Qiagen® Investigator® DIPplex Kit: Validation and Use with Parentage Section Cases Involving Degraded/Inhibited Samples or Complex Relationships

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Abstract

Human identification by deoxyribonucleic acid (DNA) analysis in the field of forensic science is most commonly completed by utilizing short tandem repeat (STR) amplification kits. In recent years, amplification kits have been developed targeting other regions of DNA that can provide valuable information. The Qiagen® Investigator® DIPplex kit targets thirty deletion-insertion polymorphisms (DIPs), commonly referred to as insertion-deletion polymorphisms or indels. Indels are biallelic DNA length polymorphisms characterized by the presence (insertion) or absence (deletion) of a certain DNA sequence made up of one or more nucleotides. Indels have benefits similar to that of single nucleotide polymorphism (SNP) analysis, but what makes them even more beneficial is that they can be analyzed using the same methods as STRs with technology already present in forensic DNA testing laboratories. All amplicons are less than 160 base pairs (bp), which is relatively short and makes the DIPplex kit ideal for highly degraded DNA samples. The number of indels in the DIPplex kit and their low mutation rates make them suitable for relationship testing and body identification cases.

An internal validation of the Qiagen® Investigator® DIPplex kit was completed at the Marshall University Forensic Science Center (MUFSC) DNA Laboratory. The studies for this validation included sensitivity and stochastic studies, precision and accuracy, repeatability and reproducibility, mixture analysis, contamination assessment, and known and non-probative samples. All validation studies were completed on both the Applied Biosystems® 3500 Genetic Analyzer as well as the Applied Biosystems® 3130*xl* Genetic Analyzer.

At the MUFSC DNA Laboratory, the parentage section performs testing in body identification cases and questioned relationship cases. The use of an indel kit as a supplement to the STR profile could provide valuable information in cases involving either a

degraded/inhibited sample or complex relationship determination. Samples from these cases were identified and analyzed in order to determine if the use of the DIPplex kit in addition to the STR profile would improve the statistics as hypothesized.

When used as a supplement to STR testing, the DIPplex kit provided valuable information. When a degraded/inhibited bone sample was analyzed using DIPplex, it provided a more complete profile than the STR profile. When complex relationship cases were analyzed using DIPplex, it improved the statistics for the case. In the future, analyzing more degraded/inhibited samples and more complex relationship cases would provide a more thorough understanding of the pros and cons of using this kit as a supplement to STR testing.

Introduction

Short Tandem Repeat (STR) typing is presently considered the method of choice for identification in the forensic field, but genetic variation in the form of DNA polymorphisms can be used for testing as well [8]. Human DNA polymorphisms can be split into two groups: those based on substitutions of single nucleotides (single nucleotide polymorphisms or SNPs) and ones based on insertion or deletion of one or more nucleotides (insertion-deletion polymorphisms or indels). The use of SNPs has been valuable in certain applications, such as analysis of highly degraded samples [8]. Unfortunately, because they are sequence polymorphisms, SNPs must be typed by complex and expertise-demanding methods [4] and the current SNP assays require many steps and expensive high-throughput technologies [2]. Indels are short biallelic length polymorphisms [3] and are also referred to as deletion-insertion polymorphisms, or DIPs [3]. With indels, the difference between alleles is based on size rather than detecting nucleotide substitution (like with SNPs) and these size differences are readily resolvable using the same methods as STRs: simple end-labeled PCR primers and capillary electrophoresis [7].

Indels can combine the desirable characteristics of STRs and SNPs [1] and have many features that make them a strong supplement or stand-alone tool for human identity testing. Indels have short amplicon size, low mutation rates, and a lack of stutter [7]. Short amplicons are good for the analysis of highly degraded DNA, low mutation rates are good for relationship testing and disaster victim identification, and the lack of stutter makes peak assignments easier [1]. Additionally, indels have high multiplexing capability [4], simple analytical procedures, the ability to be analyzed using technology already present in most crime laboratories [7], and are spread widely throughout the genome [3].

The Qiagen® Investigator® DIPplex Kit (Qiagen®, Hilden, Germany) allows for multiplex amplification of thirty biallelic autosomal indels plus amelogenin. The indels in this kit are distributed over nineteen autosomes and all amplicon lengths are shorter than 160 base pairs (bp) [6], while most STR kits have amplicon lengths of 100-400 bp. The target amount of DNA for most STR kits is between 0.5-1.0 nanograms (ng). The optimal target range of DNA for the DIPplex kit is 0.2-0.5 ng [9], but in a previous study using DIPplex, full and partial indel profiles resulted from 0.062 ng and 0.016 ng of DNA, respectively [7].

At the Marshall University Forensic Science Center (MUFSC) DNA Laboratory, the parentage section routinely encounters cases involving complex relationships or body identification cases with bone that is degraded/inhibited. The use of an indel kit, such as DIPplex, as a supplement to the STR testing is hypothesized to improve the statistics in complex relationship cases or provide more information in cases with degraded/inhibited samples.

Due to the age and nature of a bone sample, partial STR DNA profiles are often reported when using STR kits. DNA extracts that produced partial DNA profiles were amplified using the DIPplex kit to determine if the smaller base pair sizing and increased sensitivity of the kit would

improve the discriminating power. Relationship cases can involve incest and/or related alleged fathers and in either of these situations, or a combination of them, the shared DNA between individuals could lead to a male relative not being conclusively excluded or even multiple men not being excluded as the father due to the lack of three inconsistencies in the pattern of inheritance. An incestual scenario of this type was amplified with DIPplex to determine how the additional information provided would impact the results and accompanying statistical analysis. To further the concept of a complex relationship case being impacted by the use of indels, DIPplex was also performed on a case involving the question of half-sibship to identify the level of influence on the statistic.

In order to use the DIPplex kit with challenging parentage section cases at the MUFSC DNA Laboratory, an internal validation of this kit was completed. The validation studies were performed on both the eight capillary Applied Biosystems® 3500 Genetic Analyzer (3500) (Life Technologies™, Foster City, CA) and the sixteen capillary Applied Biosystems® 3130*xl* Genetic Analyzer (3130*xl*) (Life Technologies™, Foster City, CA). The studies for this validation include those found in the Scientific Working Group on DNA Analysis Methods (SWGDAM) Validation Guidelines for DNA Analysis Methods: sensitivity and stochastic studies, precision and accuracy, repeatability and reproducibility, mixture analysis, contamination assessment, and known and non-probative samples [10].

Materials and Methods

For each amplification set-up using the DIPplex kit, a total of 25 µL was added to each well: 10.6 µL of master mix and a combination of 14.4 µL of nuclease-free water and DNA. The master mix contained 5 µL of Reaction Mix A, 5 µL Primer Mix DIPplex, and 0.6 µL MultiTaq2 DNA Polymerase. All samples were amplified on an Applied Biosystems® GeneAmp® PCR

System 9700 Thermal Cycler (Life Technologies™, Foster City, CA) for 30 cycles, according to the manufacturer's standard cycling protocols. This included a hot-start at 94°C for 4 minutes to activate the DNA polymerase, 30 cycles of the following: 94°C for 30 seconds, 61°C for 120 seconds, and 72°C for 75 seconds, followed by one cycle at 68°C for 60 minutes, and a final hold at 10°C [9].

For each genetic analyzer run using the DIPplex kit, a master mix was prepared by mixing 12.0 µL HiDi Formamide with 0.5 µL of DNA Size Standard 550 (BTO) per sample. Whether it was for a sample, a ladder, or a run negative, each well contained 12.0 µL of master mix. 1.0 µL of amplified product was added to wells designated for samples and 1.0 µL of DIPplex allelic ladder was added to wells designated for ladder. Each sample was run on the 3500 and 3130*xl* with a 10 second injection time and 3.0 kV injection voltage. All samples run on the 3500 were analyzed using Applied Biosystems® GeneMapper® ID-*X* Version 1.4 (Life Technologies™, Foster City, CA) and all samples run on the 3130*xl* were analyzed using Applied Biosystems® GeneMapper® ID Version 3.2.1 (Life Technologies™, Foster City, CA). For all analyses performed throughout the validation, a full sizing range and the Local Southern size-calling method were used for both the 3500 and 3130*xl*. For all other study specific parameters, see Table 1.

Table 1: Analysis Parameters for sample run on the 3500 and 3130*xl*

Analysis Parameters (AP)	Analysis Range	Smoothing	Analytical Threshold for Samples, Ladders, Positive Control (RFU)	Analytical Threshold for Negative Controls (RFU)	Analytical Threshold for Size Standard (RFU)
3500 AP 1	1,000-20,000	Light	100	100	100
3500 AP 2	1,000-20,000	Light	1	1	100
3500 AP 3	1,000-20,000	None	150	100	100
3130*xl* AP 1	2,200-6,000	Light	100	50	50
3130*xl* AP 2	2,200-6,000	Light	1	1	1
3130*xl* AP 3	2,200-10,000	None	100	50	50

Sensitivity and Stochastic Studies

Cuttings were taken from a Whatman® FTA® card (General Electric Healthcare Life Sciences, Little Chalfont, United Kingdom) spotted with blood from Dr. Terry Fenger (TF) and placed in five sample extraction tubes. These TF cards are the National Institute of Standards and Technology (NIST) traceable internal positive control at the MUFSC DNA Laboratory. These samples and five reagent blanks were extracted using the Qiagen® EZ1® Advanced XL (Qiagen®, Hilden, Germany) with the Qiagen® EZ1® DNA Investigator® Kit (Qiagen®, Hilden, Germany). The EZ1® Trace Tip Dance protocol was used and the samples and reagent blanks were eluted into 40 µL of TE buffer. After extraction, the TF samples were combined into one tube to obtain approximately 200 µL of sample extract. A Microcon® Centrifugal Filter (Merck, Darmstadt, Germany) was used to concentrate the TF sample down to approximately 40 µL.

The TF sample and reagent blanks were quantitated using the Qiagen® Investigator® Quantiplex HYres Kit (Qiagen®, Hilden, Germany) on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies™, Foster City, CA). A serial dilution of the TF sample ranging from 4.0 to 0.00195 ng/ µL was created, resulting in 12 different concentrations. The dilution series was quantitated to confirm the actual concentration was close to the desired concentration.

2 µL of the samples were loaded into their respective wells, making the desired target range from 8.0 to 0.00391 ng of DNA (Table 2).

