Internal Validation of the Applied Biosystems® AmpF*l*STR® Yfiler™ Amplification Kit on an Applied Biosystems® 3130 Genetic Analyzer

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ABSTRACT: Standard 8 of the National Quality Assurance Standards for Forensic Science Laboratories requires that an internal validation be performed before any new DNA technology can be introduced into casework. Once the validation is completed successfully, the next step will be to train the analysts to be Yfiler™ proficient. The Applied Biosystems® AmpF*l*STR® Yfiler™ PCR Amplification Kit is a multiplex assay that amplifies 17 loci located on the Y chromosome. Eight studies were performed in the validation: analytical threshold, precision, contamination, sensitivity, reproducibility, concordance, mixtures, and stutter. The results demonstrated that the internal validation was successful and that the Yfiler™ kit successfully amplified evidence samples from adjudicated casework, is male specific, and is precise.

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Introduction

Y chromosomal short tandem repeat (Y-STR) amplification targets the male component, the Y chromosome, and can be utilized in forensic casework where the male component is of interest, such as complex DNA mixtures [5] that may arise from offenses such as sexual assaults, homicides, and aggravated assaults. The majority of mixtures are composed of a high female component concentration and a low male component concentration, such as sexual assaults [2], which can lead to undetected male autosomal profiles [3,5]. By employing Y-STR analysis, the male component can be isolated via analysis of the Y chromosome, the only chromosome specific to males [6]. Analyzing loci on just one chromosome produces a haplotype, unlike a

genotype in autosomal analysis, which takes into account loci on multiple chromosomes. If the forensic sample happens to be a mixture, the resulting autosomal profile can be hard to interpret. The use of a male specific kit can isolate male profiles and aid in interpretation, especially in conjunction with a genotype produced from autosomal analysis. Since the Y chromosome is passed down the paternal line generationally, the resulting Y-STR profile is not specific to individual males, rather it is specific to male lineages. This trait allows Y-STRs to be beneficial in paternity and genealogical testing in addition to forensic casework [2]. Before any new DNA technology, be it an instrument or a chemistry kit, can be introduced into casework analysis, Standard 8 of the National Quality Assurance Standards for Forensic Science Laboratories requires a validation to be performed to demonstrate that the technology produces the desirable results, under what conditions the results are obtained, and the limitations of the technology [4,5].

The AmpF*l*STR® Yfiler™ PCR Amplification Kit may reduce time spent on mixture interpretation and increase the throughput of the Prince George's County Police Department DNA Laboratory. Y-STR amplification is of interest at Prince George's County Police Department DNA Laboratory due to the overwhelming number of cases with complex mixtures that the analysts encounter annually. The Yfiler™ kit is a multiplex assay that amplifies 17 loci located on the Y chromosome: DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385a/b, DYS393, DYS391, DYS439, DYS635, DYS392, Y GATA H4, DYS437, DYS438, and DYS448. Eight studies were performed in the validation: analytical threshold, precision, contamination, sensitivity, reproducibility, concordance, mixtures, and stutter. For each study, results were derived from peak height relative fluorescence units (RFU) values and/or allele calls. Since a developmental validation is performed by a manufacture before a product is

introduced into the market, it is predicted that the Yfiler™ kit will perform as expected in an internal validation, which tests for fewer variables than in a developmental validation.

Research Design

Analytical Threshold

An analytical threshold needs to be established in order to determine if peaks are due to noise in the baseline of an electropherogram or if they are true allelic peaks [8]. Once established, peaks that fall below the threshold are considered noise and those that fall above are considered allele peaks. The analytical threshold must be determined first in order to establish criteria to measure future results against. An analytical threshold was determined for a 5, 10, and 15 second injection time, with two different methods of calculation compared for the 5 second injection time threshold. Peak height RFU values were the measurements used in the calculations to determine the analytical threshold.

Precision and Contamination

The purpose of the precision study was to confirm correct sizing in order to reliably and accurately call alleles. A ±0.5 base pair "window" is placed around an allele, which allows for the correct detection and assignment. If the precision of this ±0.5 base pair window is skewed, then an off ladder (OL) allele or an allele that is just outside of the window can occur [1]. The precision of correct sizing directly impacts that ability to correctly assign allele calls. Yfiler™ Allelic Ladders were used to test for precision since all commonly occurring alleles for each locus are included in the Allelic Ladders. Setting up the Allelic Ladders and capillary electrophoresis (CE) run negatives in a checkerboard pattern can test for contamination. Base pair size and allele calls are analyzed for precision, whereas contamination focuses on allele calls.

Sensitivity

The purpose of the sensitivity study was to determine the range of DNA concentrations that result in reliable typing [9]. The range should be a concentration below one that produces off scale peaks and greater than one that demonstrates allelic drop out below the analytical threshold that was established earlier. According to the Yfiler™ manual, the target DNA concentration to be amplified is between 0.5 and 1.0ng [1]. A sensitivity study was performed on a 5, 10, and 15 second injection time in order to see if an increase amount of time will allow more peaks to be called in samples with lower DNA concentrations. Peak height RFU values and allele calls are analyzed to determine the range of DNA concentrations that can be typed.

Reproducibility

The purpose of the reproducibility study was to show the reliability and integrity of the Yfiler™ Kit by demonstrating the results obtained can be reproduced. Samples were amplified two different times and run on the same 3130 Genetic Analyzer on different days in order to compare the resulting allele calls and peak height RFU values.

