Chapter 2

Capillary Electrophoresis and 5-Channel LIF Detection of a 26plex Autosomal STR Assay for Human Identification

Carolyn R. Hill

Abstract

Multiplex polymerase chain reaction (PCR) is a common method used for DNA typing in forensic and paternity cases. There are numerous commercial short tandem repeat (STR) multiplex assays currently available to the forensic community. These assays amplify the core Combined DNA Index System (CODIS) STR loci for entry into the US. DNA database. Additional non-CODIS loci, which are considered genetically unlinked to the CODIS loci, can be useful in resolving challenging cases such as missing persons and mass disaster victim identification, paternity testing, and immigration testing. An STR multiplex has been successfully developed with 25 non-CODIS autosomal loci plus the sex-typing locus amelogenin for a total of 26 loci in a single 26plex amplification reaction. This chapter will focus on the preparation and the use of the 26plex assay with DNA samples for the purpose of human identification.

Key words: Multiplex PCR, Short tandem repeat, STR marker, Forensic DNA, PCR primers, 26plex, non-CODIS

1. Introduction

Multiplex polymerase chain reaction (PCR) is commonly used for various forensic DNA typing purposes. This type of testing allows for simultaneous amplification of specific target regions of the genome. Currently, there are two commercial short tandem repeat (STR) assays that can amplify 16 genetic loci in one reaction and are widely used in the forensic community. The AmpFlSTR® Identifiler® (Applied Biosystems, Foster City, CA) and PowerPlex® 16 (Promega Corporation, Madison, WI) multiplex kits both include the 13 Combined DNA Index System (CODIS) STR loci that are required by the Federal Bureau of Investigation (FBI) for data entry into the national level of the US DNA database (1, 2).

Antonio Alonso (ed.), *DNA Electrophoresis Protocols for Forensic Genetics*, Methods in Molecular Biology, vol. 830,
DOI 10.1007/978-1-61779-461-2_2,

These 13 STR markers provide a random match probability of approximately 1 in 100 trillion (1, 2). With this level of discrimination, it may not seem necessary to go beyond the CODIS loci and use additional non-CODIS (NC) markers for forensic typing. Indeed, for general forensic matching of an evidence profile to a suspect profile, the 13 CODIS STR loci are currently deemed sufficient. However, additional loci can be beneficial for human identification in scenarios involving relatives such as missing persons/mass disaster victim identification, immigration testing with limited reference samples, deficient parentage testing which is often needed if only one parent and child are tested, and in cases involving incest (3–7). Forensic and kinship testing laboratories are being challenged to answer more difficult genetic questions, and it is important that the necessary tools are in place to address these issues.

We have previously characterized 26 NC reduced-size STR loci (miniSTR) and performed population analyses on US Caucasian, African American, Hispanic, and Asian samples (8, 9). These miniSTRs were designed to span unused chromosomal locations on the 22 autosomes and have been found to be genetically unlinked to the 13 CODIS loci (8, 9). Thus, the single-locus genotype probabilities for the 13 CODIS loci and the 26 NC loci can be multiplied to determine the profile match probability. Originally, assays for the 26 miniSTR markers were developed as panels of three markers (triplexes) with product sizes below 140 base pairs (bp). These assays allowed for the recovery of genetic information from degraded DNA, which is often present in skeletonized missing persons remains or mass disaster victims (3). The loci were arranged into ten miniplexes for further characterization with US population samples.

After the initial testing of the 26 NC markers, it would have been inefficient to perform additional studies with the ten separate miniplexes. Combining all 26 loci into one multiplex reaction would facilitate this task and save resources. The NC markers have moderate-to-high heterozygosity values (>0.5) and narrow allele ranges (5–15 alleles), making them ideal for combining into a large multiplex for further testing (8, 9). The new NC multiplex does not just have utility in our laboratory; it can be beneficial to the forensic community in several ways as well. A larger multiplex can be used for the generation of a high-throughput reference database. Since most of these loci no longer exist as miniSTRs after primer redesign to fit into the multiplex, the 26plex will likely have a limited utility on degraded samples. However, when high quality and quantity DNA samples are available, the 26plex assay could be useful for kinship analysis, complex criminal paternity testing, parentage testing, immigration testing, and in missing persons/mass disaster cases.

