Validation and Comparison of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit to Identifiler®, MiniFiler™, and Yfiler® for the Pinellas County Forensic Laboratory in Largo, Florida

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ABSTRACT

An internal validation of Identifiler® Plus was performed for the Pinellas County Forensic Laboratory in Largo, Florida. The Identifiler® Plus amplification chemistry from Applied Biosystems® was selected for this study because of its reportedly improved master mix.

Seven validation studies were performed, including accuracy, precision, recovery, linearity and range, mixture, carryover, and ruggedness. Additionally, the limit of detection and sensitivity samples were run on two different genetic analyzers to ascertain any discrepancies between the instruments.

The Identifiler® Plus samples displayed greater sensitivity than Identifiler® and were comparative to MiniFiler™ and Yfiler®. Results of the precision study fell within manufacturer recommendations. Samples demonstrated 100% accuracy and no contamination was present in any of the negative controls used in the validation. Samples run at different times and on different thermal cyclers were consistent with expected results and with each other. The samples run on the 3130xl genetic analyzer displayed greater sensitivity than samples run on the 3130 genetic analyzer, but limit of detection stayed the same for both instruments.

Future studies could be completed to further compare the Identifiler® Plus amplification chemistry to the Identifiler®, MiniFiler™, and Yfiler® chemistries, such as running samples on the Applied Biosystems® 3500 genetic analyzer.

INTRODUCTION

The Pinellas County Forensic Laboratory is proposing to change their Standard Operating Procedures (SOPs) to diminish the use of the Applied Biosystems® AmpFℓSTR® MiniFiler™ Amplification Kit (Life Technologies™, Foster City, California) in favor of the Applied Biosystems® AmpFℓSTR® Identifiler® Plus Amplification Kit (ID+) (Life Technologies™, Foster

City, California) for forensic DNA analysis applications. The major advantage of this system is the reduction in analyst time and sample cost. The ID+ kit includes the thirteen core Combined DNA Index System (CODIS) loci as well as two additional loci, D2S1338 and D19S433, in a 5-dye configuration. This is an improvement in comparison to the MiniFiler™ kit, which only contains 9 loci (D13S317, D7S820, Amelogenin, D2S1338, D21S11, D16S539, D18S51, CSF1PO, and FGA) (Mulero et al. 2008). The amplification chemistry combines the Identifiler® Plus Master Mix with the AmpliTaq Gold® DNA Polymerase in a single pre-mixed tube to ensure the proper ratio for optimal polymerase performance. By employing the Identifiler® Plus Amplification Kit, the Pinellas County DNA laboratory will improve turnaround time, cut down on consumable usage, and save approximately one hour per case from the amplification step alone. There will be an overall time reduction in the thermal cycler protocol which has been optimized for a faster throughput (Wang et al. 2012). Additional analyst time will also be saved at run setup.

The ID+ chemistry has undergone extensive validation studies by Applied Biosystems® and has been in use in many forensic laboratories throughout the United States since the United States Federal Bureau of Investigation's approval of this amplification kit in 2010 (Life Technologies™, 2010). The Pinellas County Forensic Laboratory conducted an internal validation study to demonstrate that the provided methods and procedures performed as expected in the DNA laboratory as specified by the FBI Scientific Working Group for DNA Analysis Methods (SWGDAM) guidelines (Federal Bureau of Investigation, 2009). Seven validation studies were performed, including accuracy, precision, recovery, linearity and range, mixture, carryover, and ruggedness. A comparison study was also performed to compare the Identifiler® Plus chemistry to the Identifiler®, MiniFiler™, and Yfiler® chemistries.

MATERIALS AND METHODS

Extraction of DNA: All extractions were performed using the Applied Biosystems® Automate Express™ and the Applied Biosystems® PrepFiler® Express Extraction Kit in accordance with manufacturer's instructions.

Quantitation of DNA: All samples in this study were quantified using the Applied Biosystems® Quantifiler® Duo DNA Quantification Kit according to laboratory protocols.

STR Amplification of DNA: DNA samples were amplified on a GeneAmp® PCR System 9700 Thermal Cycler using a target of 0.5 ng and the Identifiler® and ID+ amplification kits.

Amplification Procedure: The extracted DNA was amplified in 0.2 mL amplification-grade sample tubes using the Identifiler® and Identifiler® Plus PCR Amplification kits. Target concentrations, an amplification blank, and an amplification positive (female cell line 9947A DNA included in the kit) were each amplified in triplicate. The following concentrations were used for the reaction mix per well: 10 µL AmpFℓSTR® ID+ Master Mix and 5 µL AmpFℓSTR® ID+ Primer Set. The tubes were amplified on a 9700 thermal cycler for 28 or 29 cycles with a total volume of 25 µL per well (Applied Biosystems, 03/2012, User Guide).

Capillary Electrophoresis: The amplified product from each sample was injected on the Applied Biosystems® 3130 Genetic Analyzer (SN 19341-005) or the Applied Biosystems® 3130xl Genetic Analyzer (SN 22285-030) using the default injection parameters (3 kV for 5 seconds).

Data Analysis: Data was collected using Data Collection Software version 3.0 and analyzed using GeneMapper® *ID-X* Software version 1.3. The peak amplitude threshold (analysis threshold) value was determined in the Limit of Detection section using a peak amplitude

threshold value of 1 relative fluorescence unit (RFU). The analysis threshold determined from the limit of quantitation (LOQ) study was then used in all other sections.

Accuracy

The DNA results obtained were compared to the DNA results where there is a known, common donor, such as a known positive control (See shaded "+C" blocks of Figures 1, 2, and 3), and the samples from the sensitivity study detailed under the "Linearity" section (See shaded "Sensitivity Study" blocks of Figures 1 and 2). Data from the 28 and 29 cycle amplifications were compared to each other and to the sensitivity data from the Identifiler® Verification (2011).

Non-probative samples were created that are representative of commonly encountered casework samples. These samples were amplified using both the Identifiler® Plus and Identifiler® kits (See shaded "Non-Probative" blocks of Figure 3). A direct comparison of the non-probative samples amplified using the Identifiler® Plus kit at 28 and 29 cycles, as well as the Identifiler® kit was conducted.

Mixture samples using control DNA 007 (male) and 9947A (female) were made according to Table 1. Tubes of 007 from expired MiniFiler™ kits and 9947A from expired Identifiler® kits were added together respectively, and a 1:1 dilution was created for each control. The control DNA was diluted to ~0.1 ng/µl and 60 µl stocks were made targeting the ratios listed in Table 1. The samples were amplified using the Identifiler® Plus kit at both 28 and 29 cycles. Data from the 28 and 29 cycle amplifications were compared to each other and to the mixture data from the Identifiler® Verification (2012).

The amount of DNA targeted for amplification for the Grid for Accuracy (Non-probative) and Grid for Accuracy (Mixture) samples were determined using the Sensitivity Study (See "Linearity"). The plates were then placed on the Applied Biosystems 3130xl Genetic Analyzer

(serial # 22285-030) and the samples were injected. The data was collected and analyzed in accordance with laboratory protocols.

Table 1: Mixture Sample Preparation		
Mixture Ratio Male:Female (M:F)	007 DNA (Male) (µl)	9947A DNA (Female) (µl)
19:1	57	3
9:1	54	6
6:1	51	9
4:1	48	12
2:1	40	20
1:1	30	30
1:0	60	0
0:1	0	60
1:2	20	40
1:4	12	48
1:6	9	51
1:9	6	54
1:19	3	57

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	B	B	B	B
B	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #3	B	B	B	B
C	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #3	B	B	B	B
D	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #3	B	B	B	B
E	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	F	B	B	B	B
F	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #2	0.125ng (Sensitivity Study)	F	B	B	B	B
G	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #2	0.063ng (Sensitivity Study)	F	B	B	B	B
H	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #2	0.031ng (Sensitivity Study)	F	B	B	B	B

Figure 1: Grid for Accuracy (28 Cycles)
*B indicates blank well, F indicates Formamide

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	B	B	B	B
B	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #3	B	B	B	B
C	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #3	B	B	B	B
D	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #3	B	B	B	B
E	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	Ladder	0.016ng (Sensitivity Study)	0.25ng (Sensitivity Study	F	B	B	B	B
F	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #1	0.125ng (Sensitivity Study)	2.5ng (Sensitivity Study)	+C Thermal Cycler #2	0.125ng (Sensitivity Study)	F	B	B	B	B
G	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #1	0.063ng (Sensitivity Study)	1.25ng (Sensitivity Study)	+C Thermal Cycler #2	0.063ng (Sensitivity Study)	F	B	B	B	B
H	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #1	0.031ng (Sensitivity Study)	0.5ng (Sensitivity Study)	-C Thermal Cycler #2	0.031ng (Sensitivity Study)	F	B	B	B	B

Figure 2: Grid for Accuracy (29 Cycles)
*B indicates blank well, F indicates Formamide

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder (ID+)	NP 8 (ID+ 28 Cycles)	NP 16 (ID+ 28 Cycles)	Reagent Blank (28 Cycles)	Ladder (ID+)	NP 8 (ID)	NP 16 (ID)	Reagent Blank (Identifiler)	Ladder (ID+)	NP 8 (ID+ 29 Cycles)	NP 16 (ID+ 29 Cycles)	Reagent Blank (29 Cycles)
B	NP 1 (ID+ 28 Cycles)	NP 9 (ID+ 28 Cycles)	NP 17 (ID+ 28 Cycles)	Ladder (ID+)	NP 1 (ID)	NP 9 (ID)	NP 17 (ID)	Ladder (ID)	NP 1 (ID+ 29 Cycles)	NP 9 (ID+ 29 Cycles)	NP 17 (ID+ 29 Cycles)	Ladder (ID+)
C	NP 2 (ID+ 28 Cycles)	NP 10 (ID+ 28 Cycles)	NP 18 (ID+ 28 Cycles)	F	NP 2 (ID)	NP 10 (ID)	NP 18 (ID)	F	NP 2 (ID+ 29 Cycles)	NP 10 (ID+ 29 Cycles)	NP 18 (ID+ 29 Cycles)	F
D	NP 3 (ID+ 28 Cycles)	NP 11 (ID+ 28 Cycles)	NP 19 (ID+ 28 Cycles)	F	NP 3 (ID)	NP 11 (ID)	NP 19 (ID)	F	NP 3 (ID+ 29 Cycles)	NP 11 (ID+ 29 Cycles)	NP 19 (ID+ 29 Cycles)	F
E	NP 4 (ID+ 28 Cycles)	NP 12 (ID+ 28 Cycles)	NP 20 (ID+ 28 Cycles)	F	NP 4 (ID)	NP 12 (ID)	NP 20 (ID)	F	NP 4 (ID+ 29 Cycles)	NP 12 (ID+ 29 Cycles)	NP 20 (ID + 29 Cycles)	F
F	NP 5 (ID+ 28 Cycles)	NP 13 (ID+ 28 Cycles)	+C (28 Cycles)	F	NP 5 (ID)	NP 13 (ID)	+C (ID)	F	NP 5 (ID+ 29 Cycles)	NP 13 (ID+ 29 Cycles)	+C (29 Cycles)	F
G	NP 6 (ID+ 28 Cycles)	NP 14 (ID+ 28 Cycles)	+C (28 Cycles)	F	NP 6 (ID)	NP 14 (ID)	+C (ID)	F	NP 6 (ID+ 29 Cycles)	NP 14 (ID+ 29 Cycles)	+C (29 Cycles)	F
H	NP 7 (ID + 28 Cycles)	NP 15 (ID+ 28 Cycles)	-C (28 Cycles)	F	NP 7 (ID)	NP 15 (ID)	-C (ID)	F	NP 7 (ID+ 29 Cycles)	NP 15 (ID+ 29 Cycles)	-C (29 Cycles)	F

Figure 3: Grid for Accuracy (Non-Probative)
*B indicates blank well, F indicates Formamide

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder	Mixture 1:4 (28 Cycles)	-C (28 Cycles)	F	Ladder	Mixture 1:4 (29 Cycles)	B	B	B	B	B	B
B	Mixture 19:1 (28 Cycles)	Mixture 1:6 (28 Cycles)	-C (29 Cycles)	F	Mixture 19:1 (29 Cycles)	Mixture 1:6 (29 Cycles)	B	B	B	B	B	B
C	Mixture 9:1 (28 Cycles)	Mixture 1:9 (28 Cycles)	F	F	Mixture 9:1 (29 Cycles)	Mixture 1:9 (29 Cycles)	B	B	B	B	B	B
D	Mixture 6:1 (28 Cycles)	Mixture 1:19 (28 Cycles)	F	F	Mixture 6:1 (29 Cycles)	Mixture 1:19 (29 Cycles)	B	B	B	B	B	B
E	Mixture 4:1 (28 Cycles)	Mixture 0:1 (28 Cycles)	F	F	Mixture 4:1 (29 Cycles)	Mixture 0:1 (29 Cycles)	B	B	B	B	B	B
F	Mixture 2:1 (28 Cycles)	Mixture 0:1 (28 Cycles)	F	F	Mixture 2:1 (29 Cycles)	Mixture 0:1 (29 Cycles)	B	B	B	B	B	B
G	Mixture 1:1 (28 Cycles)	Mixture 1:0 (28 Cycles)	F	F	Mixture 1:1 (29 Cycles)	Mixture 1:0 (29 Cycles)	B	B	B	B	B	B
H	Mixture 1:2 (28 Cycles)	Mixture 1:0 (28 Cycles)	F	F	Mixture 1:2 (29 Cycles)	Mixture 1:0 (29 Cycles)	B	B	B	B	B	B

Figure 4: Grid for Accuracy (Mixture)
*B indicates blank well, F indicates Formamide

Precision

Precision Study (Allelic Ladders):

Allelic ladders provided with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit were set up according to manufacturer's protocols, triple injected (See shaded "Precision Study" blocks of Figure 5), and analyzed according to laboratory protocols.

The base pair size estimated for each allele at every locus of the allelic ladders using the GeneScan™ 500 LIZ® size standard was exported into Microsoft® Excel. Statistics were generated for all 16 injected ladders; the statistics were analyzed together as well as separately for the injections within each of the 16 capillaries. The statistics generated include: minimum base pair (bp) size, maximum bp size, average bp size, and standard deviation of bp size. These statistics were compared to the statistics generated in the Precision Study (Allelic Ladders) in the Identifiler® verification (2012).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Ladder	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
B	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (28 Cycles	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
C	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (28 Cycles	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
D	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (29 Cycles	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
E	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (29 Cycles	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
F	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Ladder	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
G	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	F	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
H	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	F	F	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B

Figure 5: Grid for Precision
*B indicates blank well, F indicates Formamide; Columns 1 and 2 were triple injected

Precision Study (250 base pairs):

Samples containing the GeneScan™ 500 LIZ® size standard from the allelic ladder precision study detailed above were injected and analyzed in accordance with laboratory protocols. The estimated base pair sizes were exported into Microsoft® Excel, and statistics were calculated. Statistics generated include: minimum base pair (bp) size, maximum bp size, average bp size, difference in bp size, and standard deviation of bp size. These statistics were compared to the statistics generated in the Precision Study (Allelic Ladders) in the Identifiler® verification (2012).