Concordance

The purpose of the concordance study was to show the reliability and integrity of the Yfiler™ Kit by demonstrating the results obtained can be reproduced on separate instruments. Samples were amplified once and run on two different Genetic Analyzer 3130s on separate days. Allele calls and peak height RFU values were analyzed for consistency.

Mixtures

The purpose of the mixture study was twofold: first to show that the Yfiler™ kit is able to detect male mixtures, and secondly that male DNA can be detected in male-female mixtures. The concentrations at which individual Y-STR profiles can be reliably typed were analyzed for

both male-male and male-female mixtures. Allele calls and peak height RFU values were analyzed.

Stutter

The purpose of the Stutter Study was to determine the stutter ratio for the Prince George's County Police Department DNA Laboratory and compare the stutter ratios to those provided in the Applied Biosystems® AmpF*l*STR® Yfiler™ PCR Amplification Kit User's Manual [1]. In order to determine stutter ratios, allele calls and peak height RFU values were analyzed.

Materials and Methods

Kits

Table 1 lists the reagents used for each step of the analysis.

Table 1: List of reagents and manufacturer used for each analysis step

Procedure	Reagents	Manufacturer, City, State
Extraction	EZ1® DNA Investigator Kit	Qiagen®, Valencia, CA
Quantitation	Quanitfilier® Human Kit, glycogen	Applied Biosystems®, Foster City, CA
Amplification	AmpF*l*STR® Yfiler™ PCR Amplification Kit, AmpliTaq Gold® DNA Polymerase	Applied Biosystems®, Foster City, CA
Separation	AmpF*l*STR® Yfiler™ Allelic Ladder, Hi-Di™ Formamide, GeneScan™ 500 LIZ™ Size Standard, Performance Optimized Polymer (POP) 4, and 10X Run Buffer	Applied Biosystems®, Foster City, CA

Instrumentation

Table 2 lists the instruments used for each step of the analysis.

Table 2: List of instruments and manufacturer used for each analysis step

Procedure	Instruments	Manufacturer, City, State
Extraction	BioRobot EZ1® Workstation	Qiagen®, Valencia, CA
Quantitation	7500 Real-Time PCR System	Applied Biosystems®, Foster City, CA
Amplification	GeneAmp® PCR System 9700	Applied Biosystems®, Foster City, CA
Separation	3130 Genetic Analyzer	Applied Biosystems®, Foster City, CA
STR Analysis	GeneMapper® *ID* software version 3.2.1	Applied Biosystems®, Foster City, CA

Preparation of Samples

Samples were extracted using utilizing carrier RNA. Samples were then quantified using a half reaction and then diluted as necessary to obtain a DNA concentration of 1.0ng/µL, unless otherwise noted.

DNA Amplification

The total reaction volume was 25µL and was composed as described in **Table 3** with a DNA concentration target of 1.0ng in a total of 10uL, unless otherwise noted.

Table 3: Reagents and volumes needed for each Yfiler™ amplification reaction

Reagent	Volume per Reaction	
PCR Reaction Mix	9.2µL	Master Mix - 15µL
Primer Set	5.0µL	
AmpliTaq Gold® DNA Polymerase	0.8µL	
Template DNA	Up to 10µL combined	
Autoclaved Sterile H_2O		

If less than 10µL of DNA extract was used, autoclaved sterile water was used until 10µL was obtained. Amplification was performed per the Yfiler® user's manual protocol **(Table 4).** All amplifications utilized one Control DNA 9947a male specificity negative control (1:10 dilution), one Control DNA 007 male standard positive control, and one amplification negative.

Table 4: Amplification program for 9700 Thermal Cycler according to the Yfiler™user's manual

Initial Incubation Step	Cycle (30 cycles)			Final Extension	Final Hold
	Denature	Anneal	Extend		
Hold	Cycle			Hold	Hold
95°C 11 min	94°C 1 min	61°C 1 min	72°C 1 min	60°C 80 min	4°C ∞

Separation

Samples were set up on a 96 well reaction plate once unless otherwise noted. Samples were prepared by combining 8.7µL of Hi-Di™ Formamide, 0.3µL of GS-500LIZ, and 1µL of amplified DNA sample or Yfiler™ Allelic Ladder for a total reaction volume of 10µL. POP-4

and 10X Run Buffer were utilized for separation, which occurred with a 5 second injection time, unless otherwise noted, at 3kv on a 3130 Genetic Analyzer using collection software version 3.0.

STR Analysis

Data was analyzed using GeneMapper® *ID* software version 3.2.1 with the analysis parameters listed in **Table 5**.

Table 5: GeneMapper® *ID* software v3.2.1 analysis parameters

<table>
<tr><td>Size Calling Range Min.</td><td>75</td><td colspan="2">Size Standard</td><td colspan="3">CE_G5_HID_GS500</td></tr>
<tr><td>Size Calling Range Max.</td><td>450</td><td colspan="2">Size Calling Method</td><td colspan="3">Local Southern</td></tr>
<tr><td>Baseline Window</td><td>51 pts.</td><td colspan="2">Smooth Options</td><td colspan="3">Light</td></tr>
<tr><td>Min. Peak Half Width</td><td>2 pts.</td><td colspan="2">Peak Detection Algorithm</td><td colspan="3">Advanced</td></tr>
<tr><td>Polynomial Degree</td><td>3</td><td>Minus Stutter Distance</td><td>Tri</td><td>Tetra</td><td>Penta</td><td>Hexa</td></tr>
<tr><td>Peak Window Size</td><td>15 pts.</td><td>From</td><td>2.25</td><td>3.25</td><td>4.25</td><td>5.25</td></tr>
<tr><td>Slope Threshold for Peak Start</td><td>0.0</td><td>To</td><td>3.75</td><td>4.75</td><td>5.75</td><td>6.75</td></tr>
<tr><td>Slope Threshold for Peak End</td><td>0.0</td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Threshold – 5 seconds SWGDAM