A single amplification, five-dye multiplex has been developed to combine 25 of the NC STR loci (see Note 1) plus the sex-typing marker amelogenin (10) in one reaction to enable rapid analysis of reference samples (11, 12). A concordance evaluation was performed with the multiplex to compare genotypes obtained with the previously characterized miniSTR loci to determine null alleles present with the newly designed primer sets (13). The multiplex was also tested with ~400 father/son sample pairs to determine the individual mutation rates of each STR locus (13). Testing on an extended family sample study was performed to examine the potential statistical improvements to adding additional loci to the analysis (13, 14).

To make the information regarding the 26plex available to the forensic community, Standard Reference Material (SRM) 2391b (the PCR-based DNA Profiling Standard) has been updated with certified and reference values for all 26 of the new loci described (15, 16). Bins and panels for genotyping using GeneMapper ID v3.2 (Applied Biosystems) have been designed (17) to allow for a five-dye single amplification multiplex for rapid reference sample typing.

2. Materials

2.1. DNA Samples

The 26plex should be used with human DNA samples at a target amount of 1 ng; however, DNA concentrations as low as 100 pg can be amplified with this multiplex assay. Samples with higher concentrations can be diluted to 1 ng using TE^{-4} buffer (10 mM Tris, 0.1 mM EDTA, pH 8) (see Notes 2 and 3).

2.2. PCR Reagents and Instrumentation

1. 10× GeneAmp® PCR Gold buffer (Applied Biosystems).
2. 25 mM GeneAmp® $MgCl_2$ (Applied Biosystems).
3. 10 mM dNTPs (USB Corporation, Cleveland, OH).
4. 5 U/μL Taq Gold DNA Polymerase (Applied Biosystems).
5. 3.2 mg/mL Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO) (see Note 4).
6. Forward primers are labeled with fluorescent dyes (Applied Biosystems), and reverse primers are unlabeled (Qiagen Operon, Alameda, CA). Primer sequences are listed in Table 1 (see Note 5).
7. TE^{-4} buffer (10 mM Tris, 0.1 mM EDTA, pH 8).
8. Cary Spectrophotometer (Varian, Santa Clara, CA).
9. GeneAmp® 9700 Thermal Cycler (Applied Biosystems).

Table 1
26plex PCR primers used in this assay

Locus name	Forward dye label	Primer sequence (5′–3′)	(Primer), μM
D1GATA113	PET	F - acattaagcacatgcctctttgt R - **G**atgaactcattggcaaaagga	1 1
D1S1627	NED	F - catgaggtttgcaaatactatcttaac R - **G**ttttaattttctccaaatctcca	2 2
D1S1677	6FAM	F - gcagtcagcttgattgatcc R - **GTTTCTT**agaatgcaatagcaaatatcagaatg	3 3
D2S441	NED	F - caaaaggctgtaacaagggcta R - **G**ttcactctccttcccaaatgtt	1 1
D2S1776	VIC	F - ttacctgtgagtatgtgtgcgta R - ggtgctaggtgtgctcagga	1.5 1.5
D3S3053	VIC	F - tgacacaaatggaccaagaca R - **GTTTCTT**gagagagcccttgaaatagca	2 2
D3S4529	NED	F - cccaaaattacttgagccaat R - **G**agacaaaatgaagaaacagacag	0.75 0.75
D4S2364	VIC	F - ctaggagatcatgtgggtatgatt R - **G**cagtgaataaatgaacgaatgga	0.75 0.75
D4S2408	NED	F - agctgacatcttaccacatgttc R - **G**tgcttggcatatattaagacactgta	2 2
D5S2500	VIC	F - gtttactgataaaccaaatgatgtgc R - **G**taacttaaagggtaaatgtttgcag	2 2
D6S474	6FAM	F - ggttttccaagagatagaccaatta R - **G**tcctctcataaatccctactcatatc	1.5 1.5
D6S1017	NED	F - agatgggaacgatgcagaca R - gcataaatggatgggtggat	2 2
D9S1122	VIC	F - gggtatttcaagataactgtagatagg R - **G**cttctgaaagcttctagtttacc	0.75 0.75
D9S2157	NED	F - gatcacgccacggta R - **G**ttctccatttcaaagatcat	5 5
D10S1248	6FAM	F - cagtaaaaagcaaacctgagca R - gcttggcaaagagcagatg	1 1
D10S1435	VIC	F - cacgttgggtttcctgactt R - **G**cccagctacttgggatgcta	1 1
D11S4463	6FAM	F - ctgtcccaaggctgagtgtt R - **GTTTCTT**cgcagggcaaataaaaagaa	3 3
D12ATA63	6FAM	F - aggtggcagtgagctgtaac R - GTTtcttggattttgagggccta	1 1