Recovery

Data from the sensitivity study was imported into Excel and the percent recovery for 28 and 29 cycles was calculated. Percent recovery was determined by dividing the number of observed alleles by the number of expected alleles for a given full profile. Data from the 28 and 29 cycle amplifications were compared to each other and to the recovery data from the Identifiler®, MiniFiler™, and Yfiler® verifications (2012, 2010, and 2009, respectively).

See also Linearity

Linearity and Range

Sensitivity Study:

A DNA extract was obtained with greater than 10 ng/µl of total human DNA and re-quantified according to laboratory SOPs. Based on total human quantitation results, the samples were prepared as follows: 2.5, 1.25, 0.5, 0.25, 0.125, 0.063, 0.031 and 0.016 ng/µl. The diluted samples were amplified for 28 and 29 cycles in triplicate on one thermal cycler. They were also

run once on each of the other two thermal cyclers (Table 2 contains quantities of DNA and TE^{-4} added for amplification with the Identifiler® Plus kit).

The samples were then placed on the Applied Biosystems® 3130 Genetic Analyzer or the Applied Biosystems® 3130xl Genetic Analyzer and injected (See shaded "Sensitivity Study" blocks of Figures 1 and 2). The samples were injected and analyzed according to laboratory protocols.

Heterozygous ratios for each input amount and amplification cycle number were determined at all possible loci by dividing the lower RFU height at the locus by the higher RFU height, then multiplying by 100. Data from the 28 and 29 cycle amplifications were compared to each other and to the Sensitivity Study data from the Identifiler® verification (2011).

Using the sensitivity and percent recovery (See "Recovery") data, the ideal input range for the 28 and 29 cycle amplification procedures was determined.

A homozygous threshold was determined for each amplification cycle number using a locus in which dropout of one allele was observed in only one of the three replicates for a given target amount of DNA. The homozygous ratio ensures that DNA analysts do not call an allele a true homozygote if there is the possibility of allelic dropout. The homozygous threshold was determined by obtaining the standard deviation from the 3 replicates of the detected allele. The standard deviation was multiplied by 3 and added to the RFU height of the observed allele from the sample which exhibited dropout.

Table 2: Quantities of DNA and TE^{-4} added for amplification with the Identifiler® Plus kit

Target DNA Input (ng)	Sample Name	DNA Concentration (ng/µl)	Volume of DNA (µl)	Volume of TE-4 (µl)	Input DNA (ng/µl)
2.5	S1	2.18	1.2	8.8	2.5
1.25	S2	0.944	1.3	8.7	1.25
0.5	S3	0.34	1.5	8.5	0.5
0.25	S4	0.221	1.1	8.9	0.25

Table 2: Quantities of DNA and TE-4 added for amplification with the Identifiler® Plus kit					
Target DNA Input (ng)	Sample Name	DNA Concentration (ng/µl)	Volume of DNA (µl)	Volume of TE-4 (µl)	Input DNA (ng/µl)
0.125	S5	0.0865	1.4	8.6	0.125
0.063	S6	0.0323	2	8	0.063
0.031	S7	0.0254	1.2	8.8	0.031
0.016	S8	0.00409	3.9	6.1	0.016

Concentration of DNA determined using the Quantifiler® Duo kit after the stocks were prepared as described above.

Limit of Detection (LOD)

Minimum Threshold Study:

Two amplification negative control samples were amplified according to laboratory protocols for 28 amplification cycles and for 29 amplification cycles. From the negative control samples, sixteen amplification negative control wells for each amplification cycle number were prepared for electrophoresis according to laboratory protocols and injected 3 times (See shaded blocks of Figure 6). The peak amplitude threshold values for each dye channel (Blue, Green, Yellow and Red) were set at 1 RFU.

Collected data with a base pair size lower than the smallest allele or greater than the largest allele of the allelic ladder was deleted. The data analyzed reflected only the area in which human DNA profile alleles are observed. Therefore, all data less than 92 base pairs (bp) was deleted from the Identifiler® Plus tables. The first peak of the analyzed data is far enough below the smallest allele in the Identifiler® Plus ladder (D19S433, allele 9 at approximately 101 bp). As a result, the smaller alleles that may have appeared would still be included in the analysis. Similarly, all data beyond 368 bp, which is larger than the largest allele in the Identifiler® Plus ladder for all colors (D2S1338, allele 28 at approximately 359 bp) was deleted from analysis.

In addition, all peaks above 100 RFUs, all peaks that can be attributed to spikes (defined as peaks of the same approximate base pair size observed in two or more colors), all peaks that

are clearly Identifiler® Plus artifacts, and all peaks that can be clearly attributed to pull-up (defined as peaks that are approximately the same base pair size or within 5 GeneMapper® data points of the true peak in another color) of the GeneScan™ 500 LIZ® size standard were also deleted from analysis. Obvious rounded, extended "peaks" and raised baseline areas clearly distinguishable as artifacts were also deleted. Peaks that may be from spikes, artifacts, or pull-up of the GeneScan™ 500 LIZ® size standards, but are not clearly distinguishable as such, were retained for analysis. Due to the retention of possible pull-up peaks, spikes, and low level artifacts in the data that was analyzed, the average background RFU value calculated for each color provided a conservative upper limit.

The maximum RFU, the average RFU, the standard deviation, and the average RFU plus 3 standard deviations [Limit of Detection (LOD)] were calculated for each dye in the 28 and 29 cycle amplifications using Excel. Statistics from the 28 and 29 cycle amplifications were compared to each other and to the LOD statistics from the Identifiler® verification (2012).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Ladder	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
B	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (28 Cycles	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
C	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (28 Cycles	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
D	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (29 Cycles	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
E	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	+C (29 Cycles	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
F	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Ladder	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
G	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Formamide	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B
H	Ladder (Precision Study)	Ladder (Precision Study)	-C (28 LOD Study)	-C (28 LOD Study)	Formamide	Formamide	-C (29 LOD Study)	-C (29 LOD Study)	B	B	B	B

Figure 6: Grid for LOD

*B indicates blank well; Columns 3, 4, 7, and 8 were triple injected

Limit of Quantitation (LOQ)

See Limit of Detection (LOD)

The data obtained from the minimum threshold study that was used to determine the LOQ of the 28 and 29 cycle amplifications were the maximum RFU, the average RFU and the standard deviation. The LOQ is defined as the average RFU plus 10 standard deviations. Statistics from the 28 and 29 cycle amplifications were compared to each other and to the LOD statistics from the Identifiler® verification (2012).

Carryover

Each negative control and reagent blank was evaluated to ensure that no extraneous DNA was detected (See "-C" blocks of Figures 1-6).

Ruggedness

The "Sensitivity Study" specimens were amplified in sets at different times as well as on different thermal cyclers (See various shaded blocks of Figures 1 and 2) and 3130 instrumentation. The percent recovery and allelic heights per DNA concentration were compared between the thermal cyclers for both 28 and 29 amplification cycles.

RESULTS

Accuracy

Full or partial DNA profiles were observed for at least one of each sample amount at or above 35 relative fluorescent units (RFU), when amplified with the AmpFℓSTR® Identifiler® Plus kit. The DNA typing results showed no unexpected and/or inconsistent alleles in any of the

samples. The DNA profiles obtained for the same DNA source were consistent with the profiles obtained in this study:

- The DNA profiles obtained from samples prepared with donor TTH (sensitivity samples) were consistent with originating from donor TTH as expected.
- The DNA profiles obtained from samples prepared with positive control 9947A were consistent with originating from positive control 9947A.
- The DNA profiles obtained from non-probative samples simulating casework samples were consistent with the profiles obtained from the same samples used in previous validations.

Precision

Precision Study (Allelic Ladders):

The allelic ladder precision samples showed a range of standard deviations from 0.03 - 0.19 bp across all capillaries and markers (See Table 3 for allelic ladders deviations and comparisons between injections).

Table 3: Allelic Ladder Standard Deviations			
Injection #	Lowest Standard Deviation	Highest Standard Deviation	Difference
1	0.03	0.15	0.12
2	0.03	0.15	0.12
3	0.03	0.17	0.14
Overall	0.03	0.19	0.16

Precision (250 bp):

See Tables 4 and 5 for 250 bp results and comparisons between injections.

Table 4: Precision and LOD Data from 250bp Fragment of the GeneScan™ 500 LIZ™ Size Standard					
	Min (RFU)	Max (RFU)	Average (RFU)	SD (RFU)	Range (RFU)
Precision - 1st Inj.	245.95	246.31	246.23	0.11	0.36
Precision - 2nd Inj.	246.08	246.40	246.23	0.10	0.32
Precision - 3rd Inj.	246.10	246.42	246.23	0.10	0.32
LOD 3130xl - 1st Inj.	246.13	246.49	246.33	0.10	0.36

Table 4: Precision and LOD Data from 250bp Fragment of the GeneScan™ 500 LIZ™ Size Standard					
	Min (RFU)	Max (RFU)	Average (RFU)	SD (RFU)	Range (RFU)
LOD 3130xl - 2nd Inj.	246.10	246.50	246.33	0.10	0.40
LOD 3130xl - 3rd Inj.	246.11	246.48	246.33	0.10	0.37
LOD 3130 - 1st Inj.	245.24	246.30	245.86	0.40	1.06
LOD 3130 - 2nd Inj.	245.17	246.29	245.86	0.40	1.12
LOD 3130 - 3rd Inj.	245.20	246.26	245.86	0.40	1.06

Table 5: Average Data from 250bp Fragment of the GeneScan™ 500 LIZ™ Size Standard					
	Min (RFU)	Max (RFU)	Average (RFU)	SD (RFU)	Range (RFU)
Precision	245.95	246.42	246.24	0.11	0.47
LOD 3130xl	246.10	246.50	246.33	0.10	0.40
LOD 3130	245.17	246.30	245.86	0.40	1.13
Sens 28 – 3130xl	246.05	246.57	246.35	0.40	0.52
Sens 29 - 3130	245.85	246.50	246.25	0.14	0.65
NP	246.17	246.57	246.40	0.11	0.40
NP2	245.72	246.19	246.00	0.12	0.47
Mixtures	246.33	246.85	246.65	0.14	0.52
Sens 28 - 3130	246.12	246.56	246.45	0.10	0.44

Recovery

Table 6 contains the percent recovery of all Identifiler® Plus alleles for the six replicate samples for each input amount of DNA.

Table 6: Percent Recovery of Identifiler® Plus Alleles		
Sample Concentration (ng/µl)	Recovery - 28 Cycles (%)	Recovery - 29 Cycles (%)
2.5	100.00	100.00
1.25	100.00	100.00
0.5	100.00	100.00
0.25	100.00	100.00
0.125	100.00	100.00
0.063	100.00	96.88
0.031	31.25	66.25
0.016	66.88	76.88

Linearity and Range

Sensitivity Study:

Table 7 contains the input DNA peak heights for samples amplified for 28 cycles on each thermal cycler.

Table 7: Sensitivity Sample Peak Heights for 28 Cycles				
DNA Concentration (ng/µl)	Thermal Cycler 1 (RFU)	Thermal Cycler 2 (RFU)	Thermal Cycler 3 (RFU)	Average (RFU)
2.5	4545.03	4707.78	4912.91	4721.91
1.25	2652.75	2993.44	3729.41	3125.20
0.5	1233.90	918.66	1138.53	1097.03
0.25	291.56	247.09	310.09	282.92
0.125	140.11	161.03	127.91	143.02
0.063	99.40	99.28	96.16	98.28
0.031	40.58	54.27	47.28	47.38
0.016	59.27	66.07	47.73	57.69

Table 8 contains the input DNA peak heights for samples amplified for 29 cycles on each thermal cycler.

Table 8: Sensitivity Sample Peak Heights for 29 Cycles				
DNA Concentration (ng/µl)	Thermal Cycler 1 (RFU)	Thermal Cycler 2 (RFU)	Thermal Cycler 3 (RFU)	Average (RFU)
2.5	6853.25	7265.06	6158.03	6758.78
1.25	4528.03	4380.94	5070.13	4659.70
0.5	1625.04	1541.68	1650.00	1605.57
0.25	416.00	423.03	363.38	400.80
0.125	164.07	212.31	203.50	193.30
0.063	149.19	159.31	164.47	157.66
0.031	59.91	86.18	74.78	73.62
0.016	85.12	95.91	75.43	85.49

Limit of Detection

Table 9 contains the maximum signal, average, standard deviation, limit of detection (LOD), and limit of quantitation (LOQ) calculated for each dye color individually for 28 cycles.

Table 9: Noise Calculations for Negative Controls Amplified for 28 Cycles using 3130xl					
Dye	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
Blue (FAM)	16	4.94	2.82	13.39	33.11
Green (VIC)	11	4.95	2.81	13.38	33.06
Yellow (NED)	22	4.99	2.90	13.70	34.01
Red (ROX)	24	5.02	2.91	13.74	34.08

Table 10 contains the maximum signal, average, standard deviation, limit of detection (LOD), and limit of quantitation (LOQ) calculated for each dye color individually for 29 cycles.

Table 10: Noise Calculations for Negative Controls Amplified for 29 Cycles using 3130xl					
Dye	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
Blue (FAM)	11	5.26	2.81	13.68	33.32
Green (VIC)	15	5.27	2.80	13.67	33.28
Yellow (NED)	23	5.31	2.84	13.88	33.74
Red (ROX)	25	5.34	2.89	14.01	34.25

Tables 11-14 compare each dye channel with the number of amplification cycles performed using the 3130xl genetic analyzer.

Table 11: Limit of Detection Comparisons for Blue Channel (FAM)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	16	4.94	2.82	13.40	33.11
29	11	5.26	2.81	13.68	33.28
Fold Difference	1.45	1.06	1.00	1.02	1.01

Table 12: Limit of Detection Comparisons for Green Channel (VIC)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	11	4.95	2.81	13.38	33.06
29	15	5.34	2.80	13.74	33.35
Fold Difference	1.36	1.08	1.00	1.03	1.01

Table 13: Limit of Detection Comparisons for Yellow Channel (NED)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	22	4.99	2.90	13.70	34.01
29	23	5.31	2.84	13.84	33.74
Fold Difference	1.05	1.06	1.02	1.01	1.01

Table 14: Limit of Detection Comparisons for Red Channel (ROX)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	24	5.02	2.91	13.74	34.08
29	25	5.34	2.89	14.01	34.25
Fold Difference	1.04	1.06	1.01	1.02	1.00

Table 15 shows the totals for each calculation for 28 and 29 cycles, as well as the combined totals using the 3130xl genetic analyzer.

Table 15: Totals for 28 and 29 Cycles on 3130xl					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	24	4.98	2.86	13.55	33.56
29	25	5.29	2.84	13.80	33.65

After the LOD and LOQ had been determined for the 3130xl Genetic Analyzer, the samples were reinjected on an Applied Biosystems® 3130 Genetic Analyzer (SN#19341-005) for purposes of comparison. Tables 16 and 17 contain the maximum signal, average, standard deviation, limit of detection (LOD), and limit of quantitation (LOQ) calculated for each dye color individually for 28 and 29 cycles, respectively.