Six amplification negatives and two Control DNA 9947a male specificity negative controls (1:10 dilution) were amplified, injected three times, and separated. For analysis, the threshold was set to 1 RFU for all dye channels, except orange, which had a threshold of 50 RFU. All peaks within ±2 base pairs of a size standard peak were filtered and deleted in order to account for peaks from spectral pull up. The average peak height and standard deviation was calculated for each dye channel except orange, as well as the minimum and maximum peak height RFU values were recorded. To these RFU values, the equation in **Figure 1** was applied, and the analytical threshold was calculated. The analytical threshold + 3σ, 5σ, 7σ, and 10σ was calculated.

Figure 1: Example equation from Section 1.1 of SWGDAM Guidelines [8]

$$AT = 2(Y_{max} - Y_{min})$$

Y_{max} is the highest peak within instrumental noise data
Y_{min} is the signal of the lowest trough
AT is the analytical threshold calculated

Threshold – 5 seconds

Seven Control DNA 007 positive controls, five samples, and five reagent blanks were amplified and separated. The baseline of each dye channel, except orange, was analyzed for the three highest peaks, disregarding those peaks in stutter position, those from spectral pull up, and the true allele. In the case of tetranucleotide repeats, peaks in ±4 base pair stutter position were ignored, as was ±2 and ±4 for DYS19, ±3 for DYS393, ±5 for DYS438, and ±6 for DYS448. For each sample, each dye channel had three peaks chosen: the highest peak before any allele calls, the highest peak after all allele calls, and the highest peak in between alleles. For reagent blanks, one peak was chosen after the 75 base pair (bp) size standard peak and before the first allele bin, one peak after the last allele bin but before the 450 bp size standard peak, and then one allele in between. The average and standard deviation was calculated for each dye channel and for the overall Yfiler® kit.

Threshold – 10 and 15 seconds

Five samples and five reagent blanks were amplified, set up in duplicate, run with a 10 and 15 second injection time, and analyzed the same way as in the 5 second injection time study.

Precision and Contamination

Twenty four Allelic Ladders and twenty four CE run negatives were set up in a checkerboard pattern in triplicate, run, and analyzed with a 150 RFU threshold. The checkerboard pattern tests for contamination. For precision, the average and standard deviation were calculated for each allele and for each marker. For contamination, the run negatives were inspected for any alleles called that were not due to spectral pull up.

Sensitivity

Three samples were diluted to concentrations of 2.0ng/µL, 1.0ng/µL, 0.5ng/µL, 0.25ng/µL, 0.125ng/µL, and 0.0625ng/µL, and two samples diluted to the pervious mentioned

concentrations starting at 1.0ng/µL. 1µL of each diluted sample was amplified, separated, and analyzed with a 150 RFU threshold. The same amplified DNA product was reset up and run with a 10 and 15 second injection time, and analyzed with a threshold of 150 RFU and 200 RFU respectively.

Reproducibility

Twenty samples and four reagent blanks were amplified, run at 10AM, and analyzed with a 150 RFU threshold. The amplification and run were repeated, with the run at 3PM on a separate day, and the results analyzed with a 150 RFU threshold.

Concordance

Twenty samples and four reagent blanks were amplified, run on the Applied Biosystems® 3130 Genetic Analyzer (B), and analyzed with a 150 RFU threshold. The same amplification product was re-set up, run on the Applied Biosystems® 3130 Genetic Analyzer (A), and analyzed with a 150 RFU threshold.

Male – Male Mixtures

Three male samples were diluted to concentrations outline in **Table 6**. From these dilutions the following male to male ratios were created: 1:1, 1:3, 1:5, 1:10, 1:20, 20:1, 10:1, 5:1, 3:1, and 1:1:1 (**Table 7**).

Table 6: Sample key

Sample	Concentration (ng/µL)	Letter
YF_MS_10_098_R01A1	1.0	A
YF_MS_11_212_R01A1	1.0	B
YF_MS_11_084_R02A1	0.10	C
YF_MS_11_084_R02A1	1.0	C*

Table 7: Male-male mixture ratios

Ratio	A (µL)	B (µL)	C* (µL)
1:1	2	2	---
1:3	2	6	---
1:5	2	10	---
1:10	2	20	---
1:20	2	40	---
20:1	40	2	---
10:1	20	2	---
5:1	10	2	---
3:1	6	2	---
1:1:1	5	5	5

The three single source 1.0ng/µL male samples and the ten mixture samples were amplified, run in duplicate, and analyzed with a 150 RFU threshold.

Male – Females Mixtures

Two female samples were diluted to the concentrations outline in **Table 8**. From these dilutions, and from dilution C and C* from **Table 6**, the following male-female ratios were created: 1:10000, 1:5000, 1:1000, 1:100, 1:1, and a male to female to female ratio of 1:1:1 (**Table 9**).

Table 8: Sample key

Sample	Concentration (ng/µL)	Letter
YF_MS_KJ	20.47	D
YF_MS_KJ	1.0	D*
YF_MS_09_571_ R01A1	1.0	E

Table 9: Male-female mixture ratios

Ratio	C (µL)	C* (µL)	D (µL)	D* (µL)	E (µL)
1:10000	1	---	48.80	---	---
1:5000	1	---	24.38	---	---
1:1000	1	---	---	99	---
1:100	2	---	---	18	---
1:1	6	---	---	6	---
1:1:1	---	5	---	5	5

The two single source 1.0ng/µL female samples and the six mixture samples were amplified, run in duplicate, and analyzed with a 150 RFU threshold.

