex PCR reaction should be free of nonspecific amplicons, and amplicons that utilize the same fluorescent dye (if used) must be distinguishable from one another. That is they must be resolvable no matter what the size of the allele present.

Select loci to include in multiplex

The loci used in the construction of the Y STR 10plex are listed in Table 1. The Y STR markers used in the construction of the Y STR 10plex were determined from an extensive review of the literature. The reasons for selecting the Y STR markers used in the construction of these multiplex PCR assays are described elsewhere [23].

Define reference sequence using GenBank

Sequences for each locus were obtained from GenBank (www.ncbi.nlm.nih.gov) using the standard nucleotide BLAST (Basic Local Alignment Search Tool) [27]. Previously published Y STR primers were chosen as the query sequences for the respective Y STR markers. BLAST search results listed the accession numbers that contained sequences producing significant alignments to the query sequences. The accession numbers are listed in order of their alignment score. The alignment score is defined as the degree of similarity between the query sequence primer and the sequence being compared [28]. The sequences with the higher alignment scores are listed first. The higher the score, the greater extent the nucleotide sequences are related.

The reference sequence and accompanying GenBank accession number for each sequence that contains a Y STR marker is defined as the one that returns the maximum alignment score for the query sequence. If a query sequence primer returned multiple GenBank accession numbers with a maximum score, the most recent entry and or sequence with the larger flanking region to the repeat was used. The reasoning behind using the larger sequences as the reference sequences will be discussed below. The repeat motifs used for each locus that are contained within each respective reference sequence were defined using the International Society of Forensic Genetics (ISFG) recommendations on using Y chromosome STRs [29].

Sequence alignments

Sequence alignments were performed for all of the queries that returned multiple GenBank accession numbers with a maximum alignment score. The alignments were conducted using the Baylor College of Medicine

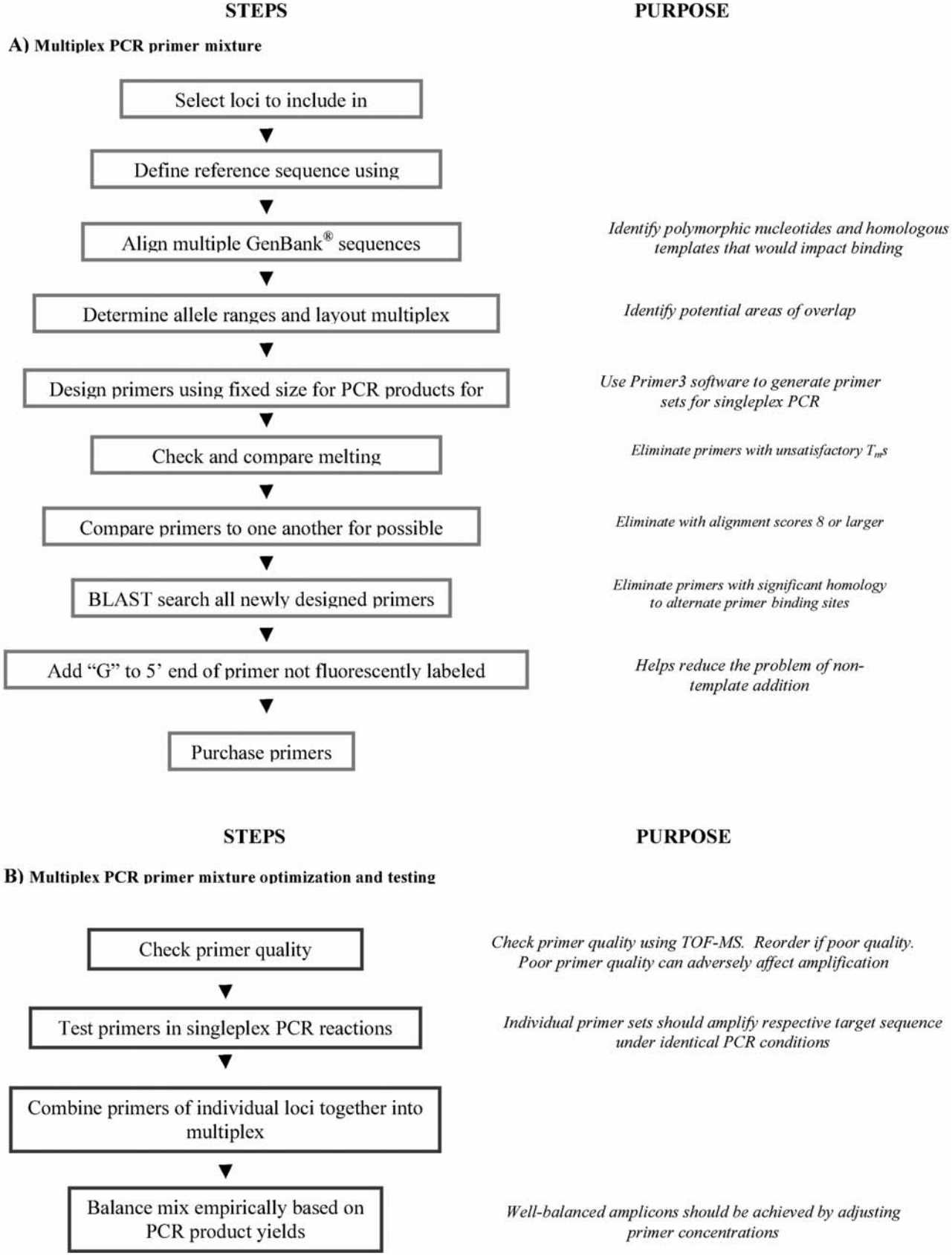

Fig. 1A,B Flowchart of the multiplex design, optimization, and testing process presented in this paper. The process is broken up into two parts. **A** Multiplex design, starting with the selection of loci to be included in the multiplex and ending with the purchasing of the desired primers. **B** Multiplex optimization and testing, starting with a check of the quality of each primer and ending with balancing the primer concentrations based upon PCR product yields