(continued)

Table 1 (continued)

Locus name	Forward dye label	Primer sequence (5′–3′)	(Primer), μM
D14S14343	PET	F - ggctctgatttccaccactg R - **G**caactcttggaagcccagtc	2 2
D17S974	NED	F - ggaacacttgagcca R - gtggactggggtaagg	2 2
D17S1301	PET	F - aagatgaaattgccatgtaaaaata R - **G**tgtgtataacaaaattcctatgatgg	2 2
D18S853	PET	F - acatatataatgtgagaaaggaggagt R - **G**ttaatgggtgcaacacacc	2 2
D20S482	PET	F - ctccattctctcacacccaat R - **G**cacttctggcttttctggttc	1 1
D20S1082	6FAM	F - acatgtatcccagaacttaaagtaaac R - **G**cagaagggaaaattgaagctg	1 1
D22S1045	6FAM	F - ccctgtcctagccttcttatagc R - **G**ctgtgcccaagttgagagaa	1 1
Amelogenin	PET	F - ccctttgaagtggtaccagagca R - gcatgcctaatattttcagggaata	2 2

The fluorescent dye labels are listed for the forward primers only. The reverse primers are unlabeled. The 5′ guanine (G) residue for "PIGtail" (GTTTCTT) sequence in each reverse primer was added to promote adenylation (3, 18) and is underlined in *bold print*. The approximate primer concentrations are listed in μM (concentration in 20 μL total volume), but must be empirically adjusted for locus-to-locus balance. Amelogenin primers that are used are from Haas-Rochholz et al. (10)

2.3. Capillary Electrophoresis Reagents and Instrumentation

1. 36 cm 3100/3130xl capillary array (Applied Biosystems), 16 capillaries.
2. Matrix Standard SD-33, G5 dye set (Applied Biosystems).
3. Hi-Di™ Formamide (Applied Biosystems).
4. GS500 LIZ internal size standard (Applied Biosystems).
5. A.C.E.™ Sequencing Buffer, 10× Running Buffer (Ameresco, Solon, OH).
6. POP™-4 or POP™-6 Polymer (Applied Biosystems).
7. 16-capillary ABI Prism® 3130xl Genetic Analyzer (Applied Biosystems).

2.4. Data Analysis Software

1. GeneMapper® *ID* v3.2 (Applied Biosystems) (see Note 6).
2. GeneMapper bins and panels (available on STRBase) (see Note 7).

3. Methods

3.1. Primer Set Preparation

1. Fluorescently labeled forward and unlabeled reverse primers are delivered lyophilized and must be reconstituted with TE^{-4} buffer (see Note 8) to the following concentrations:

   Unlabeled reverse primers = 200 µM.

   Labeled forward primers = 100 µM.
2. The unlabeled reverse primers come with specific quantification information listed in pmol and are reconstituted based on this value. Once reconstituted, they are quantified using a UV spectrophotometer absorbance reading at 260 nm. The dye-labeled forward primers do not come with specific quantification information (these primers are ordered at 10,000 pmol), so they are all reconstituted with the same amount of TE^{-4} buffer to a final concentration of 100 µM and are not quantified with a UV spectrophotometer (see Note 9).
3. The target primer mix concentration is 2 µM, but the primer concentrations should be empirically adjusted for locus-to-locus balance (see Note 10). A sample primer mix preparation sheet is included as Table 2. Note that the primer concentrations used in the primer mix calculations are for the reverse primers since those are variable. The forward primers assume a 100 µM concentration for all markers. This example primer mix set-up is for a 100 µL final volume.

3.2. PCR Reagents and Conditions

1. PCR set-up should be performed in a sterile fume hood.
2. Combine the PCR reagents listed in Subheading 2 for final concentrations of the following:

   2 mM $MgCl_2$, 1× PCR buffer, 250 µM dNTPs, 1 Unit Taq Gold, 0.16 mg/mL BSA, and 0.2 µM primer set concentration. Fill remaining volume with deionized water (diH_2O).
3. An example of a PCR template is included as Table 3. The final reaction volume in this example is 19 µL Master Mix + 1 µL DNA sample at 1 ng = 20 µL.
4. The PCR amplification conditions using the GeneAmp 9700 are: denaturation for 11 min at 95°C, amplification for 30 cycles of 45 s at 94°C, 2 min at 59°C, and 1 min at 72°C, extension for 60 min at 60°C, and a final soak at 25°C.