Table 2: NIST Traceable TF Dilution Series for Sensitivity Study

Desired Concentration (ng/µL)	Actual Concentration (ng/µL)	Desired Target (ng)	Actual Target (ng)
4.000000	3.410000	8.000000	6.820000
2.000000	1.720000	4.000000	3.440000
1.000000	0.875000	2.000000	1.750000
0.500000	0.434000	1.000000	0.868000
0.250000	0.240000	0.500000	0.480000
0.125000	0.114000	0.250000	0.228000
0.062500	0.055000	0.125000	0.110000
0.031250	0.033000	0.062500	0.066000
0.015625	0.014400	0.031250	0.028800
0.007813	0.006280	0.015625	0.012560
0.003906	0.004760	0.007813	0.009520
0.001953	0.000651	0.003906	0.001302

One amplification plate using DIPplex was set up with three repeats of the TF dilution series (Figure 1). The amplified TF dilution series and its controls were run on both the 3500 and the 3130*xl*. The TF samples run on the 3500 were analyzed with "3500 AP 1" (Table 1). All samples were checked to ensure proper allele calling. Extraneous off ladder (OL) calls were clicked off. This GeneMapper® project was utilized in determining the linear range for the DIPplex kit on the 3500. The five reagent blanks run on the 3500 were analyzed with "3500 AP 2" (Table 1). This GeneMapper® project was utilized in determining the analytical threshold for the DIPplex kit on the 3500.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DIP-SENS-TF-8.1	DIP-SENS-TF-0.06.1	DIP-SENS-TF-2.2	DIP-SENS-TF-0.01.2	DIP-SENS-TF-0.5.3	DIP-SENS-TF-0.003.3	DIP-SENS-Neg1	BLANK	BLANK	BLANK	BLANK	BLANK
B	DIP-SENS-TF-4.1	DIP-SENS-TF-0.03.1	DIP-SENS-TF-1.2	DIP-SENS-TF-0.007.2	DIP-SENS-TF-0.25.3	DIP-SENS-RB1.1	DIP-SENS-Pos1	BLANK	BLANK	BLANK	BLANK	BLANK
C	DIP-SENS-TF-2.1	DIP-SENS-TF-0.01.1	DIP-SENS-TF-0.5.2	DIP-SENS-TF-0.003.2	DIP-SENS-TF-0.12.3	DIP-SENS-RB2.1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK
D	DIP-SENS-TF-1.1	DIP-SENS-TF-0.007.1	DIP-SENS-TF-0.25.2	DIP-SENS-TF-8.3	DIP-SENS-TF-0.06.3	DIP-SENS-RB3.1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK
E	DIP-SENS-TF-0.5.1	DIP-SENS-TF-0.003.1	DIP-SENS-TF-0.12.2	DIP-SENS-TF-4.3	DIP-SENS-TF-0.03.3	DIP-SENS-RB4.1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK
F	DIP-SENS-TF-0.25.1	DIP-SENS-TF-8.2	DIP-SENS-TF-0.06.2	DIP-SENS-TF-2.3	DIP-SENS-TF-0.01.3	DIP-SENS-RB5.1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK
G	DIP-SENS-TF-0.12.1	DIP-SENS-TF-4.2	DIP-SENS-TF-0.03.2	DIP-SENS-TF-1.3	DIP-SENS-TF-0.007.3	DIP-SENS-Pos1	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK
H	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK

Figure 1: Amplification tray set-up for TF dilution series and reagent blanks

The TF samples run on the 3130*xl* were analyzed with "3130*xl* AP 1" (Table 1). All samples were checked to ensure proper allele calling. Extraneous OL calls were clicked off. Because of the results seen in this study on the 3500 genetic analyzer, only TF dilution series concentrations within 0.00390625-2.0 ng/µL were analyzed. This GeneMapper® project was utilized in determining the linear range for the DIPplex kit on the 3130*xl*. The five reagent blanks run on the 3130*xl* were analyzed with "3130*xl* AP 2" (Table 1). This GeneMapper® project was utilized in determining the analytical threshold for the DIPplex kit on the 3130*xl*.

Linearity

The TF dilution series was used to determine the linear range. The average peak heights, measured in relative fluorescence units (RFU), for TF samples per concentration were calculated along with the LOG of the RFU for each concentration in the TF dilution series. The LOG values were plotted on the Y-axis and the corresponding sample's concentration was plotted in decreasing fashion on the X-axis. A trendline was added to the graph along with the corresponding coefficient of determination, or R-squared (R^2) value. The full range of

concentrations was first plotted, and all other possible ranges were subsequently plotted on separate graphs. The results were analyzed in order to determine the linear range.

Limit of Detection

The five reagent blanks were used to determine the limit of detection (LOD), also referred to as the analytical threshold. The average peak heights were calculated for each dye color. Two graphing methods were used in order to determine the LOD. With one method, for each dye color the average peak height plus three standard deviations was plotted against the dye color. With the other method, for each dye color the maximum peak height minus the minimum peak height was multiplied by two, and this number was plotted against the dye color. These methods and results were analyzed in order to determine the analytical threshold.

Precision and Accuracy

Checkerboard patterned trays were set up separately for both genetic analyzers. The wells alternated between containing ladders and run negatives (Hi-Di Formamide and Size Standard 550 (BTO)). The tray for the eight capillary 3500 genetic analyzer contained eight ladders and eight run negatives. The tray for the sixteen capillary 3130*xl* genetic analyzer contained sixteen ladders and sixteen run negatives. The two columns on the 3500 and the four columns on the 3130*xl* were injected five times, for a total of ten injections per genetic analyzer. The trays were set up in such a way that two injections would occur in order to inject all columns filled on the tray, and the type of sample injected into each capillary alternated back and forth between ladder and run negative in order for them to be utilized in the contamination assessment.

The ladders and run negatives run on the 3500 were analyzed with “3500 AP 3” (Table 1). All run negatives were analyzed for contamination and then deleted from the project, as they were not needed for determining the precision. The ladders were checked to ensure proper allele

calling. Extraneous OL calls were clicked off. This GeneMapper® project was utilized in determining the precision for the DIPplex kit on the 3500.

The ladders and run negatives run on the 3130*xl* were analyzed with "3130*xl* AP 3" (Table 1). All run negatives were analyzed for contamination and then deleted from the project, as they are not needed for determining the precision. The ladders were checked to ensure proper allele calling. Extraneous OL calls were clicked off. This GeneMapper® project was utilized in determining the precision for the DIPplex kit on the 3130*xl*.

The sizing of the ladder peaks was compared to ensure precise sizing of each peak throughout all injections and capillaries. The standard deviation in base pair size per ladder peak was calculated and the results were analyzed to ensure that this number was less than 0.15. Variations below this threshold indicate minimal variation in sizing, and therefore precise sizing of the DIPplex kit on the respective genetic analyzer.

This study was performed a second time on the 3500 genetic analyzer. Prior to the second performance, instrument maintenance was performed. A column of ladders was set up, and this column was injected twelve times on the 3500, simulating a full tray injection. The ladders were checked to ensure accurate allele calling. The sizing of the ladder peaks was compared to ensure precise sizing of each peak throughout all injections and capillaries. The standard deviation in base pair size per ladder peak was calculated and the results were analyzed to ensure that this number was less than 0.15. The first injection sizing was also compared to the twelfth injection sizing in order to ensure that the sizing remained consistent throughout the injection of a full tray.

Repeatability and Reproducibility

The repeatability of this kit was analyzed by running the TF dilution series multiple times on the same genetic analyzer. The results of a kit are repeatable if the same results are obtained each time a sample is run on the same genetic analyzer.

The reproducibility of this kit was analyzed by running the samples involved in the validation study on both the 3500 and 3130*xl* genetic analyzers. The results of a kit are reproducible if the same results are obtained when a sample is run on multiple instruments.

Mixture Analysis

A male and female sample that were previously extracted from buccal swabs and then quantitated were selected. Both samples were diluted to a concentration of 0.1 ng/µL. These samples were used to make the following male to female ratios: 1:0, 19:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:19, and 0:1. These samples were amplified using the DIPplex kit and run on both the 3500 and 3130*xl* genetic analyzers. The mixture samples run on the 3500 were analyzed with "3500 AP 3" (Table 1). Extraneous OL calls were clicked off. This GeneMapper® project was utilized in the DIPplex mixture study on the 3500. The mixture samples run on the 3130*xl* were analyzed with "3130*xl* AP 3" (Table 1). Extraneous OL calls were clicked off. This GeneMapper® project was utilized in the DIPplex mixture study on the 3130*xl*.

The 1:0 sample was used to determine the male profile while the 0:1 sample was used to determine the female profile. These two profiles were compared in order to determine the homozygous, non-shared alleles. The peak height of the homozygous, non-shared alleles from the major contributor was divided by the peak height of the minor contributor in each mixture set in order to determine the ratio of the major contributor to that of the minor contributor. The

results were analyzed in order to determine that the DIPplex kit could recognize the presence of a major and minor contributor based on the peak heights observed.

Contamination Assessment

All negatives that were run throughout the validation were analyzed for contamination. This includes reagent blanks, amplification negatives, and run negatives. The run negatives set up in a checkerboard pattern with ladders in the reproducibility and precision studies were analyzed to ensure that the capillary was cleared of previously analyzed sample between each injection.

Known and Non-Probative Samples

Throughout the validation studies performed, all substrate types encountered in relationship testing at the MUFSC DNA Laboratory were amplified and run using the DIPplex kit. These include buccal swabs, Bode Buccal DNA Collectors™ (Bode Technology Group, Inc., Lorton, Virginia), liquid blood, blood on Whatman® FTA® cards, bone, and products of conception (POC).

Ten standard trios from past paternity proficiency tests were selected. These were all previously analyzed with an STR kit, either Promega® PowerPlex® 16 HS System (Promega, Madison, Wisconsin) or Applied Biosystems® AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies™, Foster City, CA). Five relationship cases previously analyzed and reported on at the MUFSC DNA Laboratory were also selected. These cases were adjudicated and permission to use them in the validation of DIPplex was granted by the submitting agencies. Samples were quantitated using the Qiagen® Investigator® Quantiplex HYres Kit on an Applied Biosystems® 7500 Real-Time PCR System. For each of the ten proficiency tests, the relevant previously extracted samples included DNA extracts from the mother, the child, and the alleged

father that was not excluded based on the STR results, as well as the reagent blank. The relevant previously extracted samples from non-probative cases included DNA extract from a POC for Case 1, four DNA extracts from a bone for Case 2, DNA extract from three alleged fathers (all related to the mother), a mother, and a child for Case 3, DNA extracts from two mothers and each mother's respective child for Case 4, and DNA extract from a POC that resulted in a mixed STR profile, along with the DNA extract from the mother, for Case 5. Dilutions of the DNA extracts were completed as necessary. The samples were amplified using the DIPplex kit and the DNA target was approximately 0.3 ng for each sample. An amplification positive and amplification negative were included. The amplified samples and controls were run on both the 3500 and the 3130*xl* genetic analyzers. The samples run on the 3500 were analyzed with "3500 AP 3" (Table 1). The samples run on the 3130*xl* were analyzed with "3130*xl* AP 3" (Table 1).

For the ten proficiency test trios, the combined relationship index (CRI) and the probability of paternity (POP) were calculated. All allele frequencies for the DIPplex kit were taken from a population study performed by the University of Northern Texas [7]. The Case 1 sample was analyzed in order to ensure that the DIPplex kit could produce reliable results when a POC is the substrate. Case 2 samples were analyzed to see if the DIPplex kit could provide more information for the degraded/inhibited bone samples. The CRI and POP for each alleged father was calculated in Case 3 in order to determine if the DIPplex kit could improve the statistics when used as a supplement to STR testing. A half-sibship analysis was performed for Case 4 to ensure that the DIPplex kit could be used in complex relationship cases. The Case 5 POC sample was analyzed to ensure that the DIPplex kit could identify if a mixture of related individuals was present in the sample. The resulting DIPplex profile mixture ratios of the mixed POC sample were calculated in order to compare them to the mixture ratios in their original amplification

chemistry, PowerPlex® 16 HS. The ratios for the DIPplex kit could only be calculated at locations where the mother was homozygous for one peak and the child was heterozygous.