Stutter

Samples and at least one Allelic Ladder were pulled from the previous studies' run folders, placed into new run folders, and were analyzed without the marker specific stutter ratio and with a 20 RFU threshold. Only the peaks from spectral pull up and those that did not fall in the stutter position were deleted. The ±4 stutter ratio was calculated for each sample, or the ±2, ±3, ±5, and ±6 stutter ratio where applicable. The minimum stutter ratio, maximum stutter ratio, average stutter ratio, the standard deviation, and average + 3σ were calculated for each locus. The resulting stutter ratios for each locus were then compared to the stutter ratios provided by Applied Biosystems®.

National Institute of Standards and Technology (NIST) Standard Reference Material (SRM)

NIST standard reference material 2391b Genomic 1 was amplified, separated, and analyzed with a 150 RFU threshold.

Results and Discussion

Threshold – 5 seconds SWGDAM

Table 10 shows the results from the threshold study done according to the recommendation from SWGDAM. The highest calculated analytical threshold value was 108, in the green dye channel. The AT + 10σ was 143.62, and the analytical threshold was set to 150 RFU.

Table 10: Statistics and analytical threshold (AT) + σ calculated for noise in 24 negative controls

Dye	Average	STDEV	Min.	Max.	AT	AT+3σ	AT+5σ	AT+7σ	AT+10σ
BLUE	1.99	1.40	1	15	28	32.19	34.98	37.78	41.97
GREEN	2.70	3.56	1	55	108	118.68	125.81	132.93	143.62
YELLOW	5.07	1.70	1	16	30	35.11	38.52	41.93	47.05
RED	5.45	2.43	1	29	56	63.29	68.15	73.01	80.30

Threshold – 5 seconds

The baseline of each dye channel, except orange, was analyzed for the three highest peaks, disregarding those peaks in stutter position, those from spectral pull up, and true alleles (**Table 11** and **Table 12**). 51 peaks total for each dye channel overall were recorded. The average and standard deviation for each dye channel and for the overall Yfiler™ kit was calculated. The threshold for a 5 second injection time was set at 150 RFU (**Table 13**).

Table 11: Sample POS_007_060512_KJ_G10; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	27	25	10
GREEN	80	40	5
YELLOW	49	38	11
RED	39	22	11

Table 12: Reagent Blank 12_571_MVS_RB3_E04; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	13	11	4
GREEN	46	11	4
YELLOW	16	11	9
RED	10	25	8

Table 13: 5 second injection time threshold study results

Dye	Average	STDEV	Ave+σ	Ave+3σ	Ave+5σ	Ave+7σ	Ave+10σ
BLUE	16.69	11.56	28.25	51.37	74.50	97.63	132.31
GREEN	32.82	31.42	64.24	127.07	189.91	252.74	346.99
YELLOW	19.75	13.00	32.74	58.74	84.74	110.73	149.73
RED	18.73	11.12	29.85	52.09	74.34	96.59	129.96
OVERALL	23.56	19.71	43.28	82.70	122.13	161.56	220.70
					141.84		

Threshold – 10 seconds

The baseline was analyzed in the same way as in the 5 seconds study (**Table 14** and **Table 15**). 48 peaks total in each dye channel were recorded. The average and standard deviation for each dye channel and for the overall Yfiler® kit was calculated. The threshold for a 10 second injection time was set at 150 RFU (**Table 16**).

Table 14: Sample POS_007_060512_KJ_G10; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	47	36	23
GREEN	141	31	3
YELLOW	75	57	12
RED	65	48	8

Table 15: Reagent Blank 12_571_MVS_RB3_E04; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	15	19	3
GREEN	86	18	4
YELLOW	11	9	7
RED	12	45	8

Table 16: 10 second injection time threshold study results

Dye	Average	STDEV	Ave+σ	Ave+3σ	Ave+5σ	Ave+7σ	Ave+10σ
BLUE	20.75	15.94	36.69	68.56	100.43	132.30	180.10
GREEN	45.06	47.69	92.75	188.13	283.52	378.90	521.97
YELLOW	20.88	18.59	39.46	76.64	113.82	151.00	206.77
RED	25.90	17.11	43.00	77.22	111.43	145.64	196.97
OVERALL	30.45	29.66	60.10	119.41	178.72	238.04	327.00
				149.07			

Threshold – 15 seconds

The baseline was analyzed in the same way as in the 5 and 10 seconds study (**Table 17** and **Table 18**). 48 peaks total in each dye channel were recorded. The average and standard deviation for each dye channel and for the overall Yfiler® kit was calculated. The threshold for a 15 second injection time was set at 200 RFU (**Table 19**).