3.3. Capillary Electrophoresis on the ABI 3130xl Genetic Analyzer

1. Prior to testing, a 36-cm capillary array must be installed on the 3130xl with POP-4 or POP-6 polymer and 1× A.C.E. buffer (see Note 11).
2. A 5-dye matrix should be run as the G5 spectral calibration under the "G5 filter" with the five dyes of 6FAM, VIC, NED, PET, and LIZ.

Table 2
An example primer mix preparation sheet

Locus	Locus name	Stock reverse concentration (μM)	Reverse singleplex (μL)	Dye-labeled forward (μL)	Water (μL)
1	D1GATA113	143.6	0.70	1	98.30
2	D1S1627	195.9	1.02	2	96.98
3	D1S1677	98.1	3.06	3	93.94
4	D2S441	141.8	0.71	1	98.29
5	D2S1776	137.9	1.09	1.5	97.41
6	D3S3053	103.1	1.94	2	96.06
7	D3S4529	147.4	0.51	0.75	98.74
8	D4S2364	149.0	0.50	0.75	98.75
9	D4S2408	152.4	1.31	2	96.69
10	D5S2500	184.3	1.08	2	96.92
11	D6S474	190.9	0.79	1.5	97.71
12	D6S1017	153.6	1.30	2	96.70
13	D9S1122	184.4	0.41	0.75	98.84
14	D9S2157	79.5	6.29	5	88.71
15	D10S1248	154.2	0.65	1	98.35
16	D10S1435	154.8	0.65	1	98.35
17	D11S4463	161.7	1.86	3	94.64
18	D12ATA63	192.8	0.52	1	98.48
19	D14S1434	181.4	1.10	2	96.90
20	D17S974	78.4	2.55	2	95.45
21	D17S1301	212.5	0.94	2	97.06
22	D18S853	214.2	0.93	2	97.07
23	D20S482	180.0	0.56	1	98.44
24	D20S1082	200.0	0.50	1	98.50
25	D22S1045	146.1	0.68	1	98.32
Amel	Amelogenin	187.8	1.06	2	96.94
			33.21	44.25	22.54

The unlabeled reverse primers are quantified using a UV spectrophotometer absorbance reading at 260 nm. The dye-labeled forward primers are not quantified and are all reconstituted with TE^{-4} to 100 μM. The primer concentrations used in the primer mix calculations are for the reverse primers. The forward primers use 100 μM for all calculations. This example primer mix set-up is for a 100 μL final volume. The target primer mix concentration is ~2 μM, but should be empirically adjusted for locus-to-locus balance

Table 3
An example of a PCR prep sheet for ten reactions

Stock Concentration	26plex	Desired PCR concentration	Volumes to add	Final volumes
	Total volume of reaction =20µl			n=10 reactions
25 mM	Mg concentration	2 mM	1.6 µL	16 µl
2 µM	Primer concentration	0.2 µM	2 µL	20 µl
5 U/µL	Units of Taq	1U	0.2 µL	2 µl
10 mM	dNTP concentration	250 µM	0.5 µL	5 µl
10×	PCR buffer	1×	2 µL	20 µl
3.2 mg/mL	BSA	0.16 mg/mL	1 µL	10 µl
	Water to add		11.7 µL	117 µl
	Master Mix (MM) volume		19 µl	190 µl
	Volume of added template (µL)	1 µl		
	Add 19 µL MM + 1µL sample = 20 µL rxn			

3. Amplification products are diluted in Hi-Di formamide by adding 1 µL PCR product and 0.3 µL GS500 LIZ internal size standard to 8.7 µL of Hi-Di.
4. The samples are run on the 16-capillary 3130xl without prior denaturation of the samples.
5. The samples are injected electrokinetically for 10 s at 3 kV (see Note 12).
6. The STR alleles are then separated at 15 kV at a run temperature of 60°C.

3.4. Analysis Software

1. Data from the ABI 3130xl are exported as .fsa files and are analyzed using GeneMapper ID v3.2 (see Note 6).
2. Bins and panels have been written for use with POP-6 and POP-4 (see Note 7) and are available for download on STRBase (http://www.cstl.nist.gov/biotech/strbase/str26plex.htm#Bins-and-Panels). There are no ladders available for data analysis (see Note 13).
3. The analytical threshold is set to 50 RFU for analysis in GeneMapper ID v3.2 (see Note 14).
4. An example profile for positive control 9947A is illustrated in Fig. 1 (see Note 15).
5. More information about the 26plex assay can be found on STRBase: http://www.cstl.nist.gov/biotech/strbase/str26plex.htm.