Results- 3500

Sensitivity and Stochastic Studies- 3500

For concentrations in the TF dilution series greater than or equal to 2.00 ng/µL, there were OL calls in place of actual allele calls, but they were treated as if they were actual allele calls. The third repeat amplification of the TF dilution series with a concentration of 8.0 ng/µL was not included in the analysis because there was no defined peak shape to the called alleles and the true peaks in this sample could not be differentiated from the artifacts.

Linearity

The linear range was determined to be 0.0625 to 1.0 ng/µL (Figure 2).

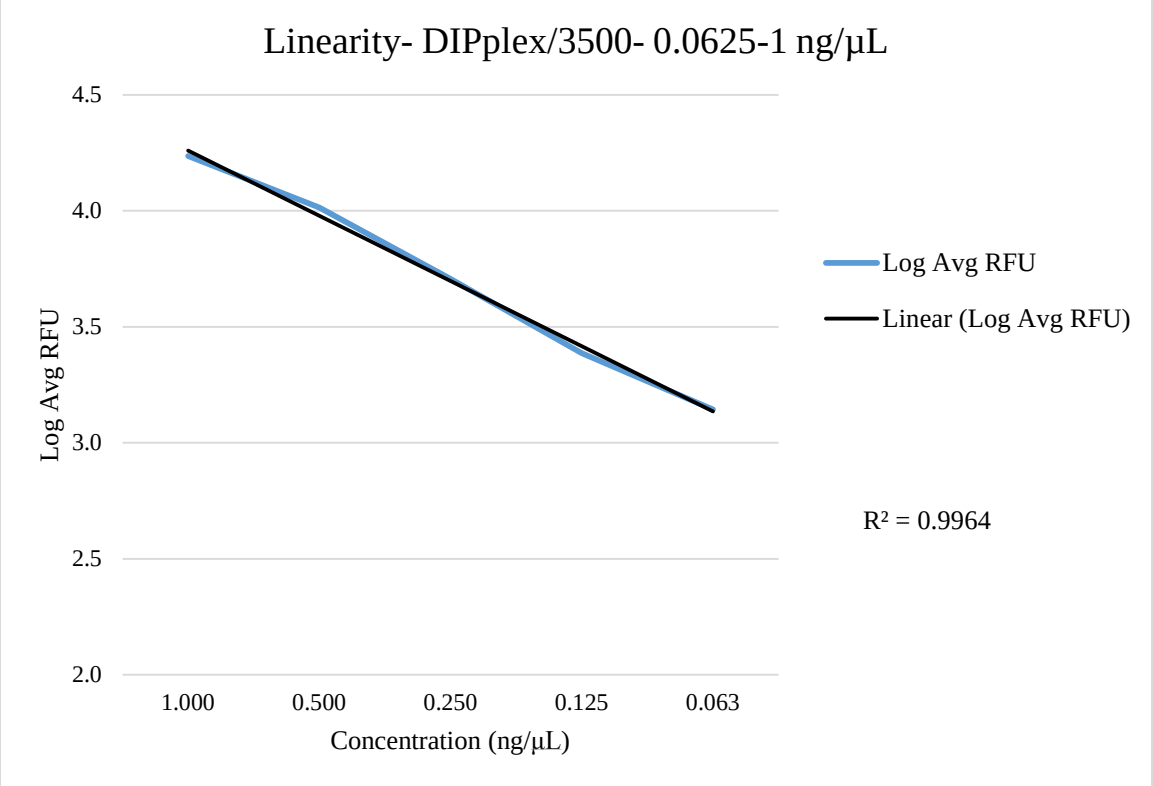

Figure 2: Linearity of DIPplex on the 3500- TF concentrations 0.0625-1 ng/µL

Limit of Detection

The results according to the Average + 3 (Standard Deviation) Method can be seen in Figure 3. The results according to the 2 (Maximum-Minimum) Method can be seen in Figure 4. The analytical threshold was set at 150 RFU.

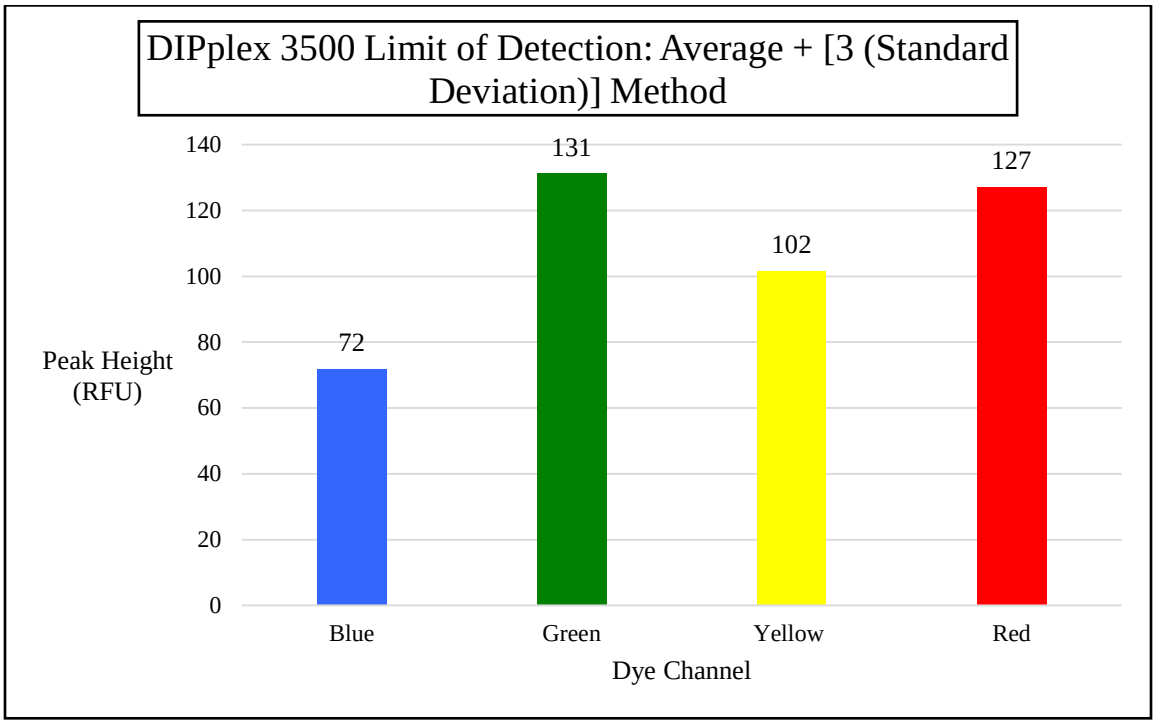

Figure 3: DIPplex 3500 Limit of Detection: Average + [3 (Standard Deviation)] Method.

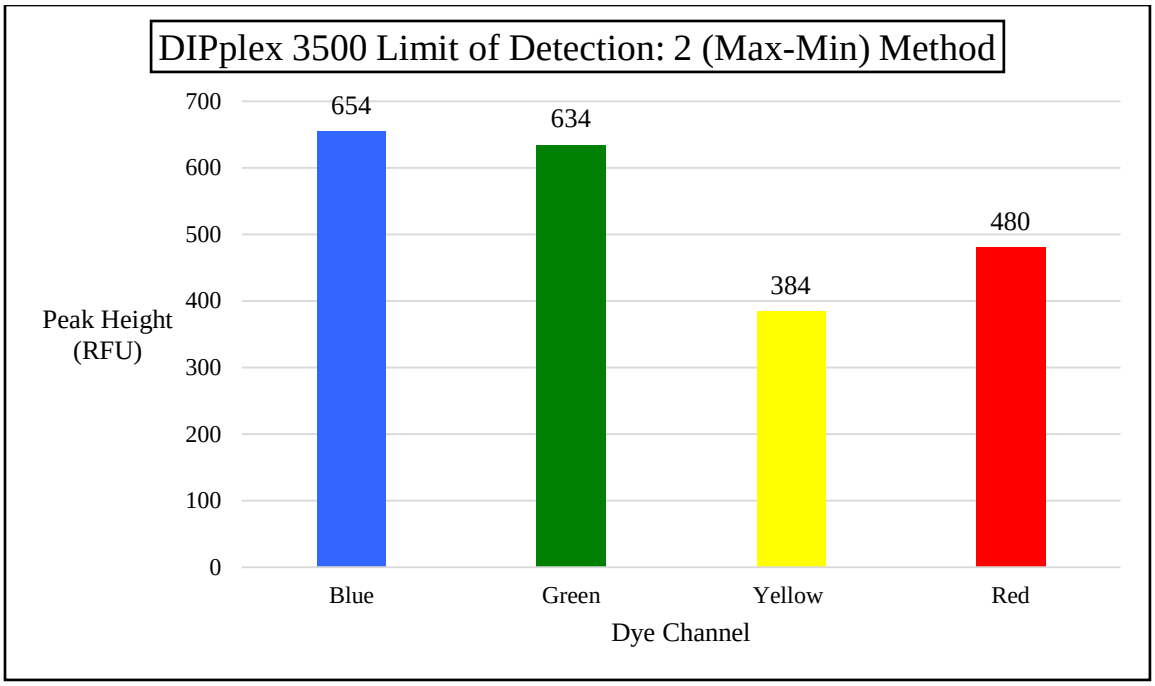

Figure 4: DIPplex 3500 Limit of Detection: 2 (Max-Min) Method

Stochastic Threshold

Using the following formula: stochastic threshold equals analytical threshold multiplied by three, the stochastic threshold was determined to be 450 RFU.

Precision and Accuracy- 3500

Out of the ten injections that were performed in this study, the first four injections had migration issues and were deleted from the project. The six remaining injections were utilized in the precision study. The ladder in the eighth capillary of the second injection utilized in this study was also deleted because of a bad injection resulting in poor resolution.

All allelic ladder peaks were called as expected. Some standard deviations in base pair size per ladder peak were not below 0.15 when the sizing was compared amongst both injections and capillaries. After the repeat of this study on the 3500, all standard deviations of base pair size per ladder peak were below 0.15 when compared amongst both injections and capillaries. The standard deviation of the base pair size per ladder peak between the first injection and twelfth

injection was also below 0.15. This was also the case when comparing all possible ranges of injections, such as the first injection compared to the twelfth injection.

Repeatability and Reproducibility- 3500

The TF profile was consistent each time it was run on the 3500 genetic analyzer. The same profiles were obtained when a sample was run on both the 3500 and 3130*xl* genetic analyzers.

Mixture Analysis- 3500

The only loci with homozygous, non-shared alleles for the male and female extracts were D77 and D56. The female profile had D77- and D56-, while the male profile had D77+ and D56+. The ratios of the peak heights for these two individuals at these two loci can be seen in Table 3 for all of the mixture ratios created. The presence of dashes (-) in the table indicates dropout of an allele and therefore the inability to calculate a ratio at that particular concentration for that sample. The resulting peak heights of the female's peaks in sample "DIP-MIX-1_19.1" indicate that there was a problem with this sample's injection.