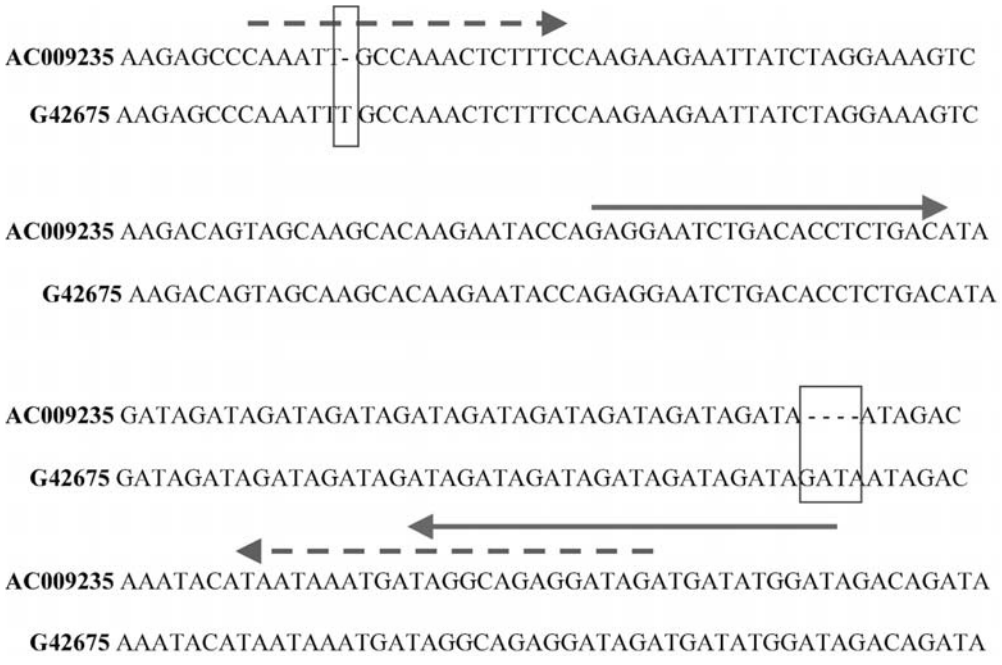

Fig. 2 Sequence alignment of the Y STR marker GATA A7.1 from GenBank accession numbers AC009235 (10 GATA repeats) and G42675 (11 GATA repeats). The alignment of sequences from multiple individuals is useful to search for possible polymorphic nucleotides that might impact primer annealing and subsequent amplification. For example, an extra "T" was observed in G42675 (see the *boxed region*). The *dotted line arrows* represent the primers from the original reference describing this marker [31]. The *solid line arrows* represent the primer positions described in this work. The *boxed areas* represent differences in sequence between the two accession numbers. The alignments were performed using the BCM Search Launcher: Multiple Sequence Alignments program located at the following URL: http://searchlauncher.bcm.tmc.edu/multi-align/multialign.html

(BCM) search launcher available at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html. Multiple sequences of the flanking region of STR loci as described above can exist due to the frequency of sequence variation inherent in human populations. Additionally, the human genome is filled (>50%) with repeated sequences including repeats derived from transposable elements, and long genomic regions which include large duplicated segments (50–500 kb) with high sequence identity (98–99.9%) [5, 6].

Sequence alignments can prove useful by identifying both multiplex potential priming sites and/or polymorphisms that may be present within the proposed primer binding site. If multiple potential priming sites exist, the primers ability to amplify the desired loci can be affected resulting in the amplification of a region of the template other than the allele of interest. The presence of polymorphisms within the primer binding regions could result in the occurrence of a null allele [30]. In the case of a null allele, a DNA template exists for a particular locus but fails to amplify during PCR due to primer hybridization difficulties related to the presence of this polymorphism.

A BLAST search using a previously published A7.1 forward primer [31] indicated exact homology within a sequence given in GenBank accession BAC Clone AC009235. A sequence alignment was then performed using the Baylor College of Medicine (BCM) search launcher located at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html, comparing the Y-GATA-A7.1 sequence contained within BAC Clone AC009235 and the sequence G42675. Sequence G42675 also contains the Y-GATA-A7.1 locus and was first deposited into the GenBank Data library by White et al. [31]. Figure 2 shows the sequence alignment for sequences contained within AC009235 and G42675. From this alignment any sequence differences between the two sequences were easily determined. In the case of A7.1 there is a T deletion (boxed sequence in Fig. 2) at position 29 for AC009235 that contains 10 AGAT repeats while the sequence within G42675 has 11 AGAT repeats. The T deletion is at a point in the sequence where the forward primer provided by White anneals [31]; thus, this might potentially cause problems with primer annealing to some DNA templates if a primer was designed to anneal where that polymorphism is located. BLAST searches and sequence alignments have proven essential to reduce the potential for non-specific binding sites for primers.