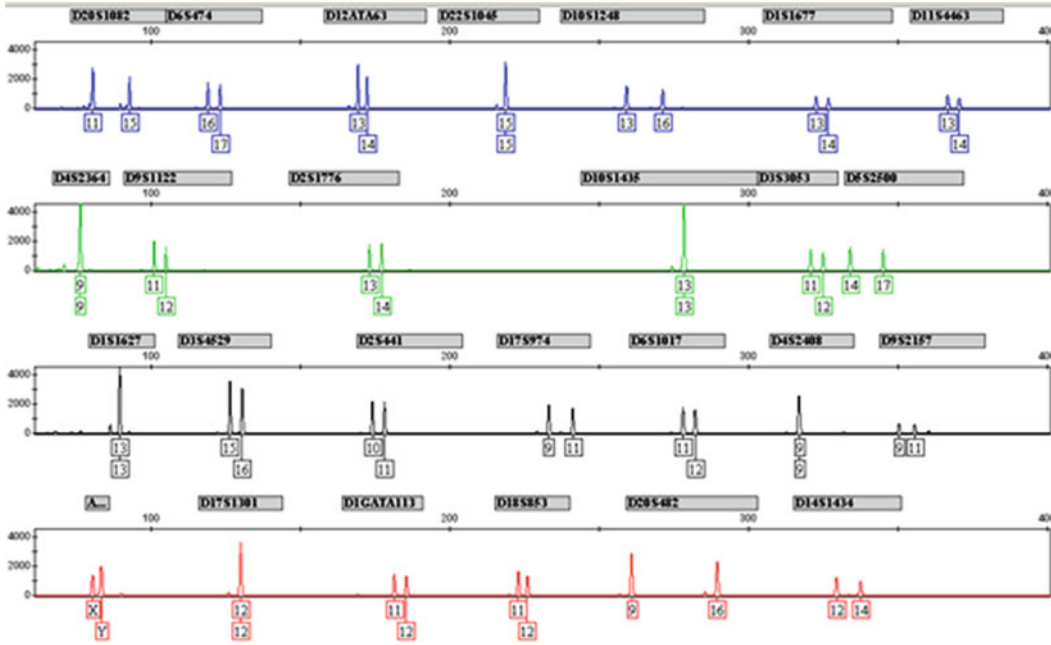

Fig. 1. The final 26plex with 25 autosomal loci plus the sex-typing locus amelogenin for a total of 26 loci. Positive control, 9947A, was used at 1 ng for 30 cycles (12). Primers for the loci were designed so that all of the amplicons present are distributed from 65 basepairs (bp) to less than 400 bp within a five-dye chemistry design with the fifth dye reserved for the sizing standard.

4. Notes

1. In the final 26plex, there were seven loci that used the original miniSTR primer sequences. The primers for the 19 remaining loci were redesigned to fit into the multiplex. Amelogenin was added and the D8S1115 was removed for a total of 26 loci. The D8S1115 was removed due to incompatible primer sequences that consistently caused artifacts to occur in the resulting DNA profiles (12).
2. A sensitivity study was performed (12) to determine the optimal amount of DNA template that can be reliably used with the 26plex. In this study, it was observed that the minimum amount of DNA that can be added without allelic drop-out is 100 pg for 30 cycles. The target amount of input DNA to be added to this assay is 1 ng. Substantial spectral bleed-through between dye channels at several loci was observed with 2 ng of DNA template.
3. At lower amounts of DNA template (<400 pg), there is a consistent artifact peak present within the D4S2364 allele range at ~70 bp (typically between 200 and 450 RFU). The size of the

artifact is proportional to the size of the authentic allele peak heights. However, this artifact peak does not interfere with allele calling. The artifact peak is more apparent with less input DNA as the locus-specific peak heights decrease. The amelogenin peak heights are lower than the allele peaks of other loci with less than 300 pg of template DNA.