Table 16: Noise Calculations for Negative Controls Amplified for 28 Cycles Using 3130					
Dye	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
Blue (FAM)	7	4.06	2.20	10.66	26.06
Green (VIC)	12	4.07	2.19	10.65	25.99
Yellow (NED)	14	4.09	2.20	10.69	26.08
Red (ROX)	17	4.12	2.23	10.82	26.44

Table 17: Noise Calculations for Negative Controls Amplified for 29 Cycles Using 3130					
Dye	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
Blue (FAM)	11	4.54	2.15	10.99	26.04
Green (VIC)	12	4.55	2.14	10.98	25.99
Yellow (NED)	14	4.57	2.15	11.02	26.08
Red (ROX)	19	4.59	2.18	11.12	26.36

Tables 18-21 compare each dye channel with the number of amplification cycles performed using the 3130 genetic analyzer.

Table 18: Limit of Detection Comparisons for Blue Channel (FAM)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	7	4.06	2.20	10.66	26.06
29	11	4.54	2.15	10.99	26.04
Fold Difference	1.57	1.12	1.02	1.03	1.00

Table 19: Limit of Detection Comparisons for Green Channel (VIC)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	12	4.07	2.19	10.65	25.99
29	12	4.55	2.14	10.98	25.99
Fold Difference	1.00	1.12	1.02	1.03	1.00

Table 20: Limit of Detection Comparisons for Yellow Channel (NED)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	14	4.09	2.20	10.69	26.08
29	14	4.57	2.15	11.02	26.08
Fold Difference	1.00	1.12	1.02	1.03	1.00

Table 21: Limit of Detection Comparisons for Red Channel (ROX)					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	17	4.12	2.23	10.82	26.44
29	19	4.59	2.18	11.12	26.36
Fold Difference	1.12	1.11	1.02	1.03	1.00

Table 22 shows the totals for each calculation for 28 and 29 cycles, as well as the combined totals using the 3130 genetic analyzer.

Table 22: Totals for 28 and 29 Cycles					
# of Cycles	Max (RFU)	Average (RFU)	SD (RFU)	LOD (RFU)	LOQ (RFU)
28	17	4.09	2.21	10.70	26.14
29	19	4.56	2.16	11.03	26.12

Limit of Quantitation
See Limit of Detection (LOD)

Carryover

All negative controls exhibited the expected negative results, with the exception of the reagent blank for five samples extracted for the non-probative study. The samples associated with this reagent blank were re-extracted with a new reagent blank, re-amplified, and underwent capillary electrophoresis on the 3130xl genetic analyzer. The electropherograms were analyzed using GeneMapper *ID-X* v1.3 and the reagent blank was clean, but the negative control associated with these samples contained a single peak at TH01 with a peak height value of 41 RFUs. To be conservative, the five associated samples were removed from analysis.

Ruggedness

Samples run at different times as well as on different thermal cyclers and 3130 instrumentation were consistent with expected results and with each other.

DISCUSSION

Once the linearity study had been completed, it was determined that 28 cycles was optimal cycle number for amplification with Identifiler® Plus due to evidence of preferential amplification in samples amplified at 29 cycles. Preferential amplification can occur if the alleles denature at different temperatures or if differential priming of synthesis of an allele has occurred (Walsh, Erlich & Higuchi, 1992). Since evidence of preferential amplification was more frequently observed in the electropherograms of samples amplified for 29 cycles, it was theorized that 28 cycles would be the ideal number of amplification cycles for this amplification chemistry; however, both cycle numbers were still used in other studies for a better overall comparison.

Accuracy

The DNA results obtained were consistent with each other and with the donor profiles. Utilizing a threshold of 35 relative fluorescence units (RFU), full or partial DNA profiles were observed for at least one of each sample concentration/type.

Samples expected to have low levels of DNA still showed accuracy, even though they did not demonstrate full profiles. A partial profile is characterized as any profile that demonstrates fewer peaks at all loci (Identifiler® Plus contains 16 loci) than a full profile, or any peaks that have a heterozygous ratio that is so low that statistics cannot be performed. Accuracy was examined using data gathered by Data Collection Software version 3.0 and analyzed with GeneMapper® *ID-X* Software version 1.3. Genotypes were exported into Excel and compared to the known profiles. The only observed differences were due to drop-out in samples with low DNA concentrations.

There were a few samples that had been amplified at 29 cycles in which peaks appeared in the stutter position, and were called because they were above the manufacturer's stutter percentage filter. It is possible that the stutter percentage filter is inaccurate for 29 cycles and should be adjusted accordingly for samples amplified for 29 cycles. Some sample had profiles containing peaks in the +4 stutter position. The laboratory typically used a 3% stutter percentage filter for +4 stutter. Sailus et al.[21] states that some of the larger loci such as D2S1337 or FGA demonstrate an elevated +4 stutter percentage. In the future, an elevated stutter percentage filter could be applied to larger loci. Since the elevated stutter was found in samples amplified for 29 cycles, it was determined that preferential amplification more than likely caused the elevated -4 stutter. Samples amplified at 28 cycles typically did not exhibit elevated stutter, which indicates that the manufacturer's stutter percentage filters were effective for 28 cycles. An analyst should evaluate each locus independently in relation to the entire DNA profile in determining if a peak is elevated stutter or a minor allele that falls in the n-4 stutter range for both 28 and 29 cycles.

A difference in heterozygous ratios was observed between 28 and 29 cycles in the mixture study. This caused misinterpretation of alleles in samples amplified for 29 cycles. Figure 7 shows the alleles at D7S820 for the 1:1 mixture sample. If the mixture alleles were resolved at a 50% or 60% heterozygous ratio, the alleles would be interpreted as a 7, 10 and 11, 12. This interpretation would be incorrect because the known profiles display a 10, 11 and 7, 12 respectively at this locus. A 40% heterozygous ratio would have to be applied to get correct possible combinations. This data indicates that preferential amplification has occurred, which interferes with accurate deconvolution of mixture alleles at 29 cycles. This demonstrates that samples amplified at 29 cycles are not suitable for mixture deconvolution.

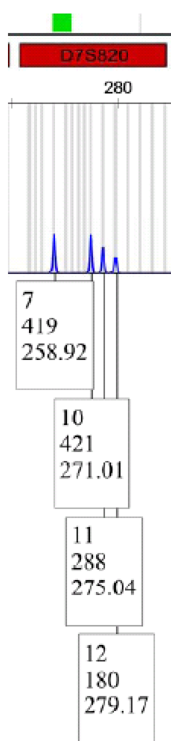

Figure 7: D7S820 locus for the 1:1 mixture ratio sample

The data indicated that for 29 cycles, the major component for a mixture could only be reliably resolved down to a 1:9 or 9:1 ratio. For 28 cycles, the data indicated that the major component could be effectively resolved down to a 1:4 or 4:1 ratio. This 1:4 or 4:1 mixture deconvolution of the major component is equivalent to what was observed in the Identifiler verification (2012).

Precision

Precision Study (Allelic Ladders):

The base pair size calculated for each allele at each locus of the Identifiler® Plus allelic ladders using the GeneScan™ 500 LIZ® size standard was exported into Microsoft® Excel. Statistics were generated for all 16 injected ladders, analyzed together as well as separately for

the injections within each of the 16 capillaries. The statistics generated include: minimum base pair (bp) size, maximum bp size, average bp size, and standard deviation of bp size.

When all three injections of 16 ladders (48 ladders in total) were compared, some of the Identifiler® Plus loci had standard deviation values that were above the manufacturer's recommended 0.15 bp value, based on needing a ±0.45 bp window (3X the standard deviation of 0.15 bp) for allele assignment using GeneMapper® *ID-X*. Smith et al. (1995) stated that the chance of an allele sizing outside +0.5 bp is relatively rare when the standard deviations are below 0.15 bp. The article also discusses that if the standard deviation exceeds 0.15 bp, it does not mean that the allele is going to size off-ladder; it means that there is an increased chance of it sizing off-ladder. Data from the non-probative and linearity sections of this verification study, using samples with known alleles and genotypes supported that the correct alleles and genotypes, were obtained for all samples in this study using more than one ladder on a plate and averaged by GeneMapper® *ID-X*.

The TH01 9.3 and 10 alleles were resolved clearly on all Identifiler® Plus ladders analyzed in this study.

The data indicated that multiple ladders should be run and spaced across the plate to ensure the alleles fall within the ±0.45 bp window. If two or more ladders are not used, the data should be checked to ensure all ladder alleles sized within the ±0.45 bp window.

As the control samples for this study were analyzed, it was observed that the positive control sample electropherograms were blown off-scale with a large amount of pull-up, stutter, and artifact peaks present. It was possible that too much DNA had been amplified, so the positive control sample electropherograms were removed from the project and the positive control (9947A) was quantitated. The positive control samples for the sensitivity study were amplified

with the correct amount of DNA as determined from the quantitation, so their electropherograms were used to confirm that the ladders in the precision study had run accurately and were sizing correctly.

Precision Study (250 bp):

Although the 250 bp fragment from the GeneScan™ 500 LIZ® size standard is a DNA fragment that is 250 bp long, it generally migrates as if it were a shorter fragment due to incomplete denaturation of the double-stranded DNA molecule. Evaluating the variation in base pair size of the 250 bp DNA fragment in the GeneScan™ 500 LIZ® size standard is important. It is used to demonstrate whether the conditions for injection, electrophoresis and collection of data over the entire length of the plate on the Applied Biosystems® 3130xl Genetic Analyzer are appropriate for the correct reporting of alleles and genotypes from samples containing human DNA. The 250 bp precision study demonstrated that the range of base pair sizes obtained across the plate differs by 0.58 bp, which is less than the suggested 1.0 bp range. Furthermore, the standard deviation of 0.11 bp was also less than the manufacturer's recommended value of 0.15 bp.

The data from the second LOD study had a range of 1.13 bp, which is outside the suggested range of 1.0 bp. The sample tray remained on the 3130xl (SN#22285-030) for 48 hours before being run on the 3130 (SN#19341-005); degradation may have occurred, which is also visible in the positive control samples for the allelic ladders. It can be assumed, if all of the samples used in other studies are considered, that if the samples had been re-injected shortly after the first run, the range of the 250 bp peak would be within 1.0 bp. Also, when multiple electrokinetic injections are performed on a sample over time, the resulting profile of the sample will change. This occurrence may have affected the range of the 250 bp peak as well.

Recovery

One hundred percent recovery of alleles was obtained for all samples above the low copy number (i.e., less than 100 pg) as expected. The results of this study were similar to the results of a previously performed Identifiler® validation. The only exception was the recovery for the 0.031 ng samples amplified for 28 cycles. It was lower for the Identifiler® Plus samples (31.25%) than the Identifiler® samples (35.56%).

The recovery results for this study were compared to the recovery results obtained from previous Identifiler®, MiniFiler™, and Yfiler® validations, as shown in Figure 8.

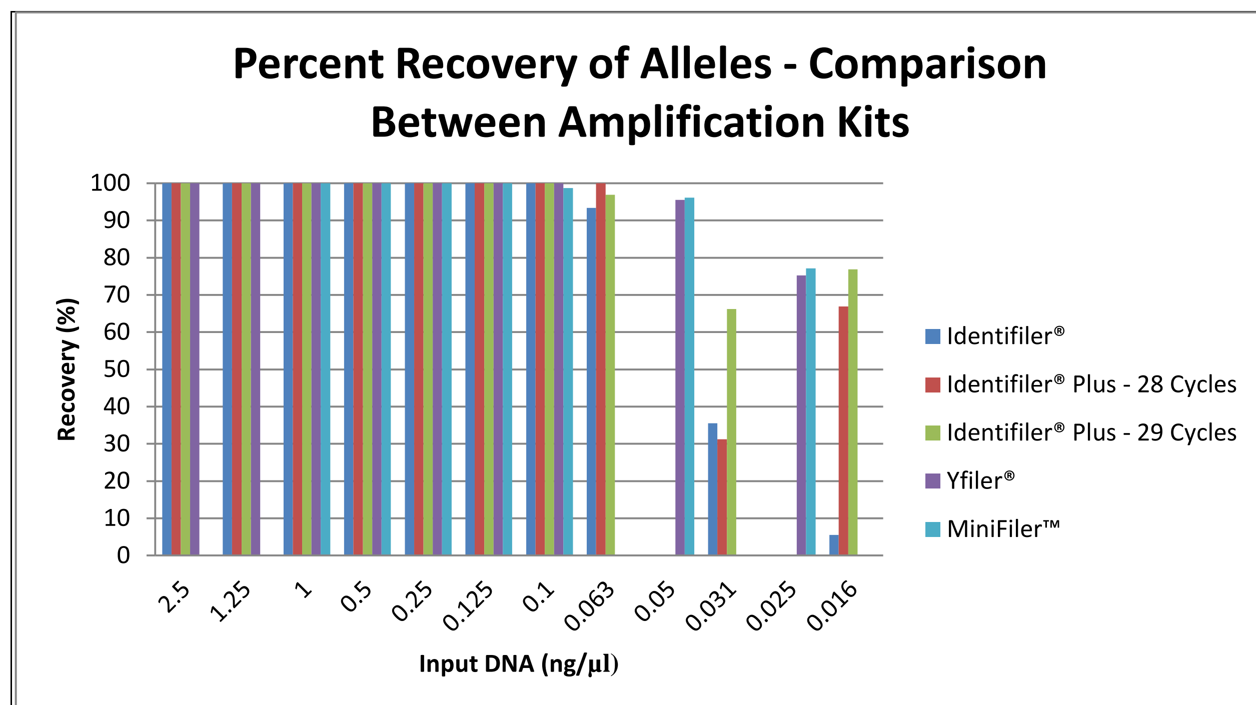

Figure 8: Comparison of Allele Recovery between Amplification Kits

Linearity and Range

Some variances in recovery were expected due to fluctuation in quantitation and amplification. However, full or partial DNA profiles were obtained for a minimum of one sample

at each concentration. Some samples showed indications of peaks above the baseline that fell within bins for ladder alleles but below the 35 RFU threshold.

The data demonstrated that all alleles for all loci were observed for the samples when 0.063 ng or more of DNA was amplified for 28 cycles with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit and injected onto the Applied Biosystems® 3130xl at 3Kv for 5 seconds. Peak heights were observed ranging from ~38 RFUs to ~194 RFUs for 0.063 ng of amplified DNA. The peak heights of the alleles detected for the three replicate amplifications at each input amount of DNA were similar at all levels of input DNA. The 2.5 ng samples injected at 3kV for 5 seconds resulted in off-scale peaks, shouldering, pull up, +4 stutter, -A peaks, and background/artifact peaks associated with the profiles. The peak heights were generally well balanced within a locus and across loci for the 0.5 ng and higher concentration of input DNA samples amplified with the Identifiler® kit. As expected, the RFUs increased as the amount of DNA amplified increased, with the exception of the 0.031 ng input samples (See Figure 9).