Table 3: DIPplex 3500 Ratios of Peak Heights for the Mixture Series

Sample Name	M: F	D77- RFU	D77+ RFU	M: F	D56- RFU	D56+ RFU	M: F
DIP-MIX-19_1.1	19:1	532	22628	42.5	-	17437	-
DIP-MIX-9_1.1	9:1	510	9499	18.6	265	6981	26.3
DIP-MIX-4_1.1	4:1	4125	17040	4.1	2539	15551	6.1
DIP-MIX-1_1.1	1:1	6478	10294	1.6	3710	6773	1.8
	F: M			**F: M**			**F: M**
DIP-MIX-1_19.1	19:1	800	-	-	737	-	-
DIP-MIX-1_9.1	9:1	6508	2606	2.5	6965	980	7.1
DIP-MIX-1_4.1	4:1	7806	5651	1.4	7112	5287	1.3

Contamination Assessment- 3500

No contamination occurred in the negative controls: run negatives, reagent blanks, or amplification negatives.

Known and Non-Probative Samples- 3500

Standard Trios

None of the alleged fathers had any inconsistencies in the pattern of inheritance when the child's profile was compared to the alleged father's profile while utilizing the mother's profile. The calculated CRI and POP for the ten standard trios for the STR kit, DIPplex kit, and the two kits combined can be seen in Table 4.

Table 4: CRI and POP for Ten Standard Proficiency Test Trios

Case ID	STR CRI	STR POP	DIPplex CRI	DIPplex POP	Combined CRI	Combined POP
CAP12B	11,435,388	99.99999125522%	3,017	99.96686593081%	34,501,041,984	99.99999997710%
CAP12C	637,389,807	99.99999984311%	754	99.86758830957%	480,732,347,936	99.99999999979%
CAP11C	172,845,739	99.99999942145%	6,126	99.98367755462%	1,058,772,277,260	99.99999999991%
CAP11B	2,282,540,690	99.99999995619%	1,291	99.92259959699%	2,946,721,084,269	99.99999999997%
CAP13A	163,756,042	99.99999938934%	10,901	99.99082772421%	1,785,173,341,620	99.99999999994%
CAP13B	1,296,547,123	99.99999992287%	670	99.85090748140%	868,329,330,347	99.99999999988%
CAP14A	294,198	99.99966009346%	331	99.69906101409%	97,465,659	99.99998997400%
CAP11A	327,669,545	99.99999969481%	1,870	99.94654775669%	612,685,974,700	99.99999999984%
CAP13C	3,358,573	99.99997022546%	3,264	99.96937212746%	10,962,382,743	99.99999999088%
CAP10C	17,576,422	99.99999431056%	831	99.87986120568%	14,612,520,709	99.99999999316%

Non-probative Samples

Case 1

The Case 1 POC sample resulted in a full female profile.

Case 2

The quantitation results for Case 2 can be seen in Table 5. Two samples in Case 2, Case2_Ca and Case2_Cb, resulted in partial male profiles with the DIPplex kit. These can be seen in Figure 5 and Figure 6, respectively. For both samples, peaks that fell within bins at D67-, D67+, and D81+ were not called, despite them being above the analytical threshold. Arrows and the peak heights designate these peaks in both Figure 5 and Figure 6. Samples C2_Cc and C2_Cd had no results with the DIPplex kit. The red channel was withheld from Figure 5 and Figure 6 to aid in sample anonymity.

Table 5: Case 2 Quantitation Results

Sample Name	Quant Results (ng/µL)
Case2_Ca	0.00738ng/µL
Case2_Cb	0.00493 ng/µL
Case3_Cc	0.00000 ng/µL
Case4_Cd	0.00272 ng/µL

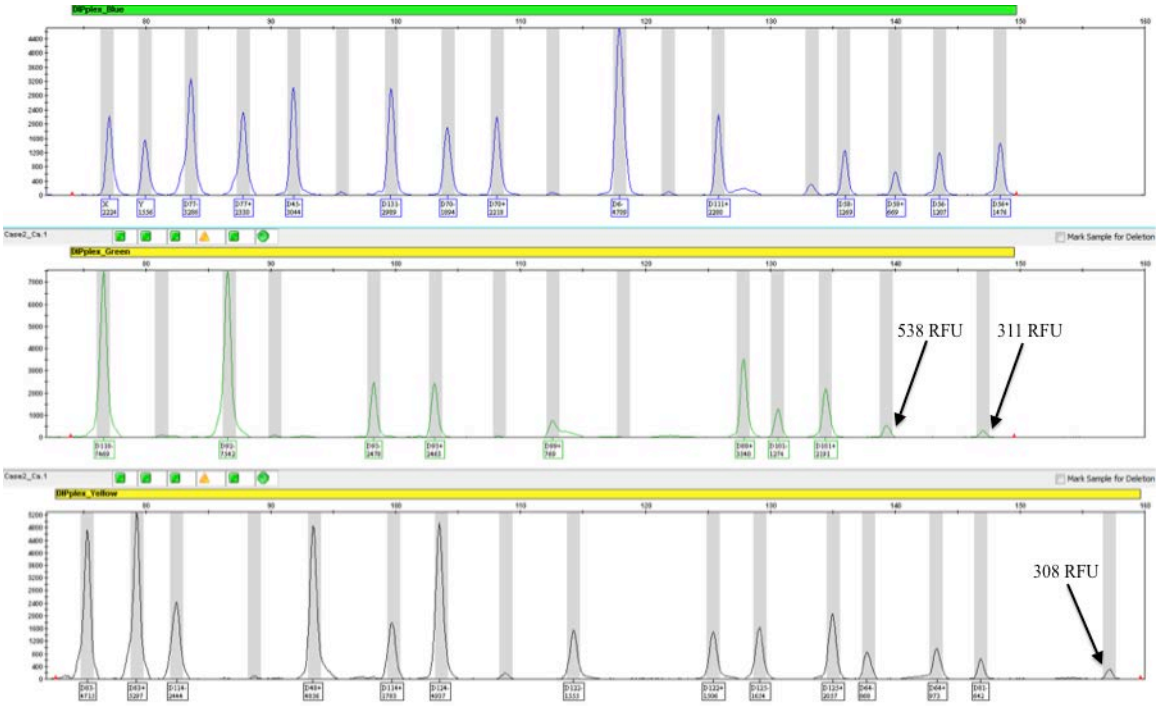

Figure 5: Case2_Ca partial profile when run in DIPplex on the 3500

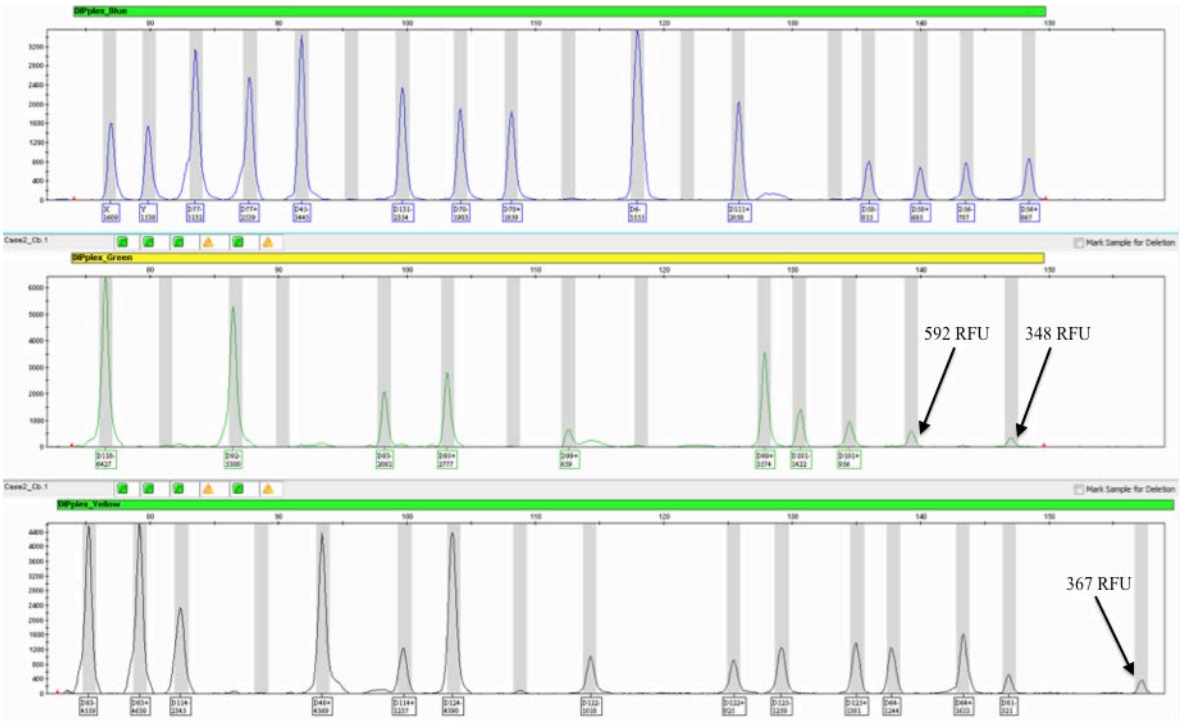

Figure 6: Case2_Cb partial profile when run with DIPplex on the 3500

Case 3

Alleged father one (AF1) had no inconsistencies in the pattern of inheritance when the child (C) was compared to AF1 while utilizing the mother (M). AF1 had a CRI of 2368.1091 and a POP of 99.9578%. Alleged father two (AF2) had two inconsistencies. Alleged father three (AF3) had three inconsistencies. The CRI and POP were not calculated for AF2 and AF3.

Case 4

The CRI for the half sibling analysis with the DIPplex results was 1707.4460. The POP was 99.9415%. These were calculated from full female profiles for both of the participants in the mother role, a full male profile for Child 1, and a full female profile for Child 2.

Case 5

The POC sample for Case 5 resulted in a mixed profile when amplified in the DIPplex kit. The imbalance of the peaks as a result of the mixture can be seen in Figure 7 at D48 and D136. The mother sample for Case 5 resulted in a full female profile.

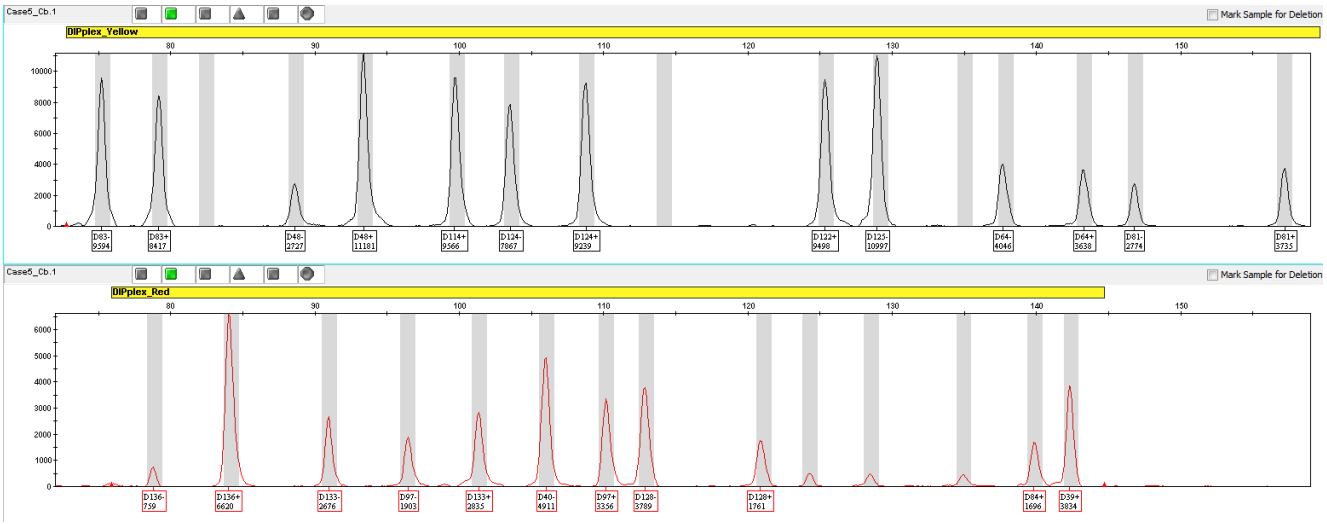

Figure 7: Case5_Cb profile when run with DIPplex on the 3500

Results- 3130*xl*

Sensitivity and Stochastic Studies- 3130xl

The second repeat amplification of the TF dilution series with a concentration of 2.0 ng/µL was not included because of poor sizing quality.