All of the other BLAST searches for the other loci listed that resulted in more than one GenBank accession number that contained the loci of interest sequence were aligned. None of the sequence alignments showed the presence of polymorphisms.

Allele and size range determination and multiplex schematic

The allele and size ranges of the Y STR 10plex Loci (see Table 1 and Fig. 3) were determined by a review of Y STR literature. The list of Y STR literature examined for the allele and size range determinations is available at STRBase [32] at the following web site: http://www.cstl.nist.gov/biotech/strbase/y_strs.htm.

The allele and size ranges for each locus listed in Table 1 were determined using the GenBank accession sequence as the standard reference point for each locus. For example, the GenBank accession number BAC Clone AC002992 contains within it the reference sequence for DYS439. The DYS439 reference sequence contains a complex repeat motif consisting of 20 GATA repeats. Template DNA containing 20 GATA repeats amplified using the primers reported by Ayub and coworkers should yield an amplicon 252 bp in length [33]. The allele range from the literature for DYS439 is 16 to 21 repeats. If these previ-

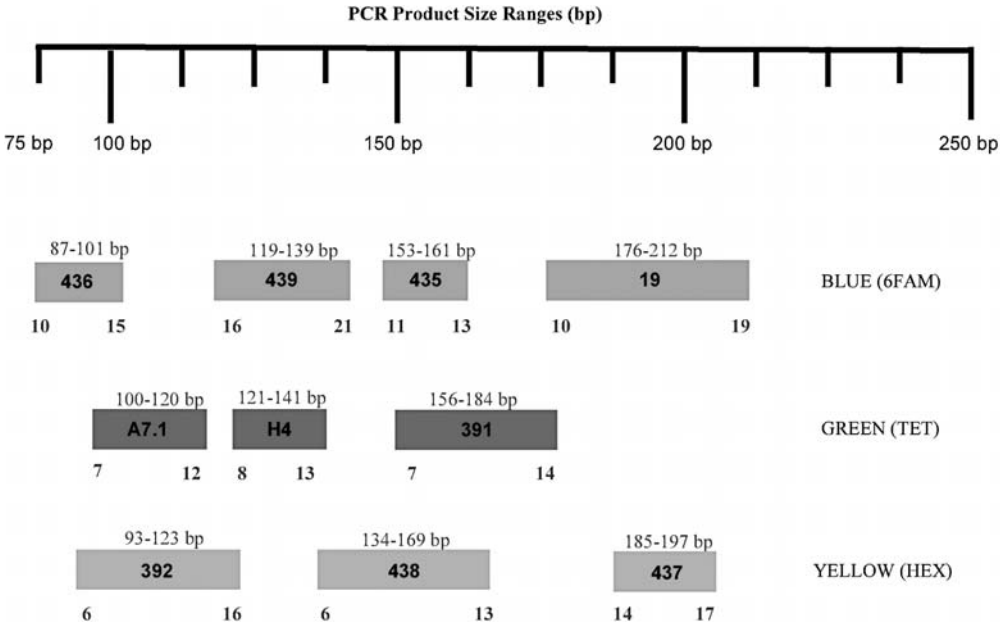

Fig. 3 Schematic of size ranges and dye label colors used in designing this Y STR 10plex assay. The length of the *box* containing the locus name represents the size ranges (*listed above box*) for the known alleles (*listed below box*). Three different fluorescent dyes are used in this Y STR 10plex that are spectrally distinguishable using Filter C on the ABI 310: 6–FAM (blue), TET (green), and HEX (yellow). Primers in this multiplex were redesigned compared to previous studies except those used to amplify DYS19 and DYS437 (see Table 1)

ously published primers were used, amplicons in the size range of 236–256 bp would be expected. In the absence of literature information on the allele range of a particular STR locus, the size range can be established empirically by running a variety of test samples.

A visual schematic (Fig. 3) of the Y STR 10plex loci was prepared. The allele and size ranges on the schematic provide for visual representation of the loci and highlight areas of possible size overlap. If an overlap in the possible alleles from two PCR products adjacent in size exists in the same dye color, then it may become difficult to assign the observed allele to its correct locus. For this reason, it would be beneficial if all of the amplicons within a single fluorescent dye color used in the Y STR 10plex had size ranges at least 10 bp apart. A 10-bp gap should accommodate the discovery of a new tetranucleotide allele and thus minimizes the need to redesign the affected primer pair. Using the schematic as the blueprint for the multiplex, primers were designed to deliver amplicons that fit the size ranges spelled out in the schematic.

Primer design

Primer3 software was used to design the individual primers sets used in the Y STR 10plex [24]. The Primer3 software allows the user to manipulate certain parameters during the design process. Some of these parameters include, minimum and maximum primer length, minimum and maximum melting temperatures, and optimal product size. For the multiplex described here the default parameters were used. Primers were designed using a fixed size for each PCR product for each locus. Not all of the primers used in the construction of the Y STR 10plex were redesigned. The primer sequences (Table 2) used in PCR amplification return amplicons that are of the sizes laid out in the multiplex schematic (Fig. 3).