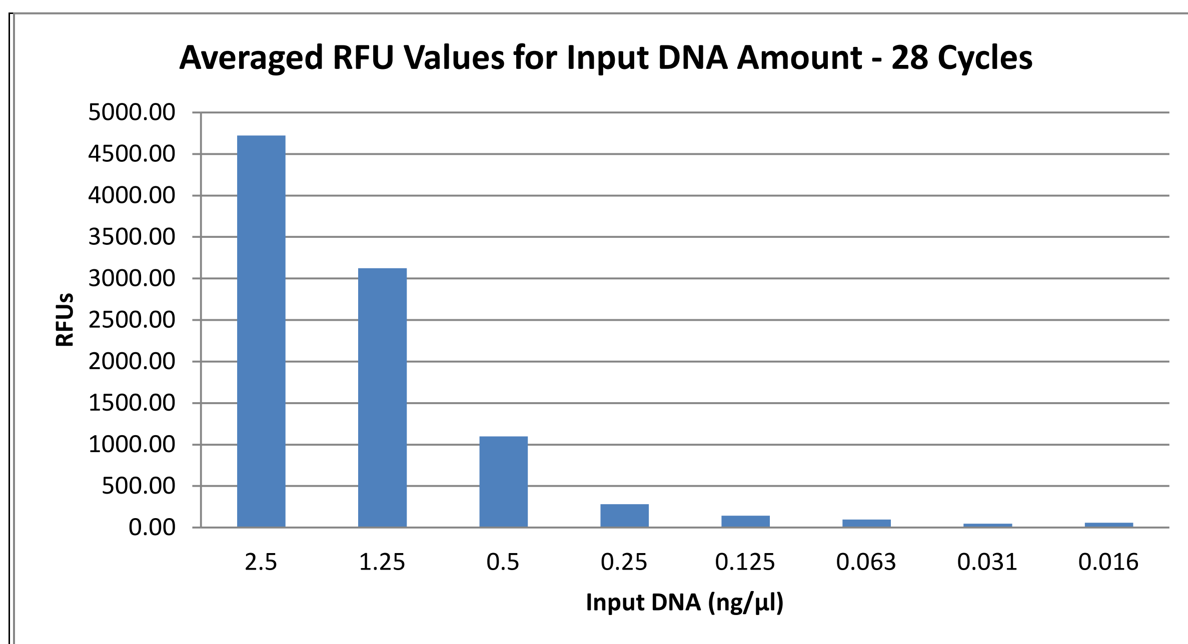

Figure 9: Comparison of Averaged Peak Heights of All Loci between Amplified Samples at 28 Cycles

Data was compared between thermal cyclers 1, 2, and 3 (Applied Biosystems® GeneAmp® PCR System 9700 thermal cycler serial # 805S7073189, 805S7073190, and 805S0131870 respectively). The averages of the peak height values were calculated per locus and graphed grouped together by input DNA, where possible (See Figure 10). Thermal cycler 3 is associated with the largest peak height values at each input DNA amount. There is no visible trend between peak height values and which thermal cycler was used.

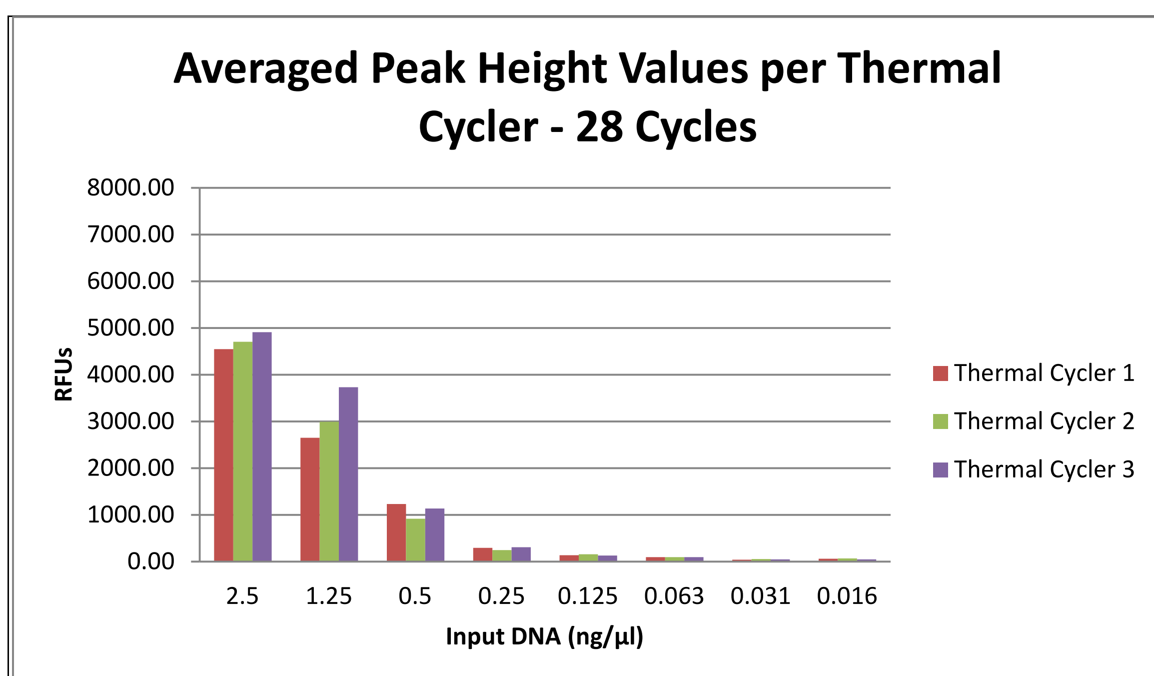

Figure 10: Comparison of Peak Heights between Thermal Cyclers for 28 Cycles

The data demonstrated that all alleles for all loci were observed in the samples where 0.125 ng or more of DNA was amplified for 29 cycles with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit and injected onto the Applied Biosystems® 3130xl at 3Kv for 5 seconds. Peak heights were observed ranging from ~37 RFUs to ~383 RFUs for 0.125 ng of amplified DNA. The peak heights of the alleles detected for the three replicate amplifications at each input amount of DNA were similar at all levels of input DNA. The 2.5 ng samples injected at 3kV for

5 seconds had off-scale peaks, shouldering, pull up, +4 stutter, -A peaks, and background/artifact peaks associated with the profiles. The peak heights were well balanced within a locus and across loci for the 0.125 ng and higher nanogram input DNA samples amplified with the Identifiler® Plus kit. As expected, the RFUs increased as the amount of DNA amplified increased (See Figure 11).

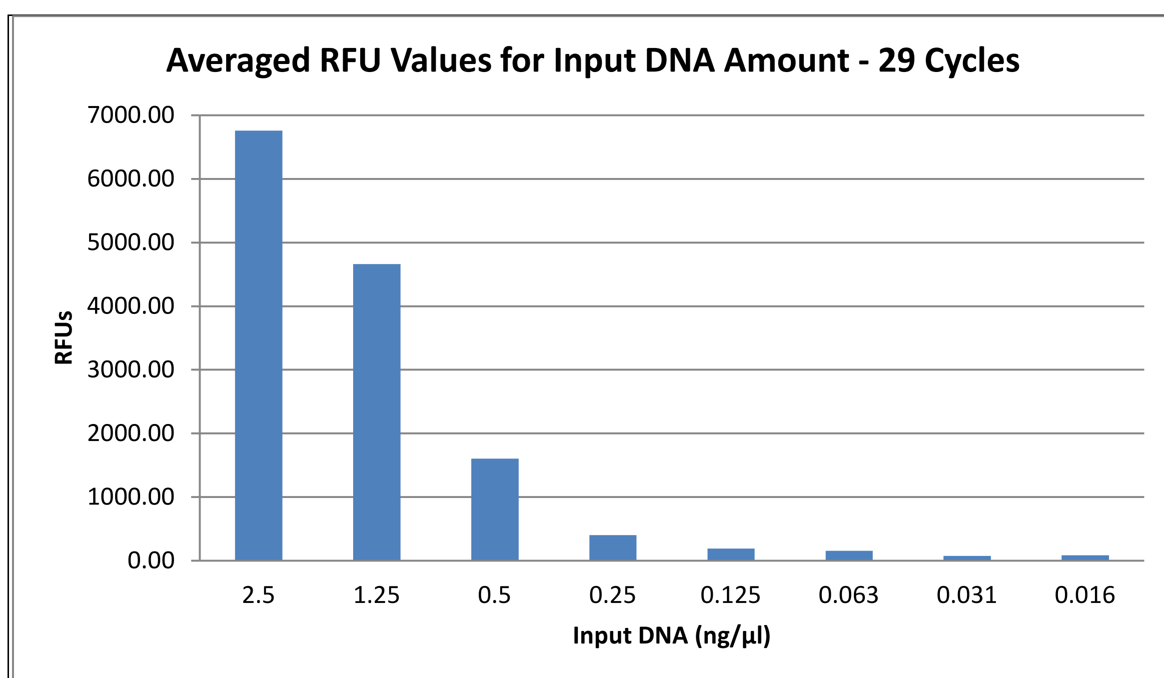

Figure 11: Comparison of Averaged Peak Heights between Amplified Samples at 29 cycles

Figure 12 compares the averaged peak heights of samples amplified at 28 and 29 cycles. The peak heights for samples amplified at 29 cycles were consistently higher than the peak heights of samples amplified at 28 cycles. However, for input DNA amounts of 0.063 ng or lower, the peak heights for both cycle numbers were low and the difference between them was negligible.

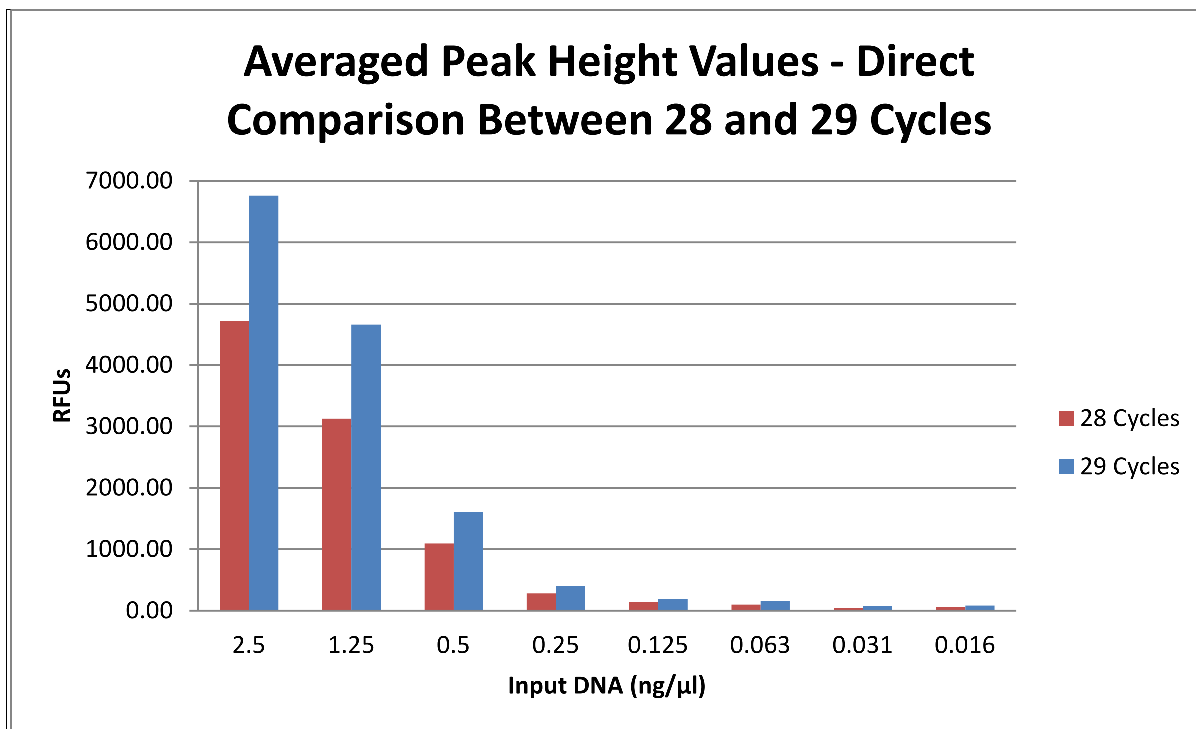

Figure 12: Comparison of Averaged Peak Heights for 28 and 29 cycles

The values were separated and analyzed according to the thermal cycler utilized during amplification. The averages were calculated per locus and graphed grouped together by input DNA (See Figure 13). As with the results for 28 cycles in Figure 9, there is no visible trend between peak height values and thermal cycler used.

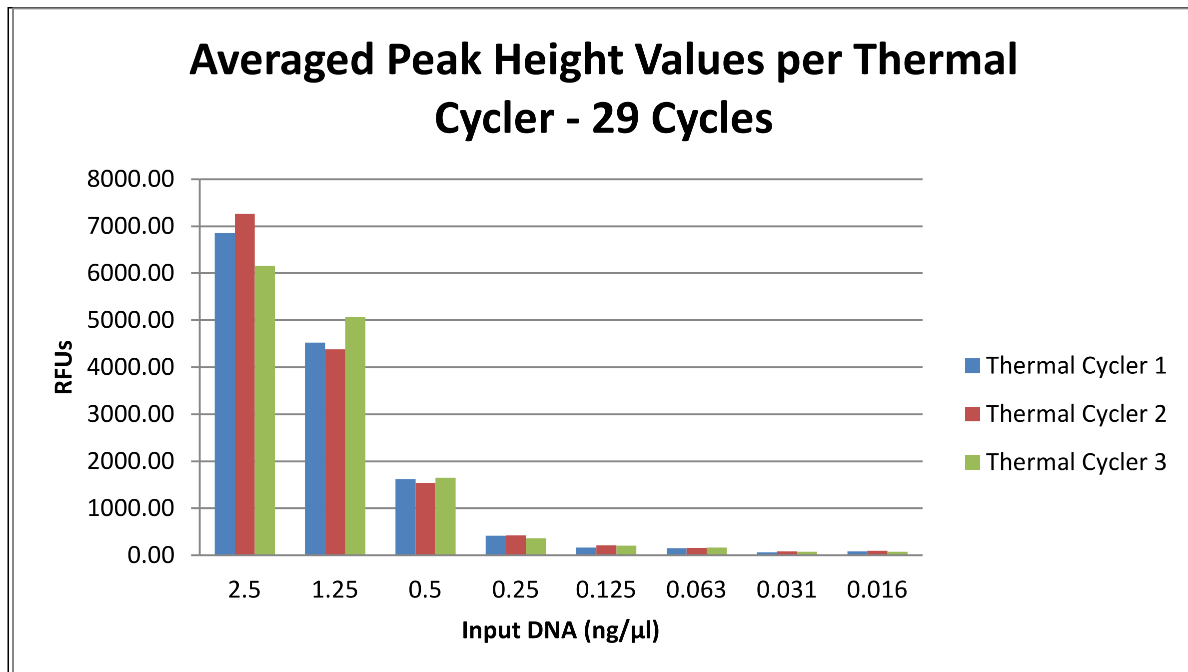

Figure 13: Comparison of Averaged Peak Heights between Thermal Cyclers for 29 Cycles

The sensitivity samples were replated and injected on the 3130 genetic analyzer for comparison purposes. Figure 14 shows the peak height values for each input DNA amount for the samples injected on the 3130 genetic analyzer.