Linearity

The linear range was determined to be 0.0625 to 0.5 ng/µL (Figure 8).

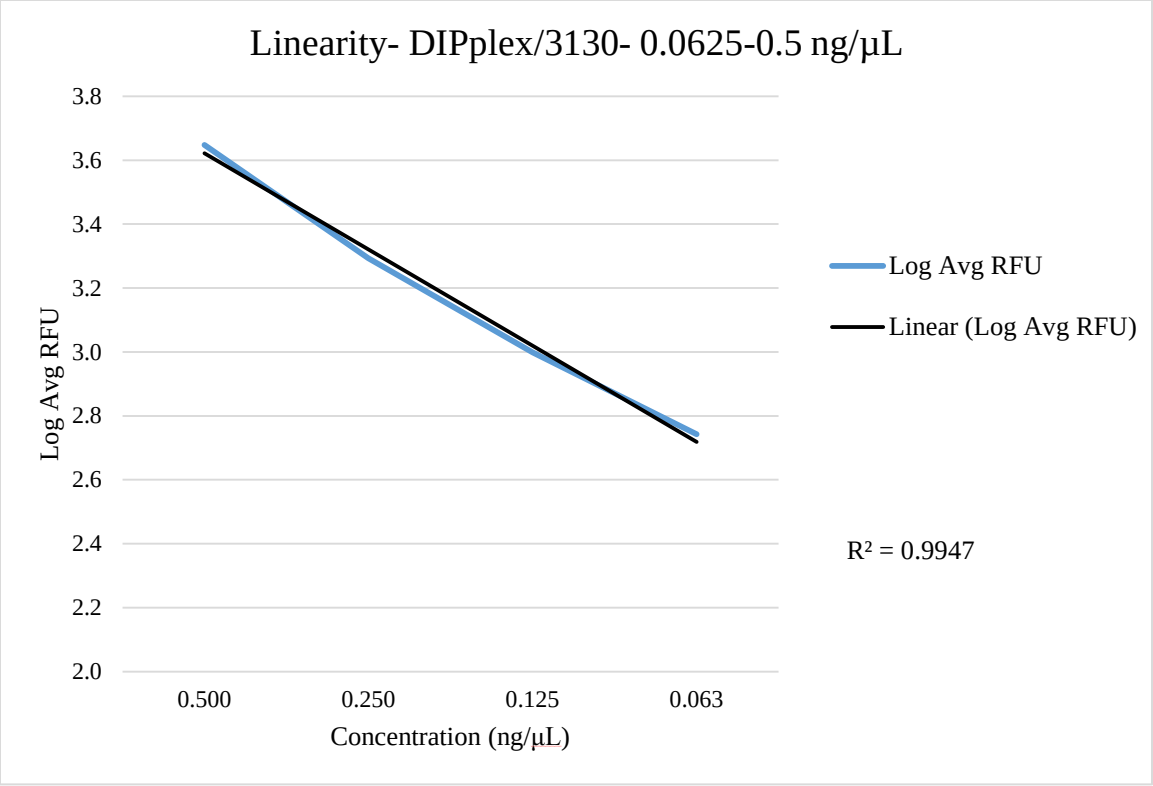

Figure 8: Linearity of DIPplex on the 3130*xl*- TF concentrations 0.0625-0.5 ng/µL

Limit of Detection

The results according to the Average + 3 (Standard Deviation) Method can be seen in Figure 9. The results according to the 2 (Maximum-Minimum) Method can be seen in Figure 10. The analytical threshold was set at 100 RFU.

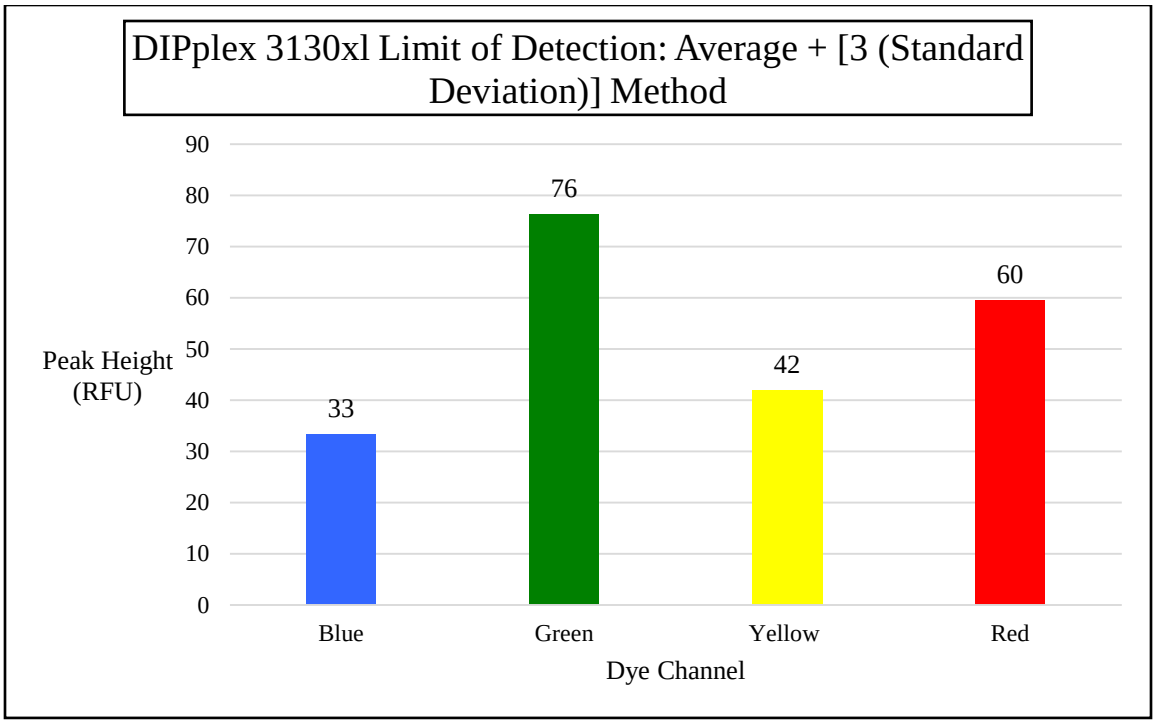

Figure 9: DIPplex 3130*xl* Limit of Detection: Average + [3 (Standard Deviation)] Method.

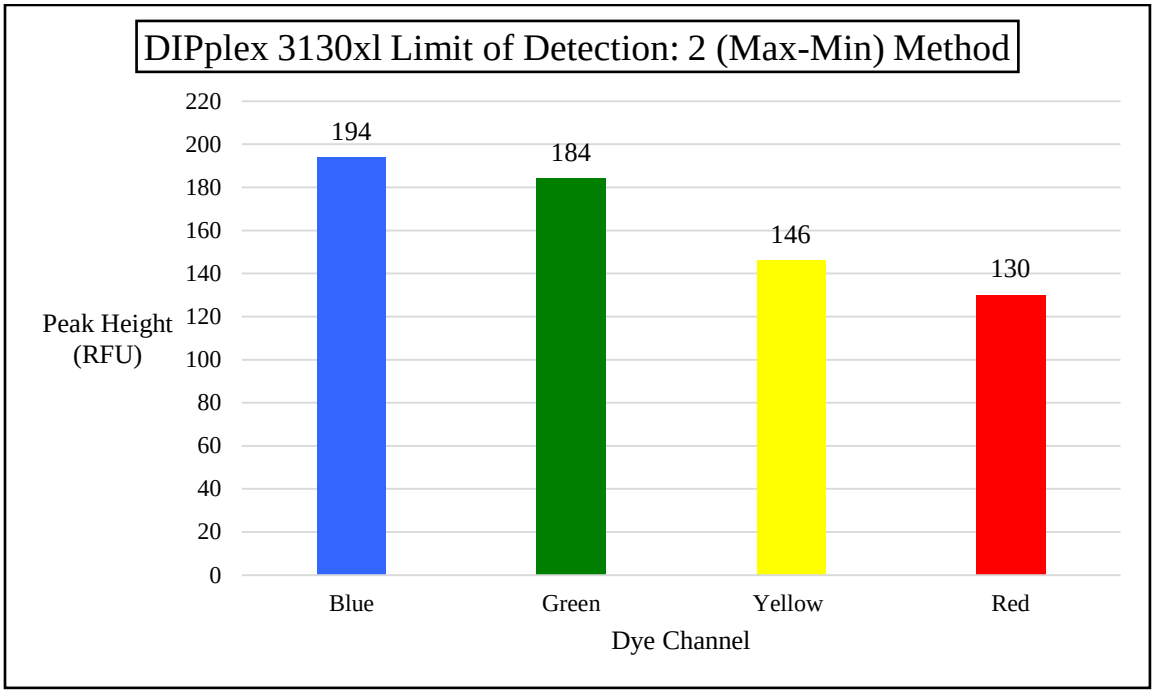

Figure 10: DIPplex 3130*xl* Limit of Detection: 2 (Max-Min) Method

Stochastic Threshold

Using the following formula: stochastic threshold equals analytical threshold multiplied by three, the stochastic threshold was determined to be 300 RFU.

Precision and Accuracy- 3130xl

Out of the ten injections that were performed in this study, the first two injections failed and were not included in the project. The eight remaining injections were utilized in the precision study.

All allelic ladder peaks were called as expected. The standard deviations of base pair size per ladder peak were below 0.15 when compared amongst both injections and capillaries.

Repeatability and Reproducibility- 3130xl

The TF profile was consistent each time it was run on the 3130*xl* genetic analyzer. The same profiles were obtained when a sample was run on both the 3500 and 3130*xl* genetic analyzers.

Mixture Analysis- 3130xl

The only loci with homozygous, non-shared alleles for the male and female extracts were D77 and D56. The female profile had D77- and D56-, while the male profile had D77+ and D56+. The ratios of the peak heights for these two individuals at these two loci can be seen in Table 6 for all of the mixture ratios created. The presence of dashes (-) in the table indicates dropout of an allele and therefore the inability to calculate a ratio at that particular concentration for that sample.