Check and compare T_m

The calculated primer melting temperature (T_m) should be comparable so that all primers anneal at similar temperatures during the PCR temperature cycling. Similar primer annealing characteristics should lead to better balance between PCR products in a multiplex reaction. Table 2 lists the T_m values for each primer sequence calculated from the Primer3 web-based software that was used to initially select the PCR primer pairs [24].

Primer3 employs the DNA oligomer melting temperature formula given below by Rychlik to calculate primer T_m [34]:

$T_m^{primer} = [\Delta H/\Delta S + R\ln(C_T/4)] + 16.6\log([K^+]/1+0.7([K^+]) -237.1]$ where ΔH and ΔS are the enthalpy and entropy of formation for the helix respectively, R is the molar gas constant, and C_T is the total strand concentration of the DNA oligomer. Because the thermodynamic values were obtained in 1.0 M Na^+, a factor was added to correct for the fact that PCR reactions are typically conducted in 50 mM K^+ [34].

In order to normalize the primer selection process, all T_m values calculations were performed keeping the counterion and total strand concentrations for all primers constant. The default Na^+ and total strand concentrations used in Primer3 are 50 mM and 0.05 μM, respectively.

Commercial and public software exists for the prediction of T_m values. However, the values for ΔH and ΔS available in the literature used in the T_m calculation may vary slightly resulting in different predicted T_m values [35, 36]. For the purpose of this multiplex primer set, the relative comparison of the T_m values calculated by Primer3 was sufficient. The average T_m of all 19 primers (with the exception of the DYS19 forward primer) is 56.3±1.3 °C. The T_m values for the same 19 primers are all within 5 °C of each other.

Previously published primers were used in the construction of Y STR 10plex multiplex if they returned the

Table 2 Primer sequences used in this study[a]

Locus		Primer sequences (5′- to -3′)	Primer conc (μM)	T_m(°C)[b]
Y19	F	**FAM**–CTACTGAGTTTCTGTTATAGT	1.00	43.0
	R	ATGGCATGTAGTGAGGACA	1.00	59.0
Y391	F	**TET**–CTATTCATTCAATCATACACCCATAT	0.50	57.5
	R	ACATAGCCAAATATCTCCTGGG	0.50	59.4
Y392	F	**HEX**–AAAAGCCAAGAAGGAAAACAAA	1.00	59.3
	R	AAACCTACCAATCCCATTCCTT	1.00	60.0
Y435	F	GGGTTGTCCAGAGAAACAGC	0.25	59.7
	R	**FAM–**CCCCCTCCTCTCGTCTATCT	0.25	59.7
Y436	F	CCAGGAGAGCACACACAAAA	0.50	59.9
	R	**FAM–**ACGAGCTGCGTTAGAGGTGA	0.50	61.1
Y437	F	GACTATGGGCGTGAGTGCAT	0.50	61.1
	R	**HEX**–AGACCCTGTCATTCACAGATGA	0.50	59.6
Y438	F	**HEX**–TGGGGAATAGTTGAACGGTAA	0.50	59.4
	R	GGAGGTTGTGGTGAGTCGAG	0.50	60.7
Y439	F	**FAM**–ACATAGGTGGAGACAGATAGATGAT	0.50	57.3
	R	GCCTGGCTTGGAATTCTTTT	0.50	60.6
A7.1	F	GAGGAATCTGACACCTCTGACA	0.50	59.3
	R	**TET**–TCCATATCATCTATCCTCTGCCTA	0.50	59.1
H4	F	**TET**–ATGCTGAGGAGAATTTCCAA	1.00	57.3
	R	CTATTCATCCATCTAATCTATCCATT	1.00	56.2

[a]*F* refers to the forward primer and *R* refers to the reverse primer for a particular locus. **FAM**, **TET**, and **HEX** are commercially available fluorescent dyes

[b]Predicted primer melting temperatures (T_m) were calculated using a total primer concentration of 0.05 μM and [Na^+] = 50 mM and equations from Rozen and Skaletsky [24]

desired PCR product size even if the primer T_m was not optimal. The DYS19 forward primer was not redesigned from ones previously published [37] because that would have generated an amplicon greater than 220 bp in length. PCR products were less than 200 bp in length to enable easier handling of degraded DNA samples. A number of experiments have shown an inverse relationship between the size of the locus and successful PCR amplification from degraded samples [38, 39, 40].

Primer–primer comparison

Excessive regions of complementarity between primers should be avoided to prevent the formation of primer–dimers, where the primers bind to one another instead of the template DNA. Primer3 does not currently have the capability for multiplex primer comparisons. The comparisons were accomplished using an algorithm developed in-house that enables a pairwise comparison of each primer in a multiplex PCR primer mixture [16]. The total number of possible primer comparisons is equal to $(2n^2 + n)$ where n is the number of primer pairs to be tested. In the case of the Y STR 10plex, there are 210 possible primer interactions, including self-interactions.

Table 3 shows three sets of primer pair interactions with the highest degree of cross reactivity. The "alignment" score reflects the number of complementary base pairs minus the number of mismatched base pairs between two primers. The G–C and A–T pairs are given equal weight. From previously reported work an alignment score of greater than 8 (default value in the Primer3 program) can lead to significant primer–dimer formation depending upon the PCR amplification conditions [41]. Of the 210 possible interactions, none of the alignment scores were greater than 7.