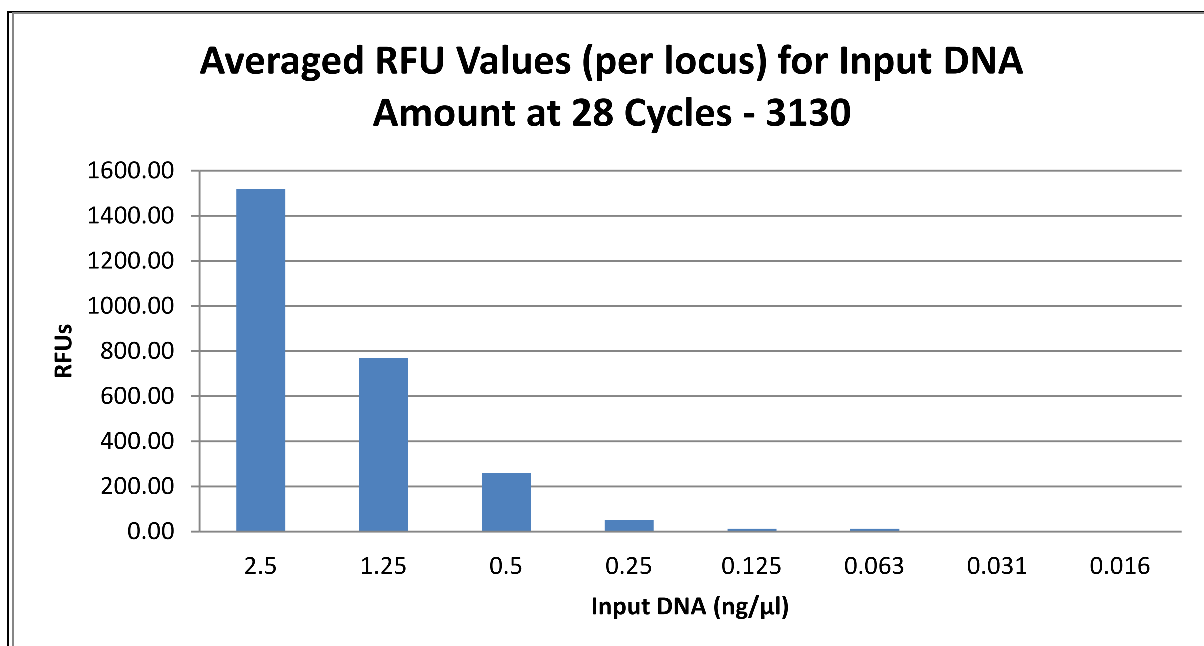

Figure 14: Comparison of Averaged Peak Heights between Amplified Samples at 28 cycles on 3130 Genetic Analyzer

Figure 15 shows the averaged peak height values for each thermal cycler at 28 cycles for samples run on the 3130 genetic analyzer. Samples amplified with thermal cycler 3 had the highest peak height values followed by thermal cycler 1, and thermal cycler 2 had the lowest peak height values.

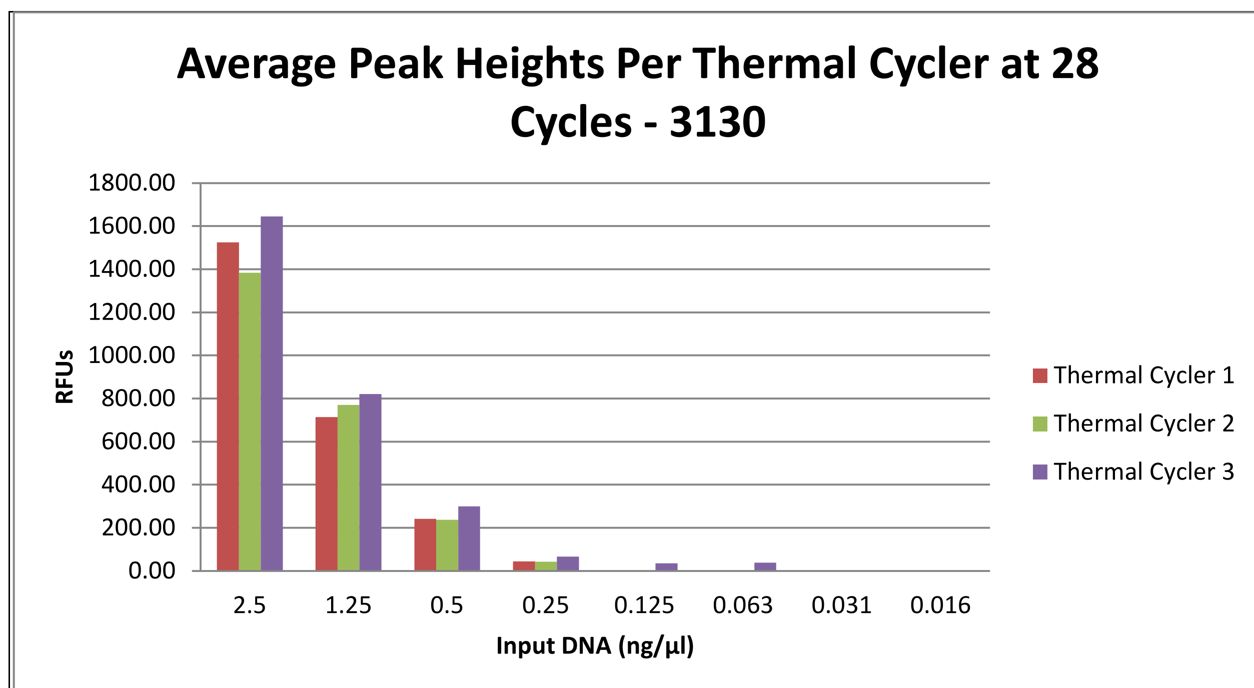

Figure 15: Comparison of Peak Heights between Thermal Cyclers for 28 Cycles on 3130 Genetic Analyzer

The average peak height values for the 3130 and 3130xl genetic analyzers are displayed in Figure 16. The samples amplified for 29 cycles and run on the 3130xl had the largest peak height values, followed by the samples amplified for 28 cycles and run on the 3130xl. The samples amplified for 28 cycles and run on the 3130 had much lower peak heights than the other two sample sets.

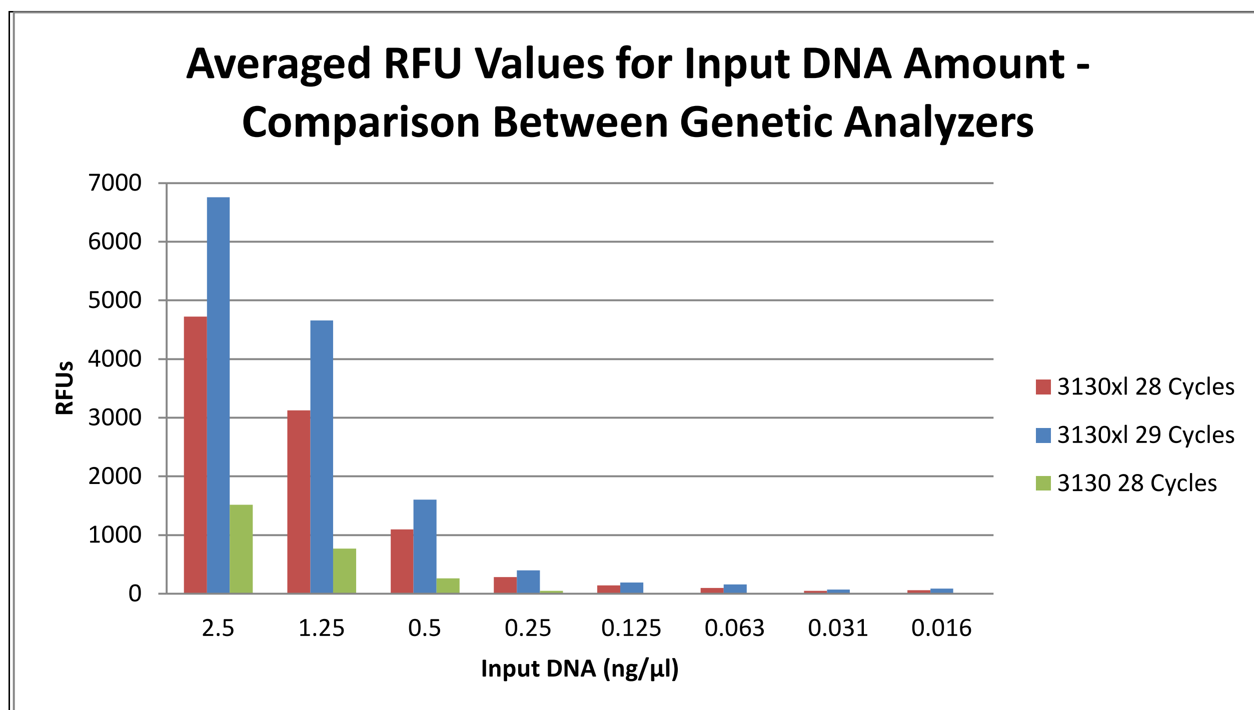

Figure 16: Comparison of Peak Heights between Genetic Analyzers

Figure 17 displays the average peak heights per input DNA amount between the four amplification chemistries. It appears that MiniFiler™ has the largest peak height values on average, followed by Identifiler Plus at 29 cycles, Identifiler®, Identifiler® Plus at 28 cycles, and Yfiler® with the smallest peak height values. However, MiniFiler™ and Identifiler® Plus both seem to be the most sensitive. The available data for MiniFiler™ did not extend down to 0.016 ng, so it is impossible to determine which kit is more sensitive unless further testing is done. It is important to note that Identifiler® Plus displays high sensitivity with 15 loci, while MiniFiler™ only amplifies 9 loci. A full profile generated with Identifiler® Plus would be more beneficial for comparison purposes.

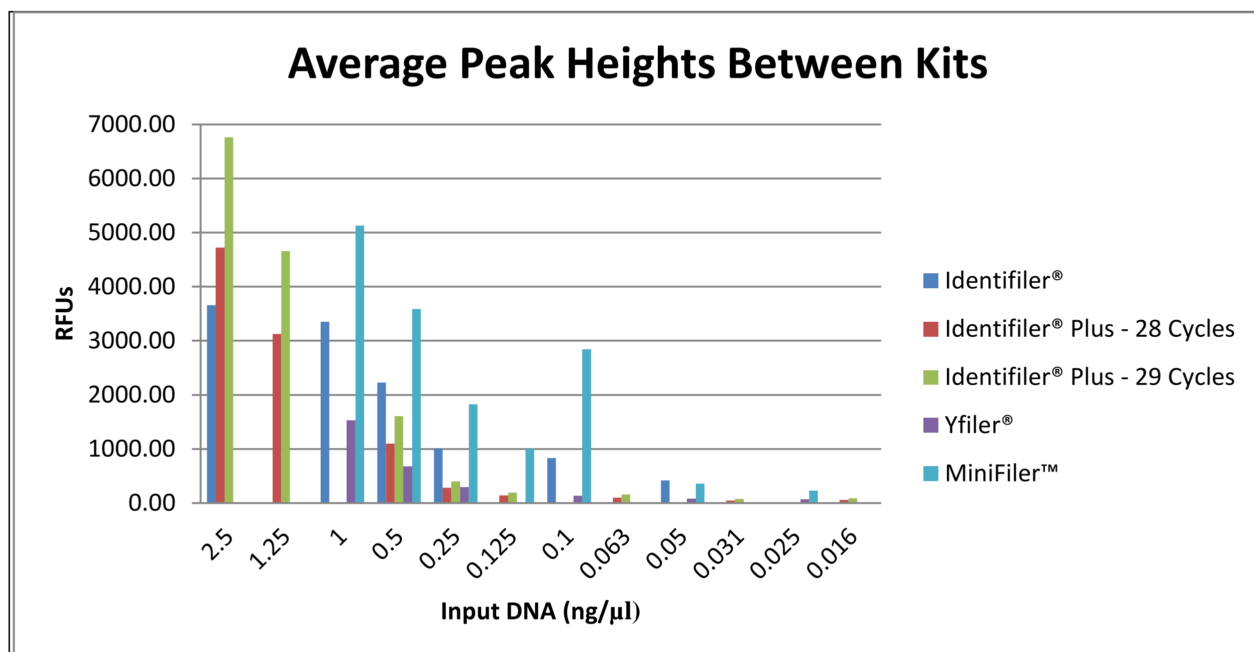

Figure 17: Average Peak Heights per Input DNA Amount – Comparison between Amplification Kits

As expected, the alleles detected for the lower amounts of DNA where partial profiles were obtained were not consistent across replicate amplifications. All alleles detected for all samples were compared to the profile of the stock sample donor and were determined to be the correct alleles. When single differing alleles were detected in a profile from a locus for which the donor was heterozygous, the two independent alleles were always correct and together comprised the complete heterozygous profile for that locus. Alleles were missing from loci spread across the Identifiler® Plus profile (including Amelogenin). Therefore, caution should be used when assigning gender to samples amplified with low amounts of input DNA. When all of the 0.031 ng samples amplified at 28 cycles on each thermal cycler were compared to each other, every locus showed dropout of at least one allele. For the 0.063 ng replicate samples, full profiles were reported and the alleles were well balanced across all loci. For the 0.5 ng replicate samples, full profiles were reported, the alleles were generally well balanced across all loci, and only slight pull up, artifacts, -A, +4 stutter and pull-up were observed at this concentration. However,

for the samples amplified for 29 cycles, the 0.063 ng replicate samples showed dropout of at least one allele at D8S1179, TH01, and TPOX. This may have been caused by preferential amplification, in which one allele is amplified more than the other allele at the same locus.

The intra-color balance was calculated for each dye channel by dividing the minimum peak height for each channel by the maximum peak height of that channel. These values were separated by cycle number for comparison. There appears to be a trend that as the input DNA amount decreases, the cycle number with the better intra-color balance shifts from 29 cycles to 28 cycles. This shift usually occurred between 0.5 ng and 0.25 ng of input DNA, with the exception of the green dye channel, in which it occurred between 1.25 ng and 0.5 ng of input DNA.

The intra-color balances calculated for Identifiler® Plus were compared to the intra-color balances calculated for Identifiler®, Yfiler® and MiniFiler™, and the results are shown in Figures 18-21. The kit that appears to have the best average intra-color balance for all dye channels is MiniFiler™, with the exception of the green dye channel, in which Yfiler® had the highest average intra-color balance. However, the MiniFiler™ intra-color balance was calculated using a non-degraded sample. Based the results of other data generated from the MiniFiler™ verification, it is suspected that for degraded samples, MiniFiler™ would have the worst intra-color balance.