4. It is important to use nonacetylated BSA because acetylated BSA interferes in the PCR process.
5. Some of the reverse primers have an additional guanine base (G) or "PIG-tail" sequence (GTTTCTT) added to the 5′ end to help promote full adenylation (3, 18) and are noted in bold and underlined in Table 1.
6. The majority of the 26plex data have been analyzed with GeneMapper ID v3.2. However, GeneMapper ID-X v1.1 and v1.2 (Applied Biosystems) can also be used to analyze the data. The bins and panels were originally written in v3.2, but have been successfully imported into both versions of ID-X.
7. The bins and panels were originally written for samples run on a 36-cm array with POP-6 polymer (12). However, the 26plex has since been run on a 36-cm array with POP-4 polymer. The bins and panels have been adjusted accordingly and both sets are available for download on STRBase. If any other 3130xl parameters are used (i.e., 50-cm array or POP-7 polymer), the bins and panels will have to be further adjusted to fit the data because there are no allelic ladders available for the 26plex at time of publication.
8. It is imperative to use TE^{-4} buffer to reconstitute the lyophilized primers instead of diH_2O. It has been our experience that using TE^{-4} buffer reduces the presence of dye artifacts that are present in the final DNA profile compared to diH_2O. The dye artifacts can be recognized as being fairly broad peaks that possess the spectrum of one of the dyes used for genotyping (12). If dye artifacts are still present, these can be removed post-PCR using gel filtration cartridges such as Edge Columns (Edge Biosystems, Gaithersburg, MD).
9. The initial primer sequences can be ordered at any concentration. The initial concentrations listed serve as guidelines only. The final concentrations are listed as approximate values due to the fact that it is not possible to initially quantify the fluorescent dye-labeled forward primers.
10. The concentrations of the primer pairs were kept the same when preparing the primer mix. If it was necessary for the concentration of a locus to be adjusted, the forward and reverse primers were adjusted equally. The balancing of the primer mix is an essential step in the 26plex protocol and great care must be made in empirically increasing or decreasing the primers to

generate balanced PCR products (as demonstrated by peak heights).

11. As mentioned in Note 6, POP-6 was originally used for the development of the 26plex. During the development of the 26-plex, we used a single 3130xl instrument for fragment analysis and sequencing. This allowed for higher resolution separations to be achieved while eliminating the need to change the instrument between fragment analysis and sequencing. In addition, a 36-cm array was used, but was installed as a 50-cm array on the instrument. This is because there is no 36 cm, POP-6 module available within the Data Collection Software v3.0 that is installed with the 3130xl instrument. There is, however, a 50 cm, POP-6 module that was used for analysis. Also, as mentioned in Note 6, a 36-cm array with POP-4 can also be used to run the 26plex – this is the recommended human identification (HID) configuration.
12. The injection time and voltage can be adjusted if off scale data and bleed-through between dye channels occur. Typically for a "low injection," the samples were injected for 5 s at 2 kV.
13. As of the time of publication, no allelic ladders are available for the 26plex data analysis. Instead, genotyping and allele sequencing were performed with Standard Reference Material (SRM) 2391b, PCR-based DNA Profiling Standard for all the components including ten genomic DNA samples, and two cell lines (12 samples total) using the 26 additional loci. Certified and reference values were assigned to all resulting alleles and the Certificate of Analysis was updated to include this new information (15). These values can serve as a way to calibrate the genotypes observed with analyzing data from the 26plex (16).
14. The analytical threshold for the 3130xl used at NIST was set at 50 RFU. The analytical threshold and stochastic thresholds should be determined by the individual laboratory according to validation studies performed using their own instruments.
15. The 26plex is a robust STR multiplex assay with balanced heterozygote peak heights and adequate interlocus peak balance. No PCR or fluorescent dye artifacts were present at 1 ng for 30 cycles that could interfere in the correct genotyping of DNA samples.

Acknowledgments

I would like to acknowledge John Butler and Pete Vallone for previous contributions in the development of the 26plex assay. In addition, I would like to thank Mike Coble, Kristen Lewis

O'Connor, Pete Vallone, and John Butler for their helpful comments and suggestions with this manuscript. I would also like to express appreciation to the Applied Genetic group members for technical assistance with the 26plex assay, in particular Margaret Kline for the DNA Sequencing work and Dave Duewer for data analysis support. I would also like to acknowledge the National Institute of Justice for their funding to our NIST Group through the NIST Law Enforcement Standards Office interagency agreement 2008-DN-R-121. The opinions and assertions contained herein are solely those of the author and are not to be construed as official or as views of the US Department of Commerce. 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 US Department of Commerce, nor does it imply that any of the materials, instruments, or equipments identified are necessarily the best available for the purpose.

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http://www.springer.com/978-1-61779-460-5

DNA Electrophoresis Protocols for Forensic Genetics
Alonso, A. (Ed.)
2012, XIII, 394 p., Hardcover
ISBN: 978-1-61779-460-5
A product of Humana Press