Table 17: Sample POS_007_060512_KJ_G10; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	66	24	25
GREEN	231	161	5
YELLOW	90	78	11
RED	106	52	12

Table 18: Reagent Blank 12_571_MVS_RB3_E04; peak height in Relative Florescence Unit

Dye Channel	Peak Before First Allele (RFU)	Peak Between (RFU)	Peak After Last Allele (RFU)
BLUE	34	41	4
GREEN	157	25	4
YELLOW	9	17	10
RED	28	86	11

Table 19: 15 second injection time threshold study results

Dye	Average	STDEV	Ave+σ	Ave+3σ	Ave+5σ	Ave+7σ	Ave+10σ
BLUE	33.50	30.71	64.21	125.63	187.05	248.47	340.60
GREEN	77.96	82.27	160.23	324.76	489.30	653.84	900.64
YELLOW	26.81	27.91	54.72	110.54	166.36	222.18	305.91
RED	42.00	30.37	72.37	133.12	193.87	254.62	345.75
OVERALL	45.07	52.04	97.10	201.18	305.25	409.33	565.44
				201.18			

Precision

3288 concordant allele calls for the Yfiler™ Allele Ladders were produced. The mean and standard deviation of the base pair size was calculated for each allele, as well as per marker **(Table 20).** The maximum standard deviation for each marker, along with the corresponding allele, is also listed in **Table 20**. All alleles were typed correctly.

Table 20: Yfiler™ precision study results

Marker	Average Standard Deviation	Maximum Standard Deviation Observed (Allele)
DYS389I	0.085	0.100 (15)
DYS389II	0.120	0.138 (34)
DYS390	0.094	0.107 (27)
DYS456	0.075	0.078 (13)
DYS19	0.082	0.095 (10)
DYS385	0.152	0.241 (25)
DYS458	0.045	0.052 (15)
DYS391	0.083	0.097 (11)
DYS392	0.134	0.176 (16)
DYS393	0.070	0.077 (13)
DYS439	0.068	0.076 (10)
DYS635	0.067	0.075 (25)
DYS437	0.065	0.074 (13)
DYS438	0.053	0.063 (13)
DYS448	0.104	0.155 (23)
DYSGATA H4	0.066	0.075 (13)
Overall Kit	0.095	0.241 (25)

The average standard deviation in sizing for each of the loci in the Allelic Ladder ranged from 0.045 – 0.152. All the average standard deviations for each marker were below the 0.15 value, except for DYS385, which had an average standard deviation of 0.152. **Table 21** shows the breakdown of the alleles for DYS385 that have a corresponding average standard deviation greater than the 0.15 value. The range of standard deviation for DYS385 is 0.110 – 0.241.

Table 21: Breakdown of DYS385 alleles above 0.15

Marker	Allele	Average Standard Deviation
DYS385	19	0.156
DYS385	20	0.160
DYS385	21	0.165
DYS385	22	0.196
DYS385	23	0.208
DYS385	24	0.235
DYS385	25	0.241

Although marker DYS392 had an average standard deviation of 0.134, which is below the 0.15 value, there were five alleles with an average standard deviation greater than 0.15. The breakdown of the DYS392 alleles is shown in **Table 22.** The range of standard deviation for DYS392 is 0.083 – 0.175.

Table 22: Breakdown of DYS392 alleles above 0.15

Marker	Allele	Average Standard Deviation
DYS392	14	0.157
DYS392	15	0.166
DYS392	16	0.176
DYS392	17	0.173
DYS392	18	0.175

Marker DYS448 also showed one allele greater than the 0.15 value, allele 23 **(Table 23).** The range of standard deviation for DYS448 is 0.066 – 0.155.

Table 23: Breakdown of DYS448 alleles above 0.15

Marker	Allele	Average Standard Deviation
DYS448	23	0.155

Contamination

The twenty four run negatives showed no allele calls or other contamination.

Sensitivity – 5 seconds

The allele call for each marker along with the corresponding peak height were recorded for each sample. Based on a 5 second injection time and a 150 RFU threshold, all ten samples with a DNA input concentration of 0.250ng produced a full profile, while six produced a full profile with 0.125ng. For 0.0625ng, partial profiles were produced with an average of 11 loci.

The RFU values for 2.0ng/µL were in the range of high 3000 – low 6000, with the RFU values showing a proportional decrease in RFU values as the DNA input concentration decreases. The DNA concentration input range the produced full profiles is from 2.0ng/µL to 0.250ng/µL.

Sensitivity – 10 seconds

The allele call for each marker along with the corresponding peak height was recorded for each sample. Based on a 10 second injection time and 150 RFU threshold, all ten samples with a DNA input concentration of 0.250ng produced a full profile, while nine samples produced a full profile with 0.125ng. For 0.0625ng, partial profiles were produced with an average of 14 loci, while two samples displayed full profiles. The RFU values for 2.0ng/µL were in the range of high mid 6000- low 9000, with the RFU values showing a proportional decrease in RFU values as the DNA input concentration decreases. Due to allelic drop out at and below 0.125ng/µL and to allelic drop in and high stutter percentages seen at 2.0ng/µL, the DNA concentration input range that produced full profiles is from 1.0ng/µL to 0.250ng/µL.

Sensitivity – 15 seconds

The allele call for each marker along with the corresponding peak height was recorded for each sample. Based on a 15 second injection time and a 200 RFU threshold, all ten samples with a DNA input concentration of 0.125ng produced a full profile, while only two samples produced a full profile with 0.0625ng. The RFU values for 2.0ng/µL were in the range of mid 7000- mid 9000, with the RFU values showing a proportional decrease in RFU values as the DNA input concentration decreases. Due to allelic drop out at 0.0625ng/µL and to off scale peaks, allelic drop in, and high stutter percentages seen at 2.0ng/µL and 1.0ng/µL, the DNA concentration input range that produced full profiles is from 0.5ng/µL to 0.125ng/µL.

Reproducibility

The allele calls from the first CE run and those from the second CE run matched, with similar RFU values demonstrated for the same samples between the two runs.

Concordance

The allele calls from the CE run on (B) and the allele calls from the CE run on (A) matched, with similar RFU values demonstrated for the same samples between the two runs.