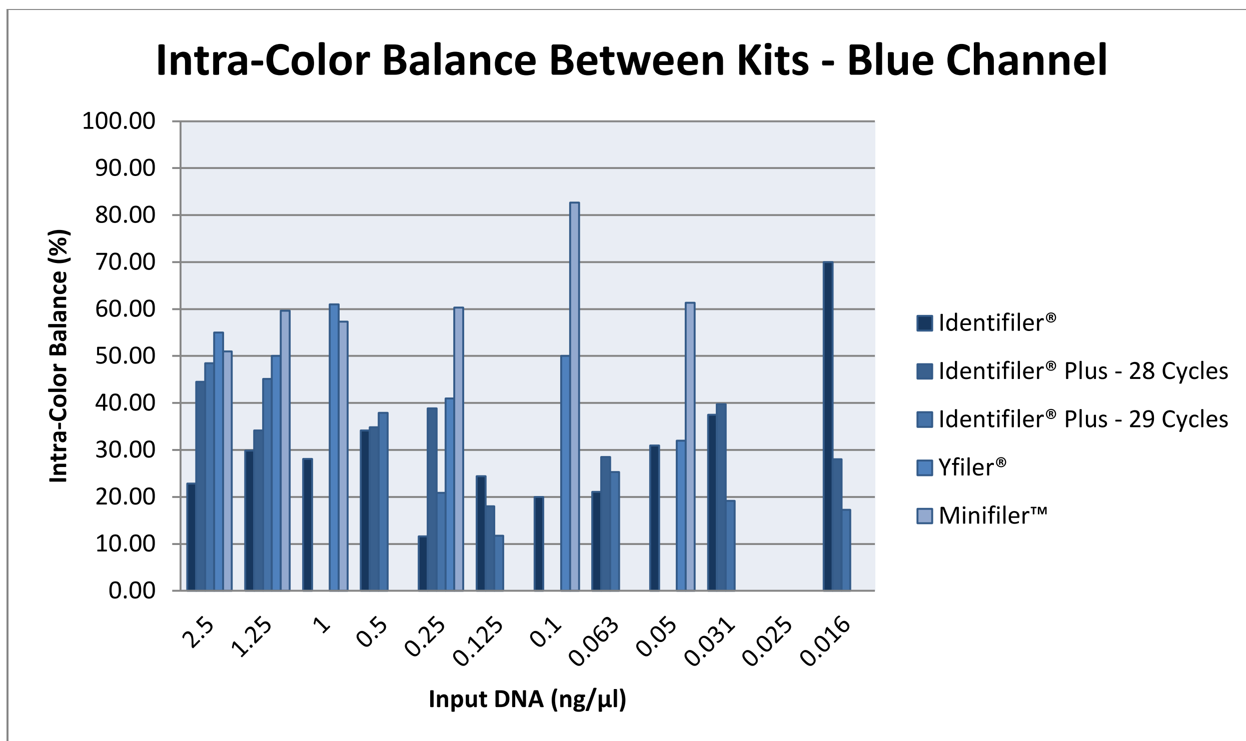

Figure 18: Intra-Color Balance comparison between kits for the blue channel

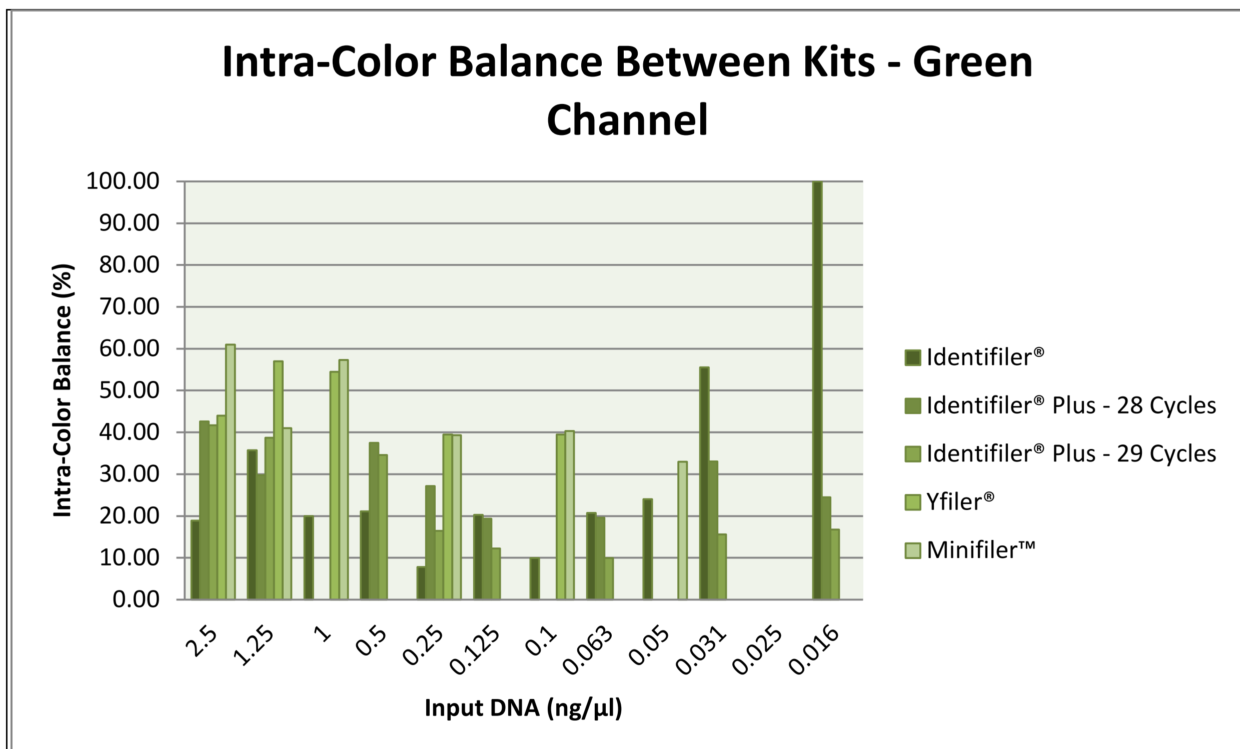

Figure 19: Intra-Color Balance comparison between kits for the green channel

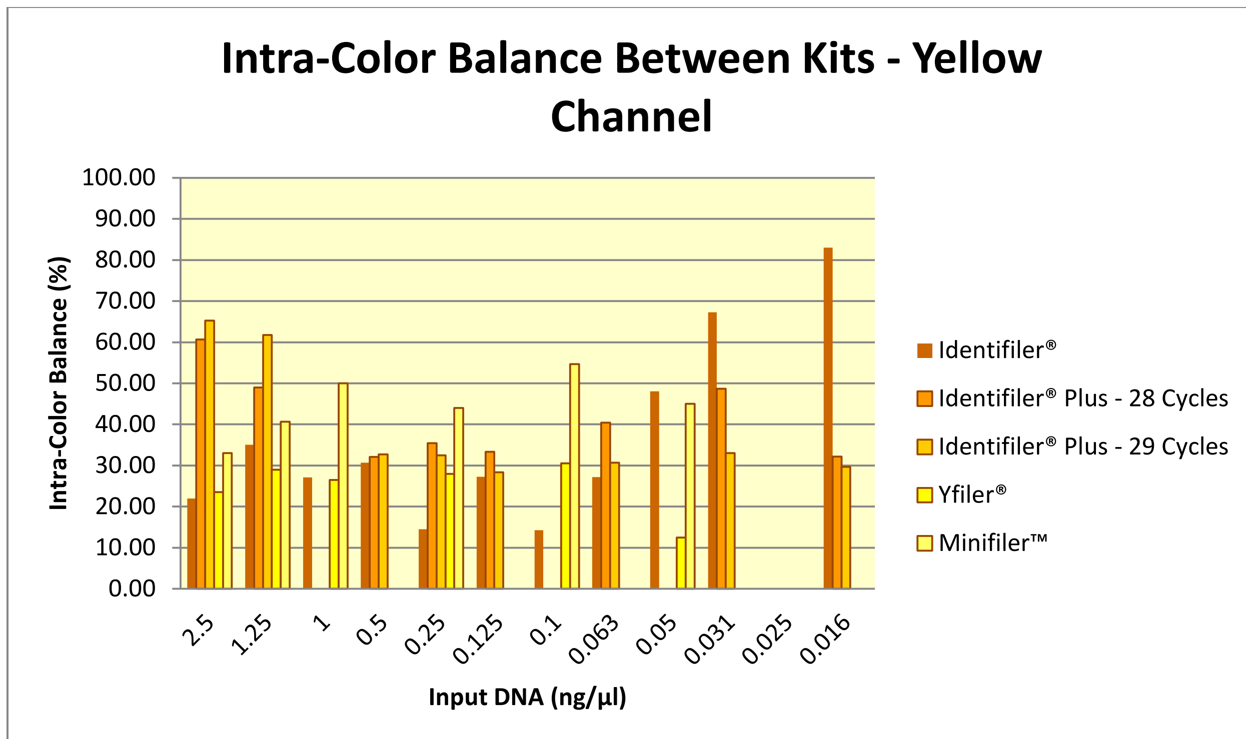

Figure 20: Intra-Color Balance comparison between kits for the yellow channel

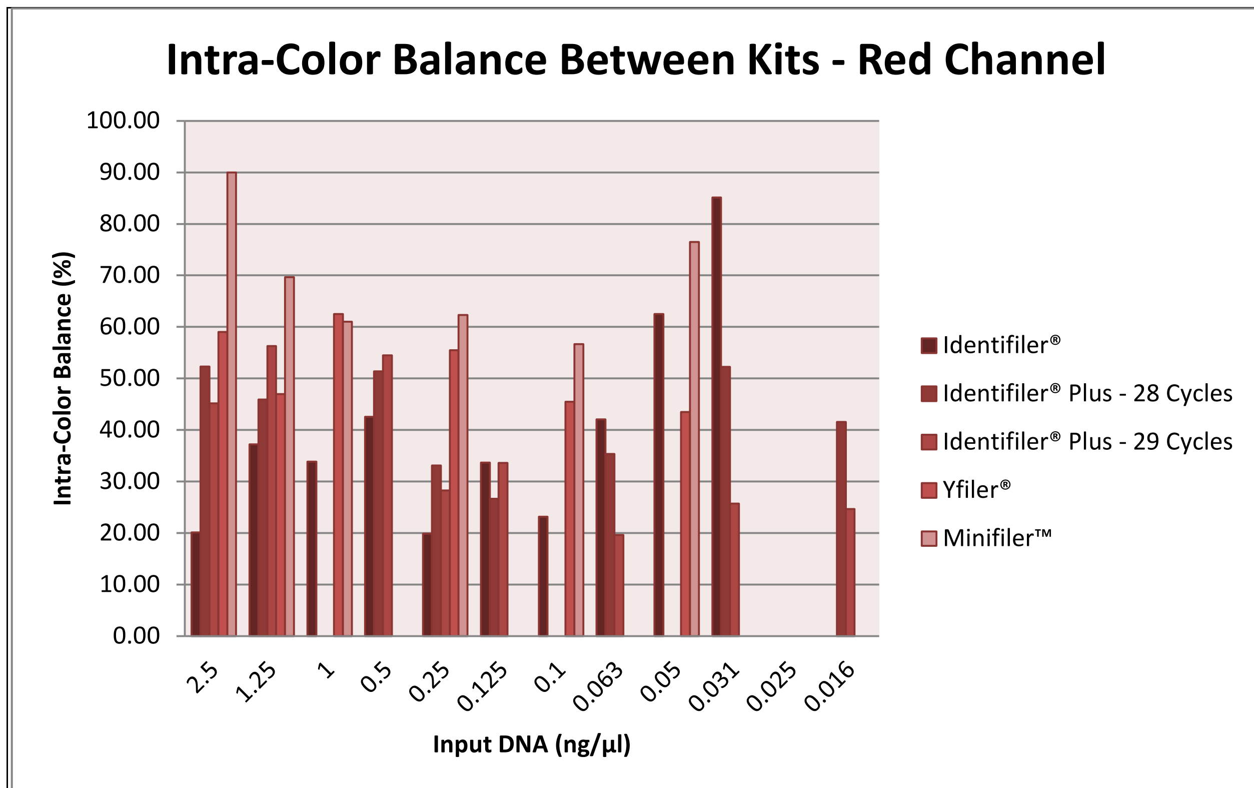

Figure 21: Intra-Color Balance comparison between kits for the red channel

In addition, allelic dropout of a 29 allele was observed at the D21S11 locus for one of the six replicates of sample S8.3-28 at the 0.016 ng DNA concentration. The RFU of the detected allele (28) was 50 RFU. A homozygous threshold was determined based on this instance to ensure that a DNA analyst would not call this a true homozygote. The homozygous threshold was determined by obtaining the standard deviation from the 3 replicates of the detected allele (28). These values were 110, 41, and 50 RFUs. The standard deviation was determined to be 37.70 RFUs. Three standard deviations were calculated and then added to the height of the 28 allele (50 RFU) to obtain the homozygous threshold. The homozygous threshold for 28 cycles was determined to be 163.10 RFU.

For the 29 cycle sensitivity samples, allelic dropout of a 14 allele was observed at the D8S1179 locus for one of the six replicates of sample S6.3-29 at the 0.063 ng DNA concentration. The RFU of the detected allele (28) was 319 RFU. A homozygous threshold was determined based on this instance to ensure that a DNA analyst would not call this a true homozygote. The homozygous threshold was determined by obtaining the standard deviation from the 3 replicates of the detected allele (28). These values were 223, 136, and 319 RFUs. The standard deviation was determined to be 91.54 RFUs. Three standard deviations were calculated and then added to the height of the 28 allele (319 RFU) to obtain the homozygous threshold. The homozygous threshold for 29 cycles was determined to be 593.63 RFU. See Table 23 for homozygous (i.e., stochastic) calculations.

Table 23: Stochastic Threshold Calculations			
Input DNA (ng)	Marker	SD (RFU)	Stochastic Threshold (RFU)
0.016	D21S11	37.70	163.10
0.063	D8S1179	91.54	593.63

Regarding the low level DNA concentrations, it is possible that some of the alleles that dropped out could be recovered and available for interpretation. This could occur the injection

time of the sample onto the genetic analyzer was increased and/or the volume of the amplified product applied to the genetic analyzer were increased, especially for the samples where peaks below 35 RFUs were observed. No visible peaks were observed at the appropriate base pair size for several of the missing alleles, indicating that very little or no amplification of that allele occurred; there may not be an option for recovering those alleles except for re-amplification using the same, or preferably a higher, amount of DNA (See CSF1PO locus in Figure 22).