Table 3 Crosschecking performed on the Y STR 10plex primers. Potential primer pair interactions are represented

Sequence Information	Potential Interaction
435-R vs. 439-R Matches = 9 alignment score[a] = 7	3-TAGTAGATAGACAGAGGTGGATACA-5 || ||||||| 5-CCCCCTCCTCTCGTCTATCT-3
436-R vs. A7.1-F Matches = 9 alignment score = 6	3-ACAGTCTCCACAGTCTAAGGAG-5 || ||||||| 5-ACGAGCTGCGTTAGAGGTGA-3
439-F vs. A7.1-R Matches = 12 alignment score = 5	3-TAGTAGATAGACAGAGGTGGATACA-5 ||||||||||| || 5-TCCATATCATCTATCCTCTGCCTA-3

[a]Level of interaction based on an alignment score which is defined as the number of complementary base pairs minus the number of mismatched base pairs between two primers

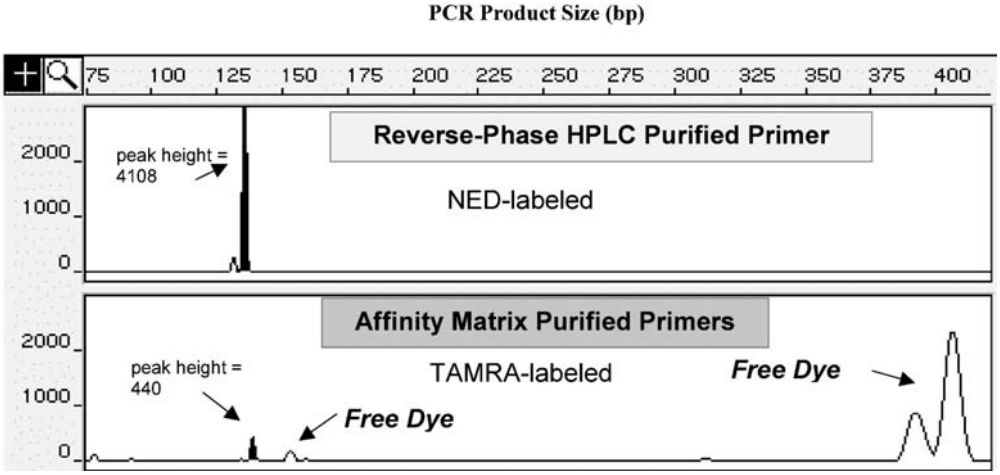

Fig. 4 Comparison of ABI 310 electropherograms from singleplex PCR reactions of the Y STR GATA H4 marker amplified with dye-labeled primers that were purified differently. The reversed-phase HPLC purified NED-labeled primer (*top panel*) produced a clean signal with a nice flat baseline. The affinity matrix purified TAMRA-labeled primer (*bottom panel*) showed multiple dye blobs in the region of 75–425 bp. These dye impurities failed to be removed after primer synthesis with the affinity matrix purification procedure and can interfere with detection of true alleles present from other locus amplified in a multiplex reaction. PCR product was generated as described in the materials and methods section

BLAST search for newly designed primers

A standard nucleotide BLAST search is performed on the newly designed primers to ensure that there are not alternate primer binding sites in the human genome other than the locus of interest. If the newly designed primer is homologous to regions other than the target sequence, it was redesigned according to the criteria above.

Primer modification ("G" tail and fluorescent dye labels)

During PCR, DNA polymerases especially *Taq* polymerase, often add an extra nucleotide to the 3′-end of the PCR product as the template strand is being copied. The addition of the nucleotide (most often an adenosine) has been termed non-template addition [42]. The adenylated PCR product is referred to as the "plus A" form of the amplicon. The adenylated amplicon is one base pair larger than the actual target sequence. The extent of adenylation is dependent on the sequence of the 5′-end of the opposing primer [42, 43]. The split peak resulting from partial adenylation becomes important especially from a measurement standpoint. Partial adenylation contributes to peak broadening in CE. Sharper peaks improve the likelihood that a system's genotyping (sizing) software can make accurate calls [25].

One study found that if the 5′-terminus of the unlabeled primer is a guanosine, complete addition of the adenosine is favored by the DNA polymerase [43]. An attempt was made to design the primer sets so that at least one primer in each pair had a 5′-guanosine. Of the 10 primer sets given in Table 2, five of these sets had primers that contained a 5′-guanosine.

Fluorescent dyes were added to the primer sequence of the primer pair that lacked a 5′-end guanosine. For those primer sets totally lacking a 5′-guanosine, the fluorescent labeled dye was arbitrarily added to either the forward or reverse primer. In the future, to maintain consistency only the forward primers will be labeled with the fluorescent dye, and all the reverse primer will be modified with the addition of a 5′-guanosine.

Multiplex PCR optimization and testing

Primer quality

High quality primers are essential to successful multiplex amplification reactions. The quality and the actual amount of the primer received depend upon such items as the coupling efficiency of the fluorescent dye and the integrity of the synthesized primer. The coupling efficiencies affect the amount of dye that is covalently linked to the 5′-end of the forward primer and vary depending upon which fluorescent dye is used. For example, TAMRA has a reported coupling efficiency of 70% [44]. The better the coupling, the higher the yield of covalently linked primer.