Male – Male Mixtures

Full profiles were obtained for both runs of the 1:3 mixtures, the 3:1, and the 5:1 mixture. The 1:5 mixture showed allelic dropout at 5 loci for one run and four loci for the other run. Full profiles were called at the 5:1 ratio. The male trio mixture showed a full profile in one of the runs, and only had drop out at DYS390 in the second run. There was a peak at the correct allele call, it was just below the analytical threshold of 150 RFU. In all mixtures, the major contributor RFU values were higher than the minor contributor values.

Male – Females Mixtures

Full male profiles were obtained from the 1:1 mixture, and partial profiles were obtained in the 1:100 mixture, with an average of 6 loci called. Peaks were present in the other male-female mixtures, but the peaks were below the analytical threshold of 150 RFU. The 1:1000 mixture showed very few allelic peaks below threshold, however the 1:5000 and 1:10000 mixtures showed more allelic peaks below threshold. The 1:1:1 male to female to female mixture gave a full male profile. The RFU values for the 1:10000 mixture were lower than the 1:5000, with the 1:1 mixture having the highest RFU values overall. The 1:1:1 mixture had lower RFU values than the 1:1, but higher than the 1:100.

Stutter

Table 24 displays each locus with the corresponding stutter percentage range, average stutter percentage, standard deviation, and average + 3σ. A total of 727 data points for used for all combined stutter, with 512 data points for all minus stutter and 215 data points for all plus stutter. Ten of the stutter values calculated from the stutter study are above the stutter values from Applied Biosystems® with six of the publish values higher than the calculated values.

Table 24: Percentage stutter values for each locus

Locus	Stutter	Number of Data Points	Stutter Range (%)	Average Stutter (%)	Standard Deviation	Average + 3σ (%)	AB Published Stutter Values (%) [1]
DYS456	-4	29	9.18-15.58	11.13	1.84	16.66	13.21
	+4	26	0.72-2.28	1.13	0.32	2.10	---
DYS389I	-4	29	4.25-8.00	6.24	1.18	9.78	11.79
	+4	6	0.49-1.54	0.90	0.50	2.40	---
DYS390	-4	29	5.57-10.28	7.83	1.43	12.11	10.4
	+4	0	---	---	---	---	---
DYS389II	-4	29	10.29-15.07	12.58	1.30	16.49	13.85
	+4	21	0.74-3.99	1.56	1.08	4.79	---
DYS458	-4	29	7.55-12.54	9.40	1.36	13.47	12.2
	+4	19	0.47-3.55	1.06	0.89	3.72	---
DYS19	-4	29	6.32-11.21	8.06	1.29	11.94	11.4
	-2	29	6.46-9.76	8.14	0.82	10.59	10.21
	+2	24	1.11-2.35	1.55	0.32	2.50	---
	+4	10	0.55-0.94	0.66	0.11	1.00	---
DYS385a/b	-4	51	5.35-14.70	9.06	2.49	16.54	13.9
	+4	19	0.55-3.29	1.40	0.78	3.74	---
DYS393	-4	29	8.54-13.68	10.37	1.60	15.16	12.58
	+4	27	0.84-3.14	1.58	0.53	3.18	---
DYS391	-4	29	4.86-8.42	6.40	0.78	8.73	11.62
	+4	3	0.41-0.47	0.44	0.03	0.55	---
DYS439	-4	29	5.27-7.95	6.30	0.59	8.07	11.18
	+4	14	0.58-2.31	1.22	0.59	2.99	---
DYS635	-4	27	0.69-8.73	5.53	2.20	12.15	10.75
	+4	1	0.71-0.71	0.71	---	2.85	---
DYS392	-3	29	7.56-12.90	9.79	1.66	14.76	16.22
	+3	29	2.16-6.65	3.81	1.23	7.49	7.9
Y GATA H4	-4	29	5.63-9.02	7.07	0.78	9.43	11.08
	+4	12	0.58-1.42	0.76	0.23	1.44	---
DYS437	-4	29	2.87-6.20	4.67	0.73	6.87	8.59
	+4	2	0.54-0.57	0.56	0.02	0.62	---
DYS438	-5	28	1.16-3.90	2.47	0.66	4.46	4.28
	+5	2	1.58-2.35	1.96	0.55	3.61	---
DYS448	-6	29	1.84-4.28	2.91	0.66	4.90	4.96
	+6	0	---	---	---	---	---

National Institute of Standards and Technology (NIST) Standard Reference Material (SRM)

The NIST standard reference material 2391b Genomic 1 produced a full Y-STR profile (**Table 25**).

Table 25: NIST SRM 2391b Genomic 1 Y-STR Profile

Marker	Allele Call
DYS456	**15**
DYS389I	**12**
DYS390	**22**
DYS389II	**29**
DYS458	**16**
DYS19	**15**
DYS385a/b	**13, 16**
DYS393	**13**
DYS391	**10**
DYS439	**11**
DYS635	**21**
DYS392	**11**
Y GATA H4	**12**
DYS437	**16**
DYS438	**10**
DYS448	**21**

Microvariants and Off Ladder (OL) Alleles

A microvariant and two OL alleles were observed in this validation. One sample from the sensitivity study had a 17.2 at 144.70 bp for locus DYS458. This microvariant was reproduced in the 1.0ng, 0.5ng, 0.25ng, 0.125ng, and 0.0625ng amplifications. Two samples from the concordance study had an Off Ladder <20 allele call at 233.79 bp for locus DYS635. These two samples were from the same individual, one from an evidence sample and one from a reference sample. The OL<20 allele call was produced in both sample profiles.