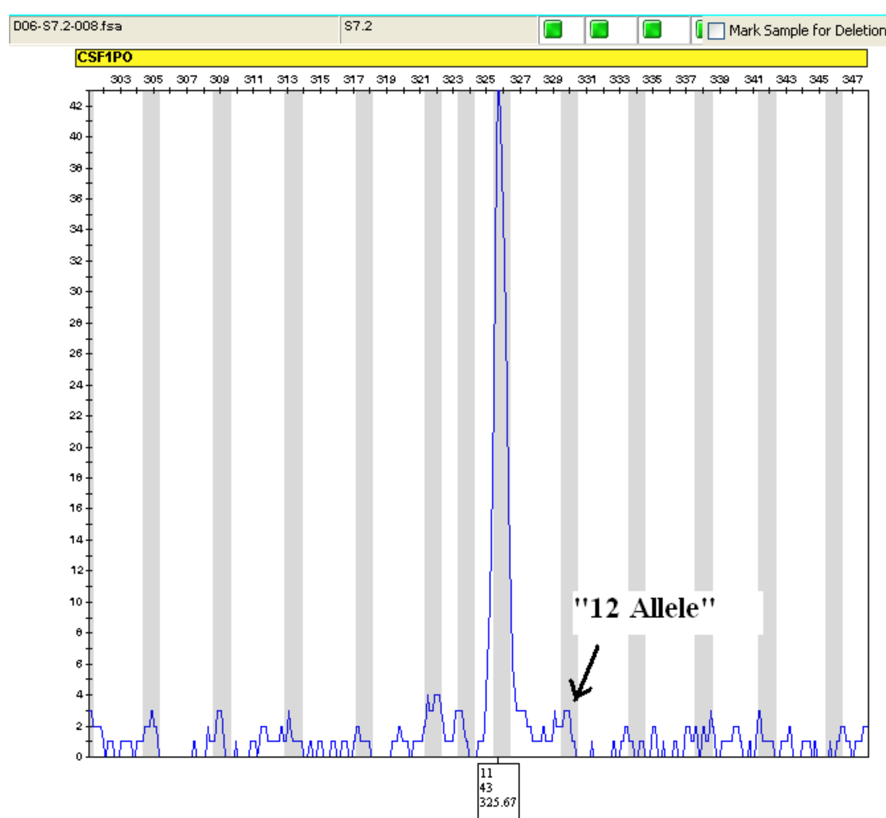

Figure 22: CSF1PO locus from the 0.031ng sample S7.2 from well D06. Note: no indication of the 12 allele. This is an example of the stochastic effects observed when small amounts of DNA are amplified.

Successful amplification of human DNA leading to the correct partial or complete DNA profiles can be achieved over a wide range of DNA concentrations. This has been demonstrated in samples quantified using the Quantifiler® Duo DNA Quantification Kit, amplified with the

AmpFℓSTR® Identifiler® Plus PCR Amplification Kit in a GeneAmp® PCR System 9700 thermal cycler using the manufacturer's recommended amplification conditions, and analyzed using the Applied Biosystems® 3130xl genetic analyzer with Data Collection Software version 3.0 and the GeneMapper® *ID-X* Software version 1.3. Based on this validation, the concentration of DNA selected for amplification could vary over a wide range depending on the condition and type of the DNA being amplified (e.g., degraded vs. high molecular weight; single-source vs. mixture; inhibited vs. not inhibited.)

Low copy number PCR has been operationally defined in Gill et al. (2006) as "the manifestation of stochastic effects leading to allelic imbalance, drop-out and increased prevalence of laboratory-based contamination." Low copy number DNA results from a low amount of genomic DNA available for PCR due to the presence or recovery of DNA from only a few cells and/or the degradation of DNA leading to the functional availability of a small amount of DNA that is sufficient in length to generate full-length fragments upon amplification. Generally, samples containing less than 100 pg (0.1 ng) of DNA (or <17 diploid cells) are considered to be "low copy number" samples. The 0.016 ng and 0.031 ng DNA samples amplified in this study support this definition. Allelic drop-out and/or imbalance between alleles and loci was apparent. All 0.063 ng DNA samples produced full profiles, indicating improved sensitivity of the amplification chemistry. Extreme caution should be used when interpreting results obtained from samples amplified with small amounts of DNA due to: 1) the loss of alleles (allelic drop-out), which may or may not be represented by a small peak below the peak amplitude threshold, 2) the imbalance of alleles and loci, which may make it difficult to determine if the DNA is from a single source or multiple donors, 3) the loss of the Y allele at the

Amelogenin locus leading to the false conclusion that the donor of the DNA is a female, and 4) the risk of contaminating alleles (allele drop-in) being present in the sample.

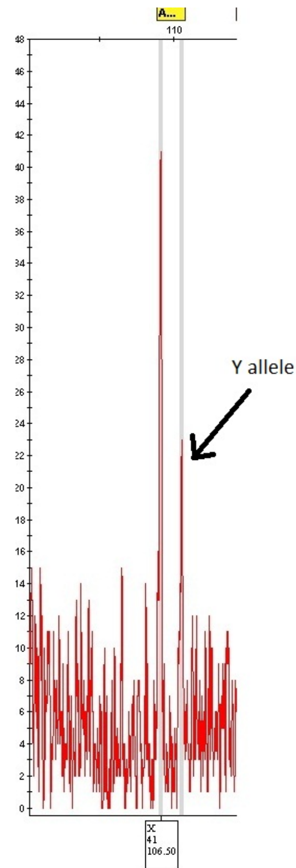

Figure 23: Amelogenin locus from one of the 0.016 ng sensitivity samples (S8.3 in well A08).

Note the presence of the Y allele below the peak amplitude threshold of 35 RFUs.

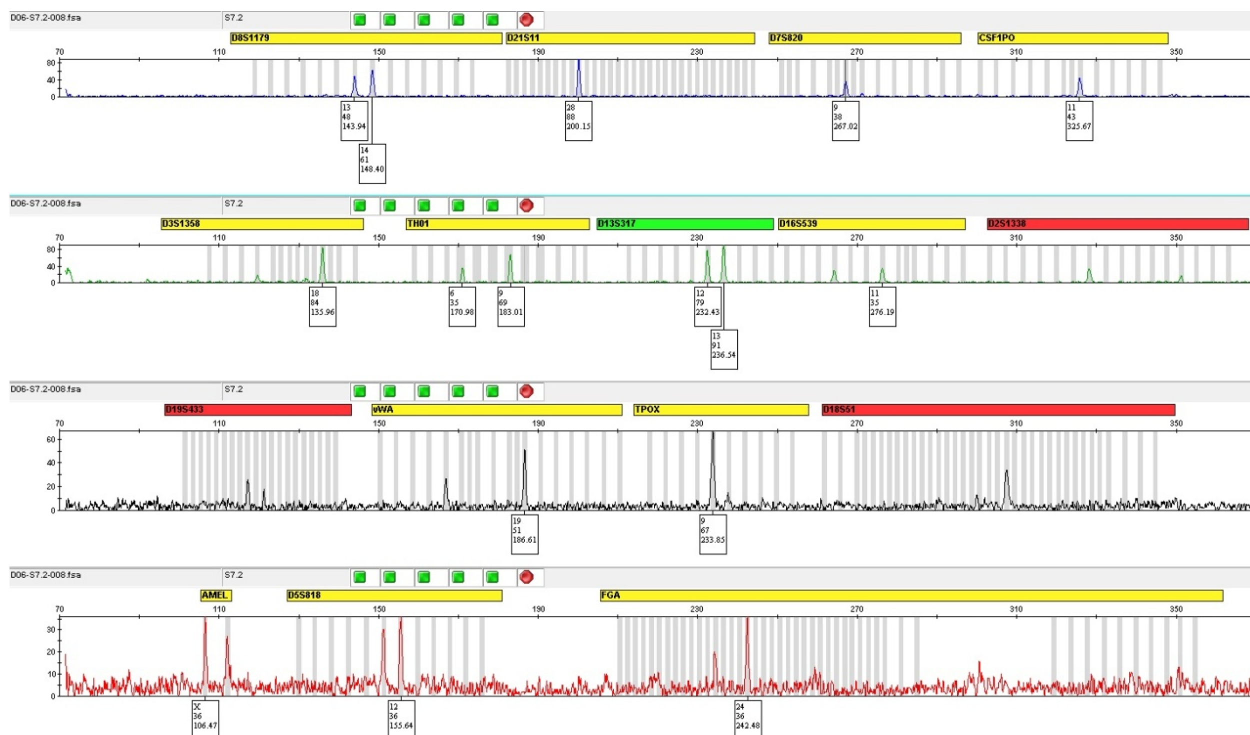

Figure 24: Peak height imbalance observed within and across loci when low amounts of input DNA were amplified. This is the 0.031 ng sample S7.2 from well D06. Note the allele imbalance and missing alleles at multiple loci.

The range aspect of the study provided a foundation for understanding the limitations of this amplification chemistry and identified artifacts that may affect analysis when very low or high amounts of DNA are amplified. This portion of the study was used by the laboratory in developing the analysis and calling thresholds as well as STR interpretation guidelines.

The sensitivity results generally supported that the normal range of 1.0 ng to less than 0.5 ng of human DNA from a single source amplified for 28 cycles with the Identifiler® Plus kit may be injected at 3kV for 5 seconds since: 1) all alleles present should be readily observed (i.e., no "drop-out" of alleles), 2) all alleles should be in the linear range of the charge coupled device (CCD) camera and therefore the data may be more quantitative, and 3) the data may be more

easily interpreted with reduced concern for the various artifacts and issues that arise with off-scale data and samples that overload the camera and 3130/3130xl capillaries.

It was important to identify an optimal amount of input template that does not lead to excessive pull-up peaks, artifact peaks and alternative stutter products that could confound mixture interpretations. Exceeding this range may cause peaks that fall in the -4 bp and +4 bp stutter positions, as well as the presence of known artifact peaks. Special attention should be paid to peaks in those positions when determining if a sample contains a mixture of two or more profiles. This is especially true if the amount of DNA amplified was greater than 1.0 ng/µl to permit high peak heights, which increases the likelihood of observing various artifacts, stutter and pull-up peaks. Having the option of amplifying a range of input DNA amounts provides the opportunity to better optimize the resulting DNA profile, with consideration of the peak heights needed to report and interpret the results while minimizing the amount of background and artifact peaks associated with PCR testing.

Limit of Detection

In the Identifiler® validation (2012), the analytical threshold for the Identifiler® amplification chemistry was set at 35 RFUs. The consistency of the data generated in this study supports the continued use of this threshold.

These observations were confirmed by mathematical analysis of the RFU peak height data obtained. The calculated values for each dye color for the Identifiler® Plus kit data are shown in Tables 8 and 9. The limit of detection (LOD) is the RFU value below which 99.7% of the background "noise" peaks should be observed. The limit of quantitation (LOQ) provides an upper limit value below which all background "noise" would generally be expected to fall.

The background noise was very low in all analyzed electropherograms. Every electropherogram had a minimum RFU value of 1, due to the application of algorithms in the GeneMapper *ID-X* software. The values typically ranged between 1 and 10 RFUs for all dye colors. Occasional peaks with values above 10 RFUs would appear arbitrarily in all dye colors at various base pair sizes. These may have been caused by spikes during capillary electrophoresis, pull-up from the GeneScan™ 500 LIZ® size standard, or artifacts present in the amplified sample. One sample (A03-NEG_28_1-001.2) contained an artifact that appeared to be similar to a dye blob in that it lacked the Gaussian curve of a regular peak. However, this "blob" was present in all dyes at approximately the same RFU. These peaks were considered extraneous and removed from analysis. No background peaks above 25 RFUs were observed for any color.

Artifacts and anomalies have been observed in all commercially available STR systems and molecular biological systems. Artifacts are typically reproducible from amplification to amplification, while anomalies are non-reproducible, intermittent occurrences that are not consistently observed in an STR system, e.g., spikes and baseline noise. These artifacts (as well as pull-up) were marked and not used for the analysis of the limit of detection or limit of quantitation.

The average background noise was lower for the 3130 than for the 3130xl, which is surprising because the 3130xl is a more sensitive instrument (See Figure 25). The plate was allowed to sit on the 3130xl autosampler for two days before being analyzed with the 3130. This may have caused degradation of any DNA present. When the negative control electropherograms were scrutinized, three samples (D04_NEG28-1_004.3, H04_NEG28-1_004.2, and D07_NEG29-1_004) showed evidence of degradation in the GeneScan™ 500 LIZ® size standard. These samples were re-run on a separate plate on the 3130.

Figure 24 compares the limits of detection between 28 and 29 cycles as well as between the 3130 and 3130xl genetic analyzers.

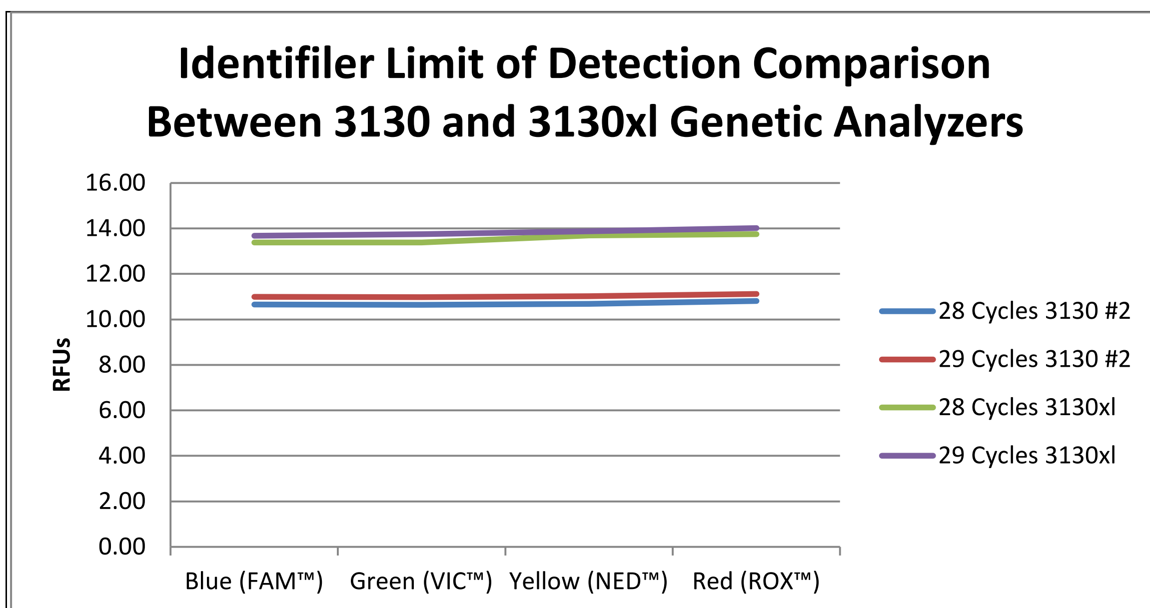

Figure 25: Identifiler® Plus LOD Comparison Between 3130 and 3130xl Genetic Analyzers

Figures 26 and 27 compare the limit of detection for Identifiler® Plus at 28 and 29 cycles with the LOD of the Identifiler® chemistry. Both kits were run on the 3130 and 3130xl genetic analyzers. The LODs for Identifiler® Plus at both cycle numbers were higher than the LOD for Identifiler®, but the results between dye channels were more consistent. This indicates that DNA profiles amplified with Identifiler® Plus will have more consistent peak heights between dye channels.