Table 6: DIPplex 3130*xl* Ratios of Peak Heights for the Mixture Series

Sample Name	M: F	D77- RFU	D77+ RFU	M: F	D56- RFU	D56+ RFU	M: F
DIP-MIX-19_1.1	19:1	100	3529	35.3	-	3155	-
DIP-MIX-9_1.1	9:1	100	3139	31.4	-	2804	-
DIP-MIX-4_1.1	4:1	702	2963	4.2	467	3056	6.5
DIP-MIX-1_1.1	1:1	945	1535	1.6	631	1184	1.8
	F: M			**F: M**			**F: M**
DIP-MIX-1_19.1	19:1	1398	-	-	1787	248	7.2
DIP-MIX-1_9.1	9:1	1052	423	2.5	1374	192	7.2
DIP-MIX-1_4.1	4:1	890	657	0.7	950	702	1.4

Contamination Assessment- 3130xl

No contamination occurred in the negative controls: run negatives, reagent blanks, or amplification negatives.

Known and Non-Probative Samples- 3130xl

Standard Trios

The 3500 data was used to calculate the paternity statistics for the ten trios. The 3130*xl* profiles for all samples involved in the ten trios were checked for concordance with those analyzed using the 3500. All profiles were concordant, except for dropout of D136- for the alleged father in case CAP14A.

Non-probative Samples

The 3500 data was used for analyzing the five cases and calculating any paternity statistics that were necessary. The 3130*xl* profiles for all samples involved in the five cases were checked for concordance with those analyzed using the 3500. All profiles for samples involved in the five cases were concordant, with the exception of six instances of dropout on the 3130*xl* and one instance of dropout on the 3500.

Discussion and Conclusion- 3500

Sensitivity and Stochastic Studies- 3500

There were OL calls in place of actual allele calls in the higher concentrations of the TF dilution series because the size standard and subsequently the sizing were not correct because of the samples' high concentrations. Due to the heights, it was obvious that the OL should be actual alleles and were treated as actual alleles.

Linearity

All R^2 values above 0.99 were considered a good trendline. The largest range with an R^2 value above 0.99 that also consistently produced full profiles was defined as the linear range. The linear range of the DIPplex kit on the 3500 was determined to be 0.0625 to 1.00 ng/µL.

Limit of Detection

According to the Average + 3 (Standard Deviation) Method, the analytical threshold could be set at approximately 70 for blue, 130 for green, 100 for yellow, and 130 for red in order to best avoid calling baseline noise as actual peaks. According to the 2 (Maximum-Minimum) Method, the analytical threshold could be set at approximately 650 for blue, 630 for green, 380 for yellow, and 480 for red in order to best avoid calling baseline noise actual peaks. Because of the large difference between the minimum and maximum peaks in the five reagent blanks, the 2 (Maximum-Minimum) Method is unreliable and therefore the Average + 3 (Standard Deviation) Method will be used. At the MUFSC DNA Laboratory, they choose to set the analytical threshold the same for all colors for convenience. They also set it slightly higher than the highest calculated threshold in order to be conservative. On the 3500, the analytical threshold for all colors was set at 150 for samples, ladders, and positive controls and 100 for negative controls and the size standard.

Stochastic Threshold

While presenting at AAFS 2011 Workshop #17, Joanne Sgueglia of the Massachusetts State Police Laboratory suggested setting the analytical threshold prior to establishing the stochastic threshold, and having the stochastic threshold satisfy two criteria: that the stochastic threshold is three-fold the analytical threshold, and that a partial profile can be obtained at 150 picograms (pg) [11]. Because these criteria were set for an STR amplification kit that has a higher target DNA input than the DIPplex kit, the ability for a partial profile to be obtained at 150 pg was not utilized as one of the criteria for setting the stochastic threshold of the DIPplex kit. The analytical threshold for the 3500 is 150 RFU, and three times that threshold results in the setting of the stochastic threshold at 450 RFU.

Precision and Accuracy- 3500

The ladders being called as expected confirmed accuracy of the DIPplex kit on 3500. During the initial setup that resulted in analysis of three columns of ladders, the standard deviations of the allelic ladder peaks' sizing indicated that the kit was not precise. Troubleshooting of this issue resulted in the conclusion that performing regular instrument maintenance of the genetic analyzer, as suggested by the manufacturer, would provide a better environment to get the expected and desired results. After the repeat of this study, the standard deviations fell below 0.15 and confirmed precision of the DIPplex kit on the 3500. The first and twelfth injection, as well as the comparisons of injections in between, fell below the 0.15 standard deviation threshold, which means that one ladder could be used to type an entire tray of samples. Applied Biosystems® recommends one ladder be used per every twenty-four samples [9].

Repeatability and Reproducibility-3500

The results when using the DIPplex kit were determined to be repeatable and reproducible. They are repeatable since the same profile was obtained each time it was run on the 3500. They are reproducible since the same profiles were obtained when a sample was run on both the 3500 and 3130*xl*.

Mixture Analysis- 3500

The DIPplex kit being biallelic resulted in only two loci that could be analyzed for the mixture study. When the peak heights of the major contributor were compared to the peak heights of the minor contributor, the major contributor's were always larger. When the female is the major contributor, the female's peak heights should be around the same RFU. In sample "DIP-MIX-1_19.1", this is not the case. This was determined to be an injection issue. This tray was not re-setup in order to obtain better results for this sample because it was not necessary for the study. The resulting ratios of the major and minor contributors' peak heights indicate that the DIPplex kit is able to detect the presence of a mixture, which was the main goal of this study since this kit will primarily be utilized for single-source profiles and the ability to recognize contamination is necessary.

Contamination Assessment- 3500

The DIPplex kit has no issues with contamination or clearing the capillary of DNA between injections when run on the 3500. While no contamination occurred, consistent baseline artifacts existed in all reagent blanks and amplification negatives, but not in the run negatives. A typical reagent blank can be seen in Figure 11 and the red channel and its artifacts for four separate reagent blanks can be seen in Figure 12. As evidenced by Figure 12, the artifacts in the red channel were seen consistently throughout all reagent blanks. This was the case for all color

channels. Qiagen® was contacted for technical assistance regarding this issue. According to technical support, artifact peaks seen in all reagent blanks and amplification negatives are not amplification products, but dye blobs caused by the fluorescent dyes. The DIPplex kit uses the technology and chemistry of the first generation Qiagen® STR kits that may lead to background noise.

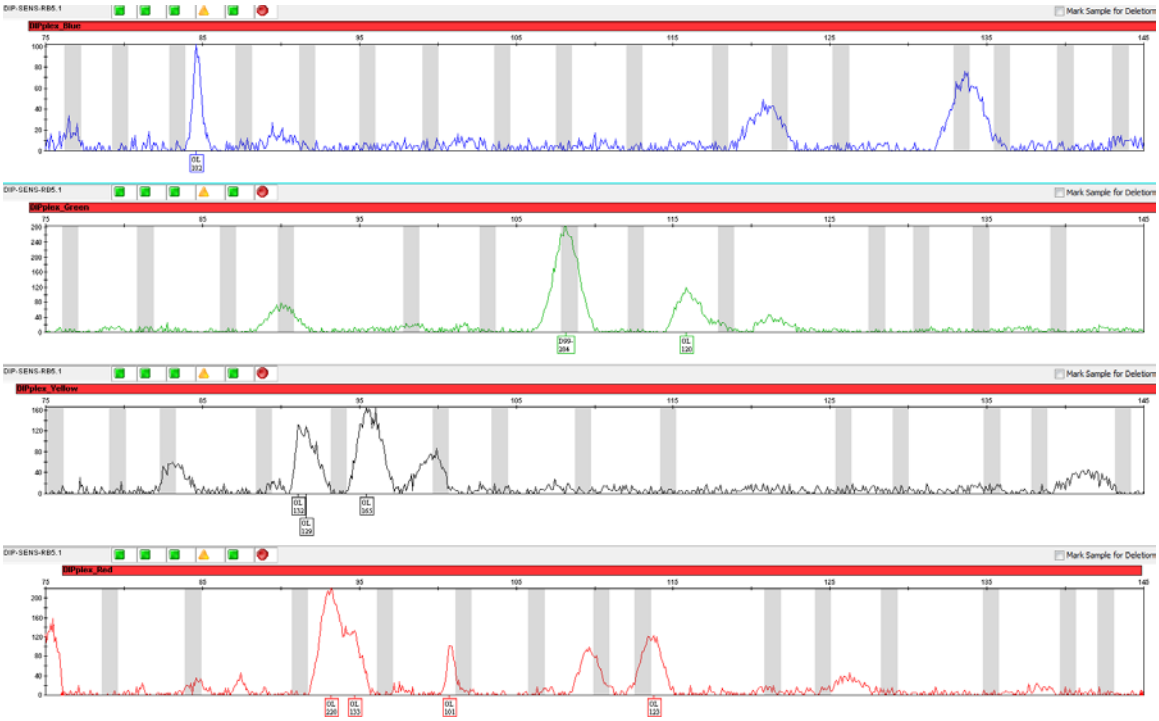

Figure 11: DIPplex 3500 Reagent Blank Artifacts

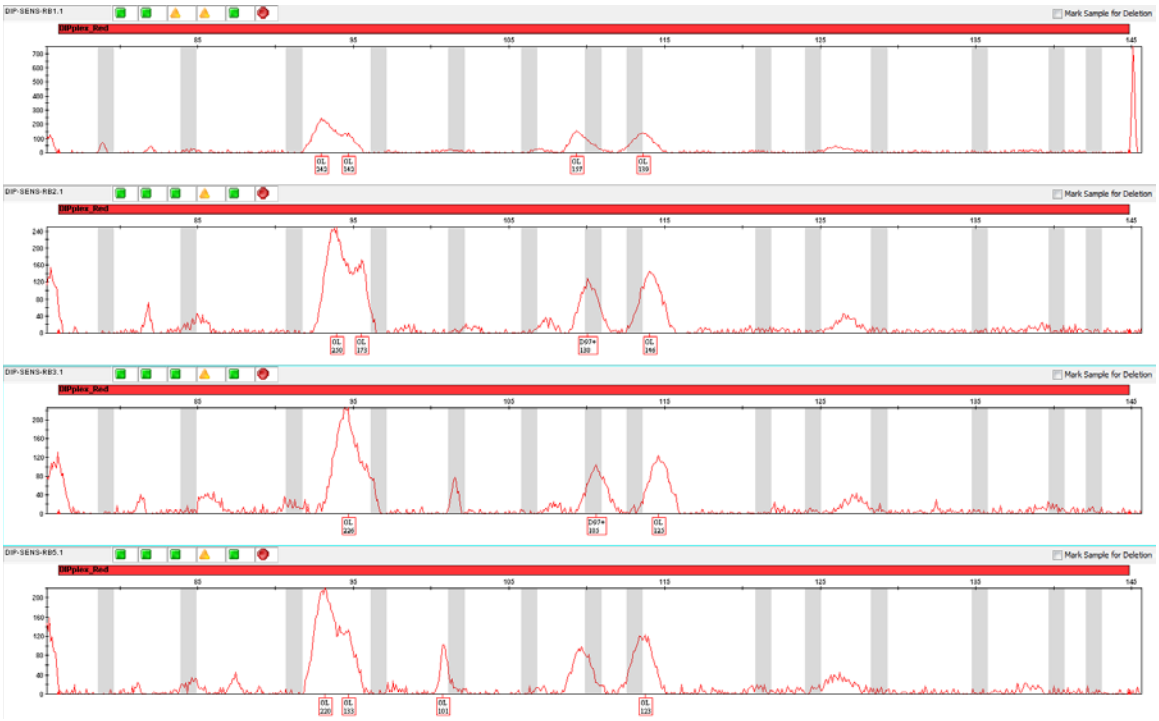

Figure 12: DIPplex 3500 Artifacts in the Red Channel of Four Reagent Blanks

Known and Non-Probative Samples- 3500

Substrate Types

The DIPplex kit is capable of amplifying and producing reliable capillary electrophoresis results for all substrate types used in paternity testing at the MUFSC DNA Laboratory including buccal swabs, Bode Buccal DNA Collectors™, liquid blood, blood on Whatman® FTA® cards, bone, and POC.