A dye reaction mixture that is not sufficiently purified may contain dye-labeled product, unreacted amino-linked oligonucleotide, excess dye species and salts. Excess dyes species, often referred to as "dye blobs", can also occur when fluorescent dyes come off their respective primers. Figure 4 shows how purity of the primers can affect amplification and detection. The electropherogram in the top panel of Figure 4 represents a PCR amplicon that resulted from a NED-labeled primer that had been purified by reversed-phase HPLC. The electropherogram shown in the bottom panel of Fig. 4 represents a PCR amplicon that resulted from a TAMRA-labeled primer and showed evidence of free dye "peaks" that failed to be removed through affinity matrix purification. The migration of free dye in the capillary can interfere with the detection of true alleles present from other loci amplified in a multiplex interaction.

Reversed-phase HPLC primer purification appears to be sufficient to remove unincorporated dye labels. While HPLC-purified primers were purchased in many cases, a quality control check was performed using time-of-flight mass spectrometry (TOF–MS). It has been shown that TOF–MS provides a rapid and accurate check on primer quality prior to using the oligonucleotides in a PCR reaction [45]. From examining the TOF–MS spectra (not shown) the molecular weight determined using the TOF–MS was compared to the calculated value determined from the mass of the dye (if present) and the sum of the masses of the nucleotides expected. Primers with either incorrect sequences or oligonucleotide failures will compete for binding sites on the DNA template. Either oligonucleotide failures or an oligomer of the incorrect sequence impact the presence and/or the length of the subsequent amplicon.

Test primers in singleplex PCR

Each primer pair was initially tested in a PCR singleplex reaction on three different DNA samples, two from male donors and one from a female. Two males were chosen in case amplification of one of the male templates failed. Variations in amplification between two male samples can arise from differences between the sequences in the primer binding region or the concentrations of the potential binding sites of the primers. The female was chosen as a negative control to ensure that the primer pair does not amplify any non-Y-chromosome regions. Admittedly, this is a small sample set. A small sample set was chosen to speed up the multiplex design process. For a legitimate evaluation of each loci of interest, many more individuals should be tested for the presence of null alleles and to assure that no amplification of these loci occurs in females.

All primer pairs were tested under identical amplification conditions including the same DNA template concentration. If amplification of a particular locus was poor, the primer concentration for that locus was increased and the amplification was repeated. Primer pairs that fail to amplify the male samples or indicate amplification in the female sample were eliminated. Ideally, these primers will be eliminated during the design process through sequence homology checks. All of the primer pairs chosen in our study successfully amplified the male sample and did not amplify the female sample.

Multiplex PCR testing

For the initial construction of the multiplex primer set all of the primer pairs were combined yielding a final concentration of 1.0 µM. This concentration value was chosen based on a review and comparison of previous Y STR multiplex studies of markers that are also used in the Y STR 10plex [13, 14]. Unlike previously published multiplex PCR primer mixture design protocols [15, 17] in which many other experimental conditions, such as $MgCl_2$ concentration, and buffer concentration were modified to achieve better amplification, only the primer concentrations were adjusted. If one PCR product was higher in peak height intensity relative to the other amplicons in the multiplex, the appropriate primer pair concentrations were lowered to generate a more balanced multiplex.

Balance mix empirically based on PCR product yields

Electropherograms, like the example shown in Fig. 5, were visually examined for each male DNA sample used to challenge the performance of the 10plex. The visual examination of the CE data permits the empirical adjustment of primer concentrations in order to balance the amplification yields [16]. When comparing the multiplex amplification yields from different primer concentrations, rela-

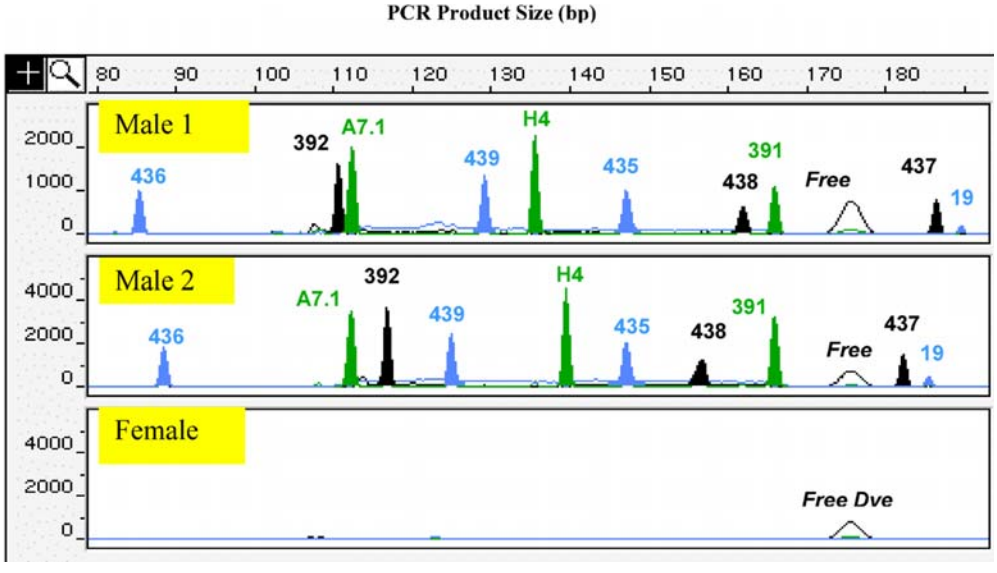

Fig. 5 Comparison of ABI electropherograms of PCR amplicons generated with the Y STR 10plex primer set listed in Table 2. The *top two panels* show the result from two different male DNA samples while the *bottom panel* is from a female DNA sample. Failure to detect PCR products from the female sample demonstrates that the primers in the Y STR 10plex are male-specific. PCR and electrophoresis conditions as described in the materials and methods

tive peak areas or heights of the amplicons were used to estimate the needed primer concentrations adjustments in order to improve the balance between the amplicon.