Discussion

Threshold

The peaks above baseline can be attributed to noise, which is due in part to the amplification components such as unincorporated primers and deoxynucleotide triphosphates

(dNTPS). As the injection time increased, the peak height RFU values increased since the amount of amplification components injected into the CE increased (**Table 11**, **Table 12, Table 14, Table 15, Table 17, Table 18**). A few peaks, some of which are noted in the Yfiler™ kit user's manual, have been reproduced just above baseline in the majority of the samples and controls [1] (**Table 26, Figure 2**). Run negatives do not show these artifact peaks, which demonstrates that the peaks are due to the nature of the amplification process (**Figure 3**). The 126 bp peak in the blue channel and the 121 bp in the green channel are present in the run negative, but at a lower RFU. For all threshold studies, the green dye channel had the highest average RFU and standard deviation.

Table 26: Characteristic Peaks of the Yfiler™ Amplification and corresponding base pair size

Table 26: Artifact peaks for Yfiler™ amplification

Dye Channel	Approximate Base Pair Size
Blue	80
Green	82 & 89
Yellow	80 & 98
Red	80, 207, 219

Figure 2: Amplification negative electropherogram showing artifact peaks + higher blue and green peak

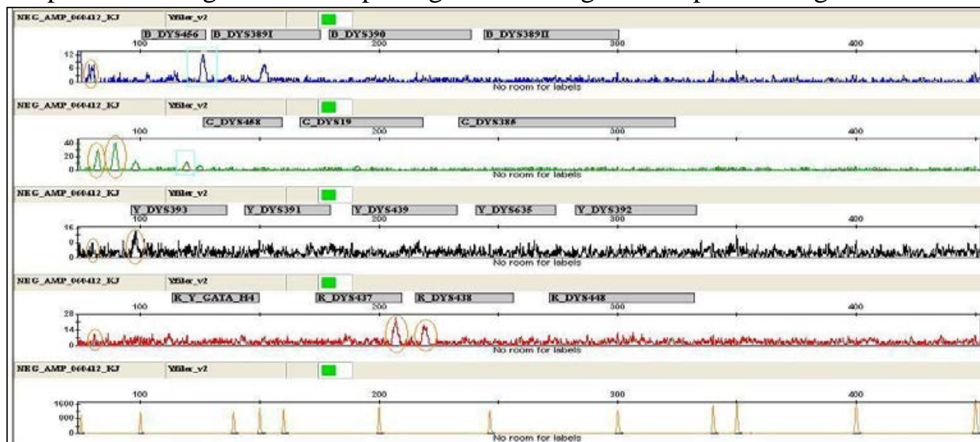
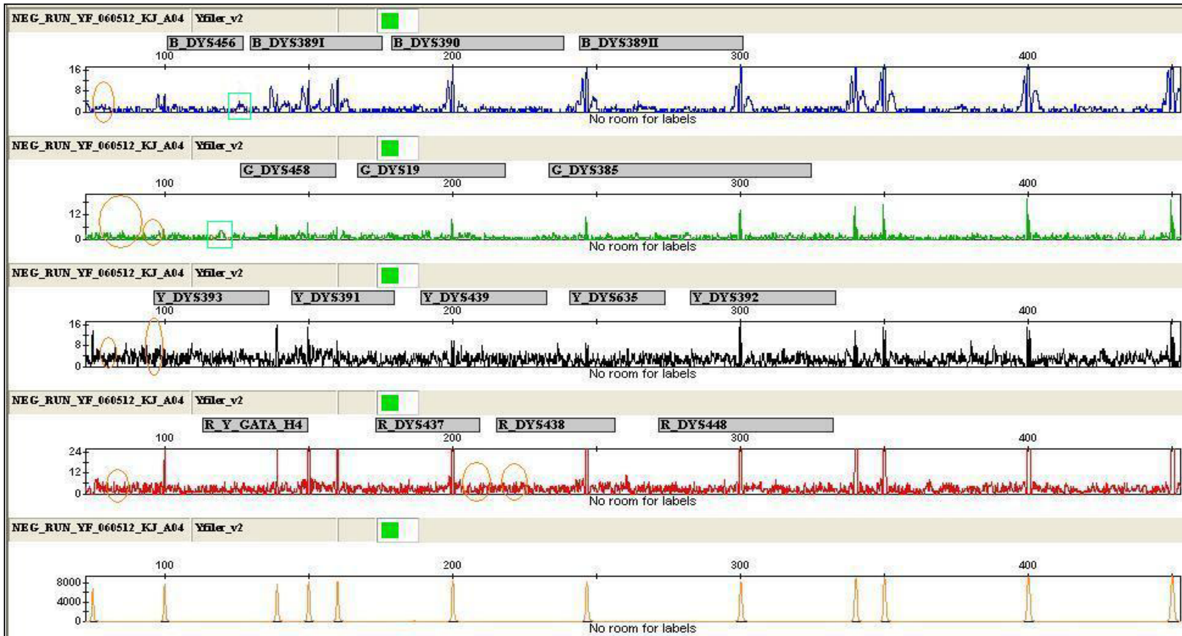


Figure 3: Run negative electropherogram showing artifact peaks + lower blue and green peak



The method to calculate the threshold provided by the SWGDAM Guidelines was only performed for the 5 second threshold study in order to be concordant with the way that Prince George's Country Police Department DNA Laboratory have validated other kits, which was to chose the three highest peaks. A threshold of 150 RFU was chosen for the SWGDAM way after discussion about which AT + σ should be utilized and the threshold from a previous Yfiler™ validation, which was 150 RFU [5]. Using either method, the analytical threshold was calculated to be 150 RFU. The three peak method takes into account data from samples other than controls, while the SWGDAM method is faster.