Standard Trios

The addition of the CRI calculated from the DIPplex data to the CRI calculated from the STR data increases the CRI significantly. The POP does not change a significant amount since it was already significant. This is because these cases are all proficiency test standard trios with no mutations or abnormalities and already have a POP at the highest reportable percentage, 99.9999%. In a case with a lower STR CRI, combining the DIPplex CRI with the STR CRI could significantly increase the overall CRI and POP.

Non-Probative Samples

Case 1

Case 1 involved one extraction of a POC with identifiable parts from a now adjudicated criminal paternity case. This sample, Case1_C, was previously analyzed with PowerPlex® 16 HS and resulted in a full profile. This case was chosen in order to ensure that the DIPplex kit could produce reliable results when a POC is the substrate type, and it was determined that it could, considering the DIPplex analysis resulted in a full profile.

Case 2

Case 2 involved four extractions of a bone sample in a body identification case. This case was previously analyzed with PowerPlex® 16 HS. With the STR kit, samples Case2_Ca and Case2_Cb resulted in partial profiles (Figure 13 and Figure 14). Samples Case2_Cc and Case2_Cd produced no results with PowerPlex® 16 HS. This case was chosen to see if the DIPplex kit could provide more information for the degraded/ inhibited bone samples. As evidenced by the DIPplex electropherograms of Case2_Ca and Case2_Cb (Figure 5 and Figure 6) compared to the STR electropherograms (Figure 13 and Figure 14), the DIPplex kit was able to provide more information despite the bone sample being degraded/inhibited.

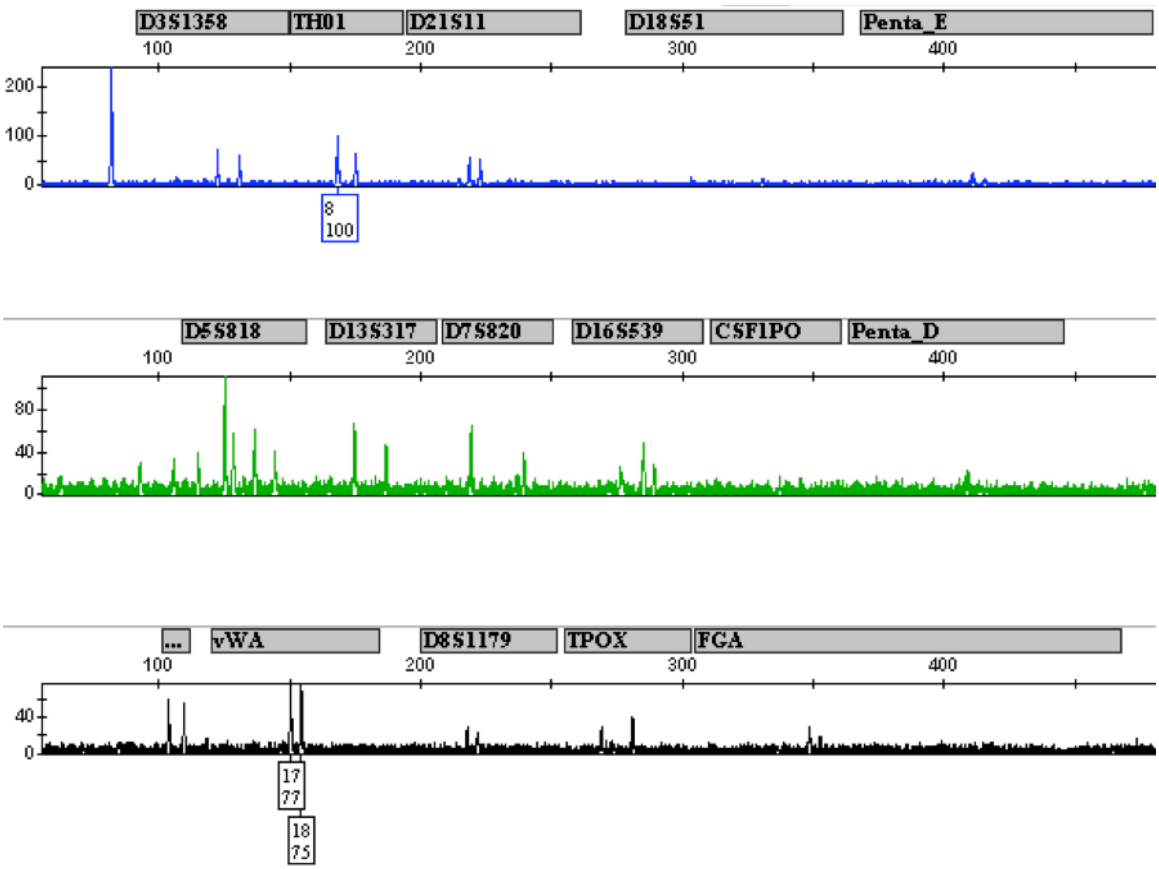

Figure 13: Case2_Ca partial STR profile

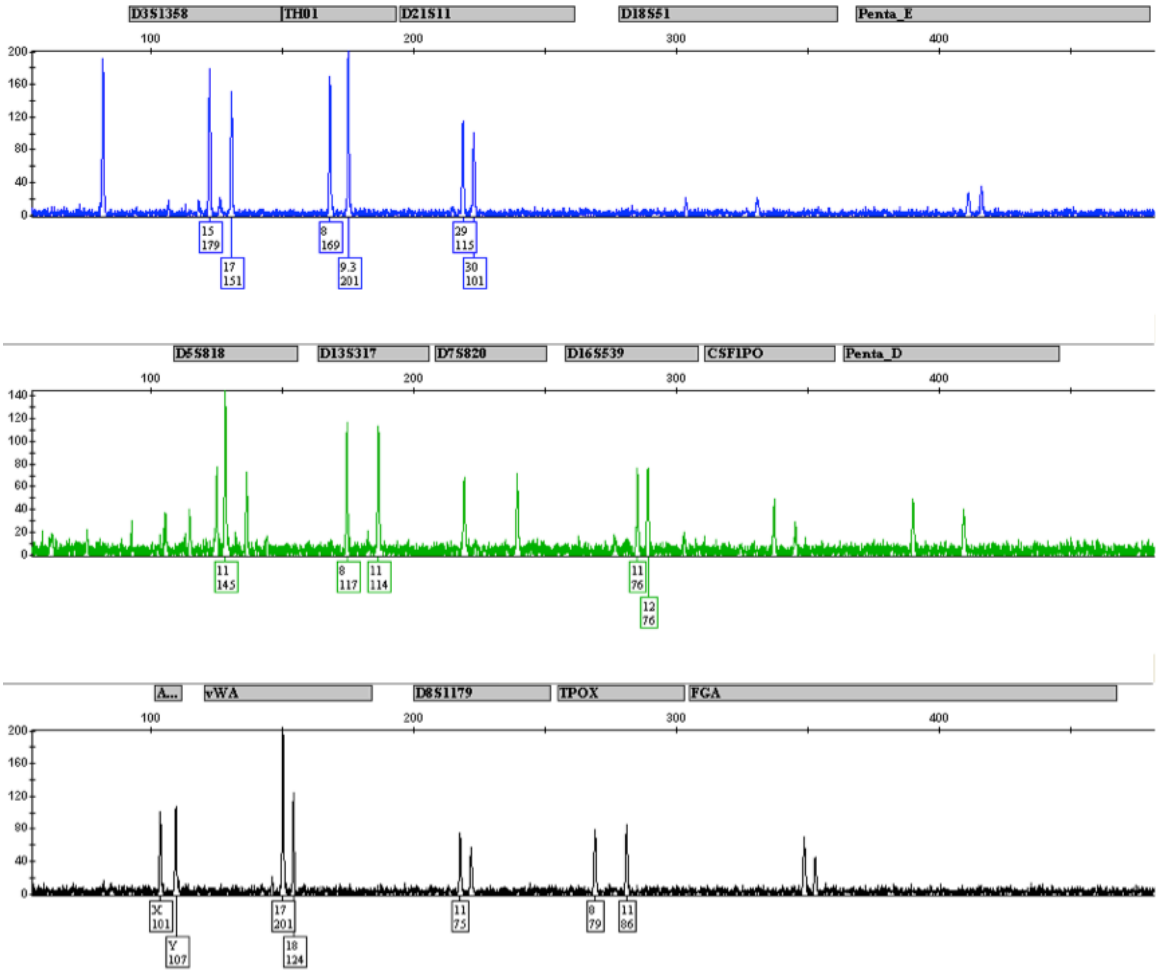

Figure 14: Case2_Cb partial STR profile

As shown in Figure 5 and Figure 6, the cause of the issue with the DIPplex profiles having peaks within bins and above analytical threshold that were not called has not yet been determined. The analysis parameters were adjusted in an attempt to have the software recognize the peaks as actual allele peaks. Qiagen® was contacted and their suggestions were completed. The issue still remains and is in the process of being resolved.

Case 3

Case 3 involved three alleged fathers (AF1, AF2, AF3), a mother (M), and a child (C) in a criminal paternity case involving suspected rape and incest. This case was previously analyzed with Identifiler® Plus. The three alleged fathers were all related to the victim. AF1 was the

brother of M and had no inconsistencies and therefore could not be excluded as the father of C. Three or more inconsistencies are required for exclusion. The CRI was 5,948,000 and the POP was 99.9999%. AF2 was the father of M and had two inconsistencies in Identifiler® Plus and therefore could not be excluded as the father of C. The CRI was 0.1107 and the POP was 9.9702%. AF3 was the brother of M and had four inconsistencies and was therefore excluded as the father of C. This case was chosen to see if the DIPplex kit could provide more information and improve the paternity statistics and results for this case. The addition of the DIPplex information increased the AF1 CRI to over 14 billion and added two additional inconsistencies for AF2 for a total four inconsistencies, which excludes AF2 as the father. AF3 was already excluded as the father with the STR results alone, but even with the DIPplex results alone, he had three inconsistencies and was therefore excluded as the father. Using the DIPplex kit as a supplement to STR testing in cases involving incest and related alleged fathers can provide enough information to improve statistics and the overall results by excluding additional alleged fathers.