The amplicon peak for DYS19 was the weakest in intensity of the 10 loci used in the construction of the Y STR 10plex. This low signal for DYS19 may arise from the T_m of its forward primer with respect to the annealing temperature used during the PCR amplification process. While amplification of DYS19 could have been improved by adjusting the annealing temperature used in the PCR thermal cycling conditions, lowering the annealing temperature may have affected the amplification of the 9 other loci being examined.

Discussion

Multiplex PCR primer selection is a complex process that can be stratified into logical steps to obtain a working primer mix. Successfully optimized multiplex PCR primer pairs should be able to amplify of all desired loci, achieve similar yields between respective amplicons, be absent of non-specific PCR amplicons, and have all amplicons labeled with a particular fluorescent dye resolvable no matter what the size of alleles present. Additionally, the introduction of this Y STR 10plx could prove valuable to the forensic community. It provides scientists with a working multiplex assay that is male specific and gives results on 10 Y STR markers simultaneously.

Increasing the number of loci simultaneously amplified is restricted by several factors. The first is size. Amplicons should be designed that are at least 75 base pairs in size. The most common algorithm used for determining the DNA fragment size is known as the local southern method, and works very well for accurate sizing of DNA fragments over the 100–450 bp size range [25]. The local southern sizing method uses two sizing peaks below and two above the amplicon to determine its size. In CE electropherograms, the 35 bp and 50 bp can sometimes not be sized. This happens because these sizing fragments are often concealed by the peaks caused by unincorporated primers. Thus, sizing at below 75 bp can be inaccurate. For this reason amplicons below 75 base pairs should be avoided if one intends to use CE and the local southern method as the primary means of sizing analysis. Larger STR alleles (>400 bp) are not desirable because of allelic dropout of larger alleles in STR markers caused by preferential amplification of the smaller alleles in the multiplex, or if highly degraded DNA samples are being examined [38, 39, 40, 46]. Second, the more polymorphic a particular locus is the larger the size range it will encompass. One may have to decide between two less polymorphic markers or one with a larger potential size range of alleles. Third, the lack of adequate instrumentation may curtail the amount of amplicons that can be simultaneously detected.

Multicolor fluorescence detection is necessary to resolve similar sized PCR products that are labeled with spectrally distinguishable dyes. Instruments, such as the ABI Prism 3100 Genetic Analyzer 16-capillary array system, with a five dye capability can expand the analysis of multiple PCR amplicons. Amplification products of similar size can be distinguished by using different fluorescent dye labels for each one. Based upon these factors, a multiplex containing 40 different primer–pairs is theoretically possible if PCR product size ranges are extended to approximately 500 bp and STR loci are selected with moderate allele ranges.

With the exception of an in-house computer algorithm used to check for primer dimers, the multiplex design approach presented here uses publicly available software tools like GenBank, BLAST, BCM sequence alignment, and Primer3. This upfront utilization of publicly available informatics can reduce the labor-intensive empirical studies common to multiplex PCR optimization. An effective and working multiplex PCR primer mixture can be constructed quickly. This multiplex design strategy is currently being used to expand the Y STR multiplex to simultaneously amplify 20 or more loci in a single reaction [23].

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References

1. Chamberlain JS, Gibbs RA, Rainer JE, Nguyen PN, Casey CT (1988) Nucleic Acids Res 16:11141–11156
2. Henegariu O, Hirschman P, Killian K, Kirsch C, Lengauer R, Maiwald K (1994) Andrologia 26:97–106
3. Shuber AP, Skoletsky J, Stern R, Handelin BL (1993) Hum Mol Genet 2:153–158
4. Kimpton CP, Fisher D, Watson S, Adams M, Urquhart A, Lygo J, Gill P (1994) Int J. Legal Med 106:302–311
5. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Yandell MG et al (2000) Science 5507:1304–1368
6. Lander ES, Linton LM, Birren B, Nusbaum C et al (2001) Nature 409:860–921
7. Evans JC, Frayling TM, Cassell PG, Saker PJ, Hitman GA, Walker M, Levy JC, O'Rahily S, Rao PV, Bennett AJ, Jones EC, Menzel S, Prestwich P, Simecek N, Wishart M, Dhillon R, Fletcher C, Milward A, Demaine A, Wilkin T, Horikawa Y, Cox NJ, Bell GI, Ellard S, McCarthy MI, Hattersley AT (2001) Am J Hum Genet 69:544–552
8. Prince JA, Feuk L, Sawyer SL, Gottfries J, Rickstein A, Nagga K, Bogdanovic N, Blennow K, Brookes AJ (2001) Eur J Hum Genet 9:437–444
9. Edwards A, Civitello A, Hammond HA, Caskey CT (1991) Am J Hum Genet 49:746–756
10. Prinz M, Boll K, Baum H, Shaler B (1997) Forensic Sci Int 85:209–218
11. Jobling MA, Tyler–Smith C (1995) Trends Genet 11:449–456