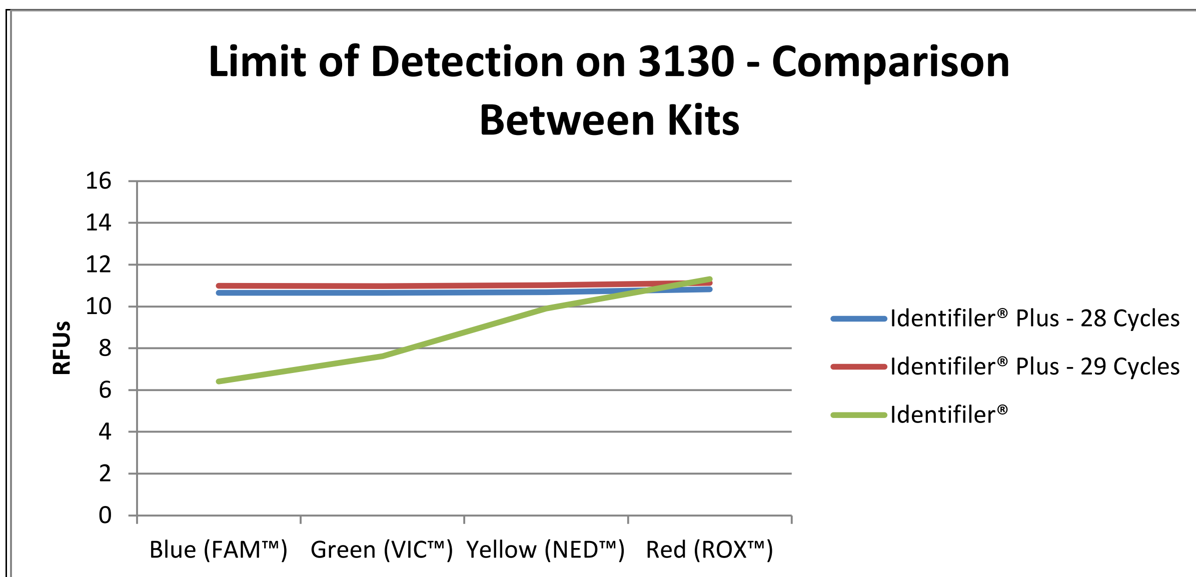

Figure 26: LOD Comparison Between Identifiler® and Identifiler® Plus on the 3130 Genetic Analyzer

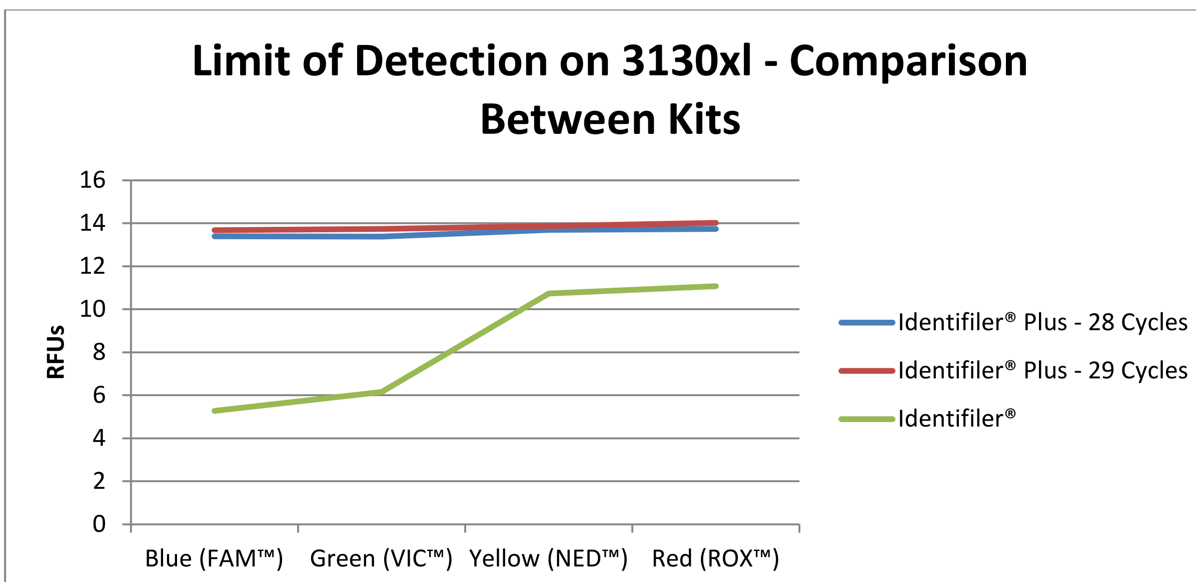

Figure 27: LOD Comparison Between Identifiler® and Identifiler® Plus on the 3130xl Genetic Analyzer

Limit of Quantitation

A limit of quantitation (LOQ) was calculated for each channel for both 28 and 29 cycles. The blue (6-FAM™) channel had the lowest LOQ for 29 cycles at 33.32 RFUs, and an LOQ of 33.11 RFUs for 28 cycles. The red (PET™) channel had the highest LOQ for both 28 and 29 cycles at 34.08 and 34.25 RFUs respectively. The green (VIC™) channel had the lowest LOQ for

28 cycles at 33.06 RFUs, and an LOQ of 33.35 RFUs for 29 cycles. The yellow (NED™) channel had an LOQ of 34.01 RFUs for 28 cycles and 33.78 RFUs for 29 cycles. The measured LOQ differences were negligible (See Figure 29).

The same calculations performed on the results for the 3130 genetic analyzer, and the channel with the lowest LOQ for both cycle numbers was the green channel, with 25.99 RFUs for both 28 and 29 cycles. The channel with the highest LOQ for both channels was the red channel, with 26.44 RFUs at 28 cycles and 26.36 RFUs at 29 cycles. The blue channel had an LOQ of 26.06 RFUs at 28 cycles and 26.04 RFUs at 29 cycles. The yellow channel had an LOQ of 26.08 RFUs for both 28 and 29 cycles.

Figure 28 compares the limit of quantitation at 28 and 29 cycles for the 3130 and 3130xl genetic analyzers. The LOQ for both cycle numbers was higher for the 3130xl than the LOQs for the 3130, which is consistent with the LODs for both instruments.

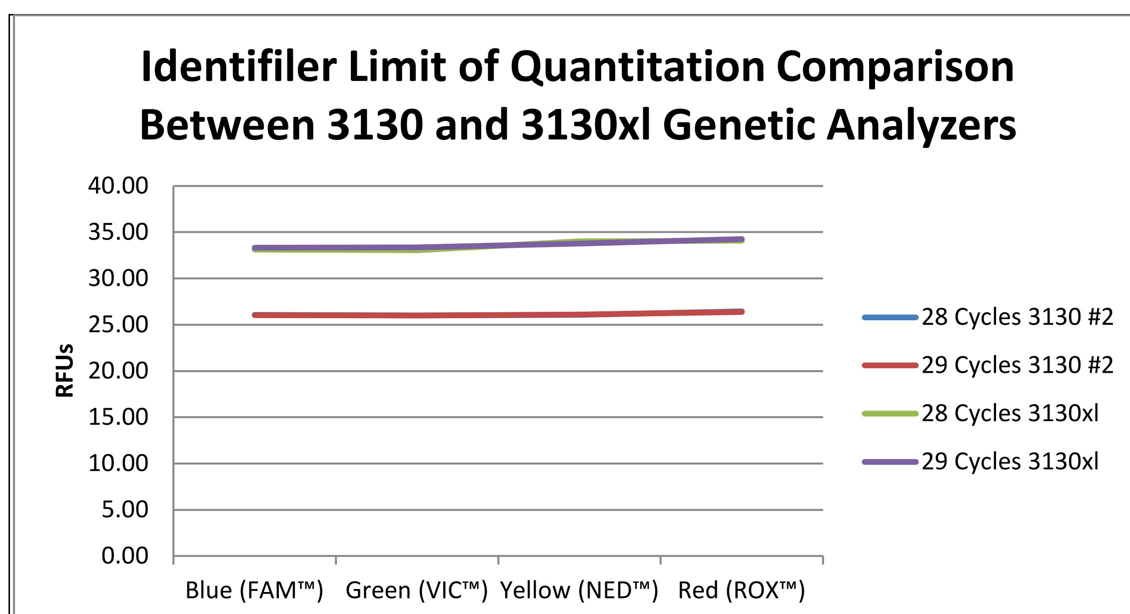

Figure 28: Identifiler® Plus LOQ Comparison Between 3130 and 3130xl Genetic Analyzers

Figures 29 and 30 compare the limit of quantitation for Identifiler® Plus at 28 and 29 cycles with the LOQ of the Identifiler® chemistry. Both kits were run on the 3130 and 3130xl genetic analyzers. The results mirror the LOD comparison in that the LOQ for Identifiler® Plus at both cycle numbers is higher than the LOQ for Identifiler®. The limits calculated for Identifiler® Plus at both cycle numbers are consistent with each other as well as across all dye channels, The manufacturer had previously reported improved inter-color balance, which is evidenced in Figures 28 and 29 and is a major improvement from the Identifiler® kit.

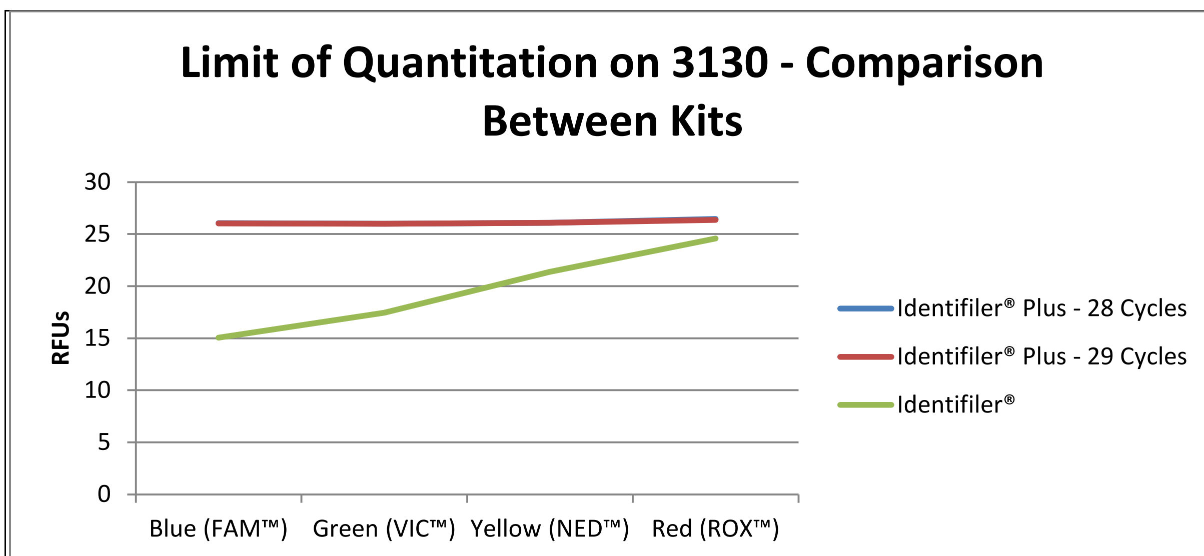

Figure 29: LOQ Comparison Between Identifiler® and Identifiler® Plus on the 3130 Genetic Analyzer

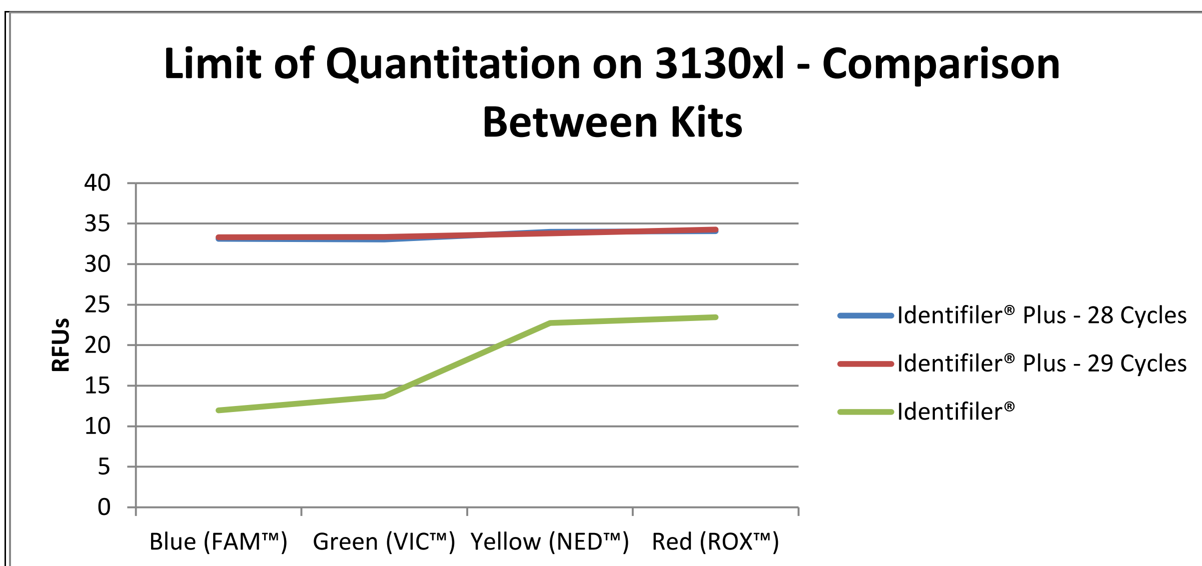

Figure 30: LOQ Comparison Between Identifiler® and Identifiler® Plus on the 3130xl Genetic Analyzer

See also Limit of Detection

<u>Carryover</u>

During analysis of the non-probative samples, it became apparent that the reagent blank for 5 samples had become contaminated. The DNA technician was determined to be the source of contamination. Since a DNA source from within the lab was identified, there was very little likelihood that the kit reagents themselves were contaminated. These samples (NP13, NP17, NP18, NP19, and NP20) were re-extracted with a new reagent blank, re-amplified, and run on the 3130xl genetic analyzer. A single peak was found in the negative control for the "6" allele at the TH01 locus with a peak height value of 41 RFUs. It could not be determined if the 6 allele came from the profile of the DNA technician, a sample associated with this negative control, or occurred as allele drop-in from a tube contaminant. It was decided that the five samples associated with the new reagent blank should be removed from analysis as a conservative measure. A 50 RFU reporting threshold (i.e. threshold in which to assign a statistical analysis)

should be established to ensure that the laboratory does not inadvertently include any unassociated profiles.

Ruggedness

Samples run at different times as well as on different thermal cyclers and 3130 instrumentation were consistent with expected results and with each other.

Future Directions

The Identifiler® Plus amplification chemistry has been compared to the Identifiler®, MiniFiler™, and Yfiler® chemistries in this validation. It was also compared on different thermal cyclers as well as two different genetic analyzers. In the future, studies should be done to examine the capabilities of Identifiler® Plus on the Applied Biosystems® 3500 genetic analyzer. It could also be compared to Promega® amplification chemistries, such as the PowerPlex® 16HS, PowerPlex® 21, and PowerPlex® Y23 amplification systems.

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