Case 4

Case 4 questioned whether two individuals share a father. Samples were provided for the two individuals in question along with both of their biological mothers. This case was previously analyzed with PowerPlex® 16 HS. It was known that child one (C1) was the child of the AF of child two (C2). The mother of C1, mother one (M1), and the mother of C2, mother two (M2), were analyzed along with the children to determine whether or not the AF could be the father of C2. The STR kit resulted in a half-sibship CRI of 38.9024 and a POP of 97.4939%. This was evidence of an indication of half-sibship.

This case was selected in order to ensure that the DIPplex kit could be used in complex relationship cases such as half sibling identification, and it was determined that it could be used. The half-sibling statistics for the DIPplex kit were of greater significance than the STR statistics. The DIPplex CRI in this case was 1707.4460 while the STR CRI was only 38.9024. The STR statistics state that there is a 97.4939% probability that C1 and C2 are half siblings, while the DIPplex statistics state that there is a 99.9415% chance that C1 and C2 are half siblings. The combined CRI for both kits is 66,423.7572 and the POP is 99.9985%.

Case 5

Case 5 involved one extraction of a POC with no identifiable parts and the DNA extract from the mother in a criminal paternity case. The POC sample, Case5_Cb, was previously analyzed with PowerPlex® 16 HS and resulted in a mixed profile. The mixture ratios, with the child being the major contributor, had an average of 3.04:1 and ranged from 1.75:1 to 4.81:1. The mixture ratios, with the child being the major contributor, had an average of 4.97:1 and a range of 1.93:1 to 11.73:1. This case was chosen in order to ensure that the DIPplex kit could identify if a mixture was present. This was achieved, and the conclusion of which DNA profile was the major component was the same whether looking at the DIPplex or STR results. The average ratios of the peak heights for the mixtures were comparable for both kits as well.

Discussion and Conclusion- 3130*xl*

Sensitivity and Stochastic Studies- 3130xl

Linearity

All R^2 values above 0.99 were considered a good trendline. The largest range with an R^2 value above 0.99 that also consistently produced full profiles was defined as the linear range. The linear range was determined to be 0.0625 to 0.5 ng/µL.

Limit of Detection

According to the Average + 3 (Standard Deviation) Method, the analytical threshold could be set at approximately 35 for blue, 75 for green, 40 for yellow, and 60 for red in order to best avoid calling baseline noise as actual peaks. According to the 2 (Maximum-Minimum) Method, the analytical threshold could be set at approximately 190 for blue, 180 for green, 150 for yellow, and 130 for red in order to best avoid calling baseline noise actual peaks. Because of the large difference between the minimum and maximum peaks in the five reagent blanks, the 2 (Maximum-Minimum) Method is unreliable and therefore the Average + 3 (Standard Deviation) Method will be used. At the MUFSC DNA Laboratory, they choose to set the analytical threshold the same for all colors for convenience. They also set it slightly higher than the highest calculated threshold in order to be conservative. The analytical threshold for all colors was set at 100 for samples, ladders, and positive controls, and 50 for negative controls and the size standard.

Stochastic Threshold

Following the methodology discussed in the "Stochastic Threshold" section under "Discussion and conclusion- 3500", the analytical threshold for the 3130*xl* is 100 RFU, and three times that threshold results in the setting of the stochastic threshold at 300 RFU.

Precision and Accuracy- 3130xl

The ladders being called as expected confirm accuracy of the DIPplex kit on 3130*xl* while the standard deviations falling below 0.15 confirms precision.

Repeatability and Reproducibility- 3130xl

The results when using the DIPplex kit were determined to be repeatable and reproducible. They are repeatable since the same profile was obtained each time it was run on the

3130xl. They are reproducible since the same profiles were obtained when a sample was run on both the 3500 and 3130*xl*.

Mixture Analysis- 3130xl

The DIPplex kit being biallelic resulted in only two loci that could be analyzed for the mixture study. When the peak heights of the major contributor were compared to the peak heights of the minor contributor, the major contributor's were always larger. The resulting ratios of the major and minor contributors' peak heights indicate that the DIPplex kit is able to detect the presence of a mixture, which was the main goal of this study since this kit will primarily be utilized for single-source profiles and the ability to recognize contamination is necessary.

Contamination Assessment- 3130xl

The DIPplex kit has no issues with contamination or clearing the capillary of DNA between injections when run on the 3130*xl*. While no contamination occurred, consistent baseline artifacts existed in all reagent blanks and amplification negatives, but not in the run negatives. A typical reagent blank can be seen in Figure 15 and the red channel and its artifacts for four separate reagent blanks can be seen in Figure 16. As evidenced by Figure 16, the artifacts in the red channel were seen consistently throughout all reagent blanks. This was the case for all color channels. As mentioned in the "Contamination Assessment- 3500" section under "Discussion and Conclusions- 3500", Qiagen® was contacted for technical assistance regarding this issue. According to technical support, artifact peaks seen in all reagent blanks and amplification negatives are not amplification products, but dye blobs caused by the fluorescent dyes. The DIPplex kit uses the technology and chemistry of the first generation Qiagen® STR kits that may lead to background noise.

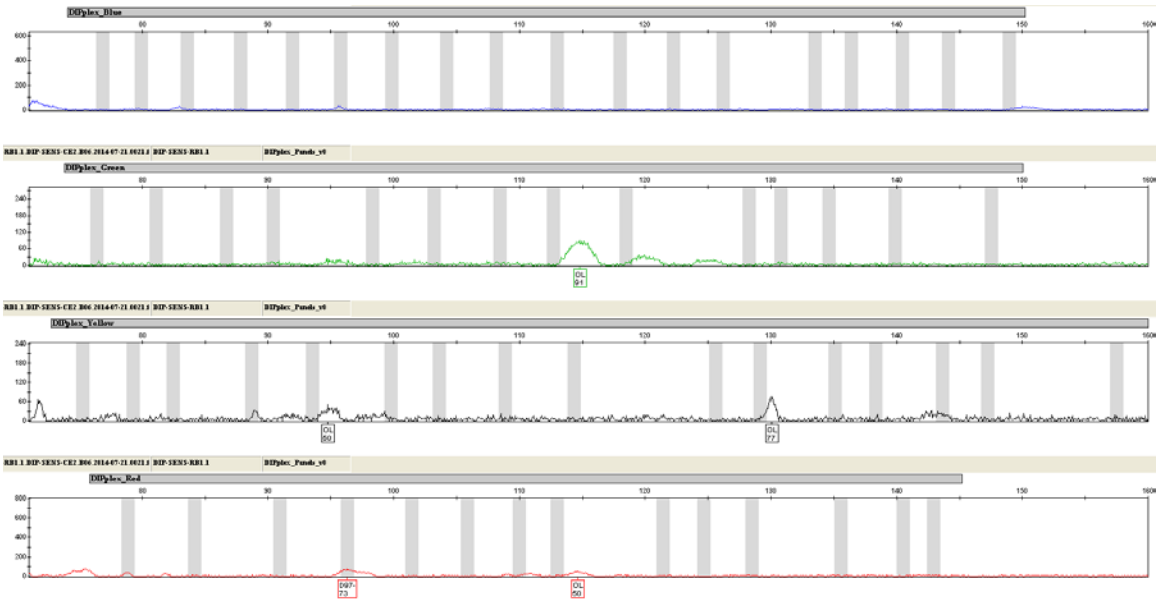

Figure 15: DIPplex 3130*xl* Reagent Blank Artifacts

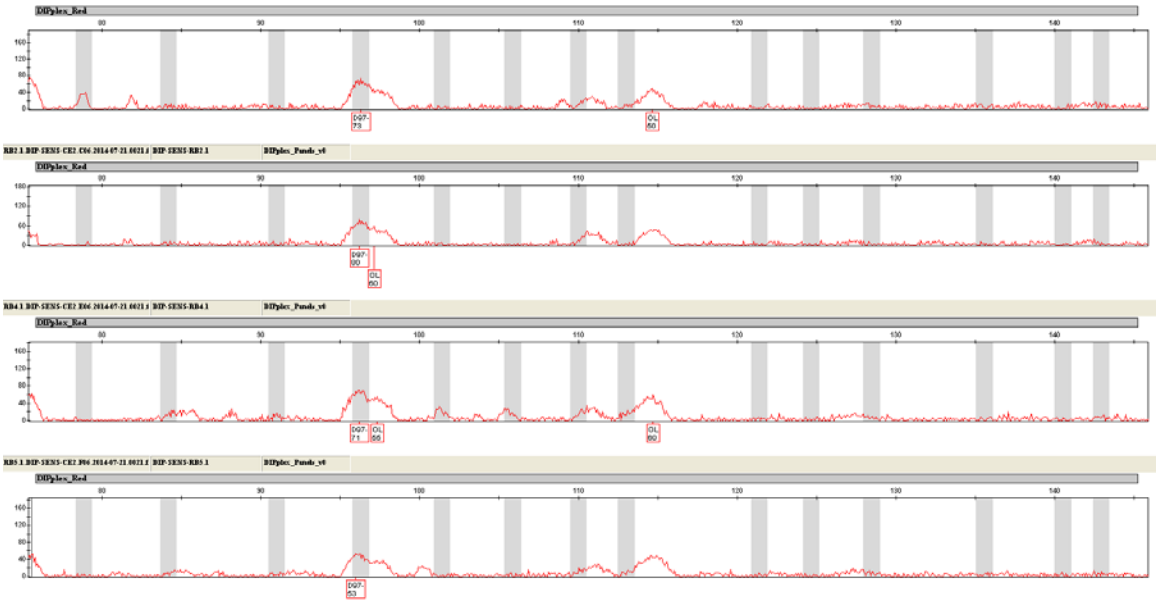

Figure 16: DIPplex 3130*xl* Artifacts in the Red Channel of Four Reagent Blanks

<u>Known and Non-Probative Samples- 3130xl</u>

<u>Substrate Types</u>

See the “Substrate Types” section under “Discussion and Conclusion- 3500”.

<u>Standard Trios</u>

See the “Standard Trios” section under “Discussion and Conclusion- 3500”.

Non-Probative Samples

See the "Non-Probative Samples" section under "Discussion and Conclusion- 3500".

Final Conclusions

According to the University of North Texas and their population research using DIPplex, there were no significant departures from Hardy-Weinberg equilibrium or significant linkage disequilibrium between the markers [7]. Because of this, the CRI for an STR kit can be combined with the CRI from the DIPplex kit, although it has been found that there is linkage between some markers. The STR marker D22S1045 and the DIPplex indel rs16363 (D136) are in very close linkage and the statistics for them should not be combined. D22S1045 is part of the European Standard Set of DNA loci, and is not present in any of the STR kits that were used on the samples in the DIPplex validation studies. Two indels, rs16363 (D136) and rs6481 (D124), in the DIPplex kit are slightly linked [5]. The linkage is minimal and therefore the statistics for both loci can be used for calculating the CRI [7].

The DIPplex kit was validated on the 3500 and *3130xl* genetic analyzers based on the successful completion of the following internal validation studies: sensitivity and stochastic studies, precision and accuracy, repeatability and reproducibility, mixture analysis, contamination assessment, and known and non-probative samples. As hypothesized, the use of the DIPplex kit as a supplement to STR testing can provide more information in cases with degraded/inhibited samples and can improve the statistics of complex relationship cases.

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