12. Kayser M, Caglia A, Corach D, Fretwell N, Gehrig C, Graziosi G, Heidorn F, Herrmann S, Herzog B, Hidding M, Honda K, Jobling M, Krawczak M, Leim K, Meuser S, Meyer E, Oesterreich W, Pandya A, Parson W, Penacino G, Perez–Lezaun A, Piccinini A, Prinz M, Schmitt C, Schneider PM, Szibor R, Teifel–Greding J, Weichhold GM, de Knijff P, Roewer L (1997) Int J Legal Med 110:125–133
13. Redd AJ, Clifford SL, Stoneking M (1997) Biol Chem 378: 923–927
14. Gusmao L, Gonzalez–Neira A, Pestoni C, Brion M, Lareu MV, Carracedo A (1999) Forensic Sci Int 106:163–172
15. Henegariu O, Heerema SR, Vance GH, Vogt PH (1997) BioTechniques 23:504–511
16. Butler JM, Ruitberg CM, Vallone PM (2001) Fresenius J Anal Chem 369:200–205
17. Shuber AP, Grondin VJ, Klinger KW (1995) Genome Res 5: 488–493
18. Schwartz LS, Tarleton J, Popovich B, Seltzer WK, Hoffman EP (1992) Am J Hum Genet 51:721–729
19. Sullivan KM, Pope S, Gill P, Robertson JM (1992) PCR Methods Applic 2:34–40
20. Ziegle JS, Su Y, Corcoran KP, Nie L, Mayrand PE, Hoff LB, McBride LJ, Kronick MN, Diehl SR (1992) Genomics 14: 1026–1031
21. Butler JM, McCord BR, Jung JM, Wison MR, Budowle B, Allen RO (1994) J Chromatogr 658:271–280
22. Butler JM (1997) Separation of DNA restriction fragments and PCR products. In: Heller C (ed) Analysis of nucleic acids by capillary electrophoresis. Vieweg, Germany, pp 195–217
23. Butler JM, Schoske R, Vallone PM, Kline MC, Redd AJ, Hammer MF (2002) Forensic Sci Int 129:10–24
24. Rozen S, Skaletsky HJ (1998) Primer3 (primer 3_www.cgi v 0.2); Code available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
25. Butler JM (2001) Forensic DNA typing. Academic Press, San Diego, pp 191–195
26. Budowle B, Baechtel FS (1990) Appl Theor Electrophor 1:181–187
27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) J Mol Biol 215:403–410
28. Karlin S, Altschul SF (1993) Proc Natl Acad Sci USA 90: 5873–5877
29. Gill P, Brenner C, Brinkmann B, Budowle B, Carracedo A, Jobling MA, de Knijff P, Kayser M, Krawczak M, Mayr WR, Morling N, Olaisen B, Pascali V, Roewer L, Schneider PM, Sajantila A, Tyler–Smith C (2001) Forensic Sci Int 124:5–10
30. Lazaruk K, Wallin J, Holt C, Nguyen T, Walsh SP (2001) Forensic Sci Int 119:1–10
31. White PS, Tatum OL, Deaven LL, Longmire JL (1999) Genomics 57:433–437
32. Ruitberg CM, Reeder DJ, Butler JM (2001) Nucleic Acids Res 29:320–322
33. Ayub Q, Mohyuddin A, Qamar R, Mazhar K, Zerjal T, Mehdi SQ, Tyler–Smith C (2000) Nucleic Acids Res 28:e8
34. Rychlik W, Spencer WJ, Rhoads RE (1990) Nucleic Acids Res 18:6409–6412
35. Owczarzy R, Vallone PM, Gallo FJ, Paner TM, Lane MJ, Benight AS (1997) Biopolymers 44:217–239
36. SantaLucia J (1998) Proc Natl Acad Sci USA 95:1460–1465
37. Roewer L, Kayser M, Nagy M, de Knijff P (1996) Adv Forensic Haemogenet 6:124–126
38. Whitaker JP, Clayton TM, Uruhart AJ, Millican ES, Downes TJ, Kimpton CP, Gill P (1995) Biotechniques 22:670–677
39. Sparkes R, Kimpton CP, Watson S, Oldroyd NJ, Clayton TM, Barnett L, Arnold J, Thompson D, Urquhart A, Gill P (1996) Int J Legal Med 109:186–194
40. Takahashi M, Kato Y, Mukoyama H, Kanaya H, Kamiyama S (1997) Forensic Sci Int 90:1–9
41. Beasley EM, Myers RM, Cox DR, Lazzeroni LC (1999) In: Innis MA, Gelfand DH, Snisky JJ (eds) PCR applications: protocols for functional genomics. Academic Press, San Diego, pp 55–71
42. Magnuson VL, Ally DS, Nylund SJ, Karanjawala ZE, Rayman JB, Knapp JI, Lowe AL, Ghosh S, Collins FS (1996) Biotechniques 21:1004–1010
43. Brownstein MJ, Carpten JD, Smith JR (1996) Biotechniques 20:1004–1010
44. Giusti WC, Adriano T (1993) PCR Methods Applications 2: 223–227
45. Butler JM, Devaney JM, Marino MA, Vallone PM (2001) Forensic Sci Int 119:87–96
46. Tully G, Sullivan KM, Gill P (1993) Hum Genet 92:637–638