Precision

Even with one average standard deviation, from locus DYS385, greater than 0.15, all alleles fell within the ±0.5 base pair window (**Figure 4**). The largest size deviation was allele 25 at -0.44 base pairs. The results for DYS385 are not unexpected because larger alleles are generally prone to migration variation since they are more sensitive to temperature and humidity changes. The results for DYS385 have been observed in another study [5]. DYS392 and

DYS448 showed an average standard deviation less than the 0.15 value, but alleles were observed above 0.15. This could be contributed to DYS392 and DYS448, along with DYS385, having the largest amplicon, amplified DNA fragments, size of their respective dye channels (**Figure 5**).

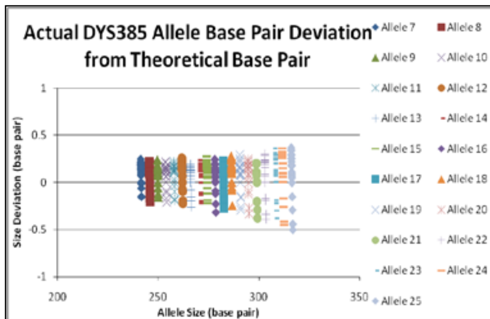


Figure 4: Size deviation of the 19 alleles of DYS385 from 24 samples

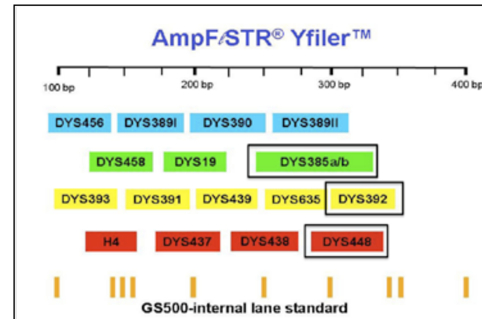


Figure 5: Loci according to the Yfiler™ Allelic Ladder [10]

Contamination

The checkerboard pattern produced an alternating pattern of Allelic Ladders and CE run negatives per injection. For example, if an Allelic Ladder was in well A01, then a run negative would be in well E01 to see if the capillary would carry over any sample and contaminate the run negative. The results showed that cross contamination did not occur.

Sensitivity

The lower the DNA input concentration, the greater the chance is that allelic dropout can occur. Increasing the injection time increases the amount of amplicons to be separated, and as such, the resulting RFU values. An increase in injection time can allow samples with low DNA input concentration to be typed, but can led to off scale peaks with higher DNA concentration samples.

Reproducibility

Reproducing the same allele calls and similar RFU values demonstrated that the Yfiler™ kit was able to accurately and reliably amplify DNA.

Concordance

Reproducing the same allele calls and similar RFU values demonstrated the concordance of the two 3130 Genetic Analyzers.

Male – Male Mixtures

The allelic drop out present in the 1:5 mixture, but not present in the 5:1 mixture could be due to the fact that the sample A showed degradation. Since sample A was the minor DNA component in the 1:5 mixture, this degradation would influence the alleles that were called. When sample A was the major component and sample B, which did not show degradation, was the minor component, full profiles were called at 5:1 ratio. The mixtures were sample A was the minor component show earlier and greater allelic dropout when compared to the mixtures were sample B was the minor component. It is probable that the degradation of sample A is one factor causing this effect. The major contributor RFU values being higher than the minor contributor values supports the fact that more major contributor DNA was amplified than minor.

Male – Females Mixtures

The 1:1000 mixture showed very few allelic peaks below threshold, while the 1:5000 and 1:10000 mixtures showed more allelic peaks below threshold. The 1:1000 mixture also showed smaller RFU values that the 1:5000 and 1:10000. Pipetting errors or dilution errors could attribute to this abnormality. Also, the 1:1000 mixture is the start of the mixtures that used the female 1.0ng/µL dilution, while the 1:5000 and 1:10000 mixtures used the neat female extract of 20.47ng/µL, which could have some effect. The RFU values were highest with the 1:1 mixture,

and decreased as the ratio increased, which shows that inhibition from the female DNA was proportional to the increase of the female DNA concentration. The RFU values for the 1:10000 mixture were lower than the 1:5000, with the 1:1 mixture having the highest RFU values overall. The 1:1:1 mixture having lower RFU values than the 1:1, but higher than the 1:100 shows that the more contributors in a mixture, the greater the role of inhibition.

Stutter

The stutter positions were ±4 base pairs from the true allele peak. In the case of DYS19, ±4 and ±2 base pair stutter positions were taken into consideration. ±3, ±5, and ±6 base pair stutter positions were taken into account for DYS392, DYS438, and DYS448 respectively, since one is a trinucleotide repeat unit, one a pentanucleotide repeat unit, and the other a hexanucleotide repeat unit. The stutter values published by Applied Biosystems® do not include any plus stutter except for locus DYS392, which has +3 stutter. It is concluded that the stutter values published from Applied Biosystems® should be followed because they are more conservative, but the calculated values should be referenced when needed. By using a more conservative stutter ratio, the chance of an allele peak being attributed to stutter instead of to a true peak is smaller, which can influence typing mixture samples.

Adjudicated Cases

Evidence and reference samples that were shown to have the same STR profile when amplified with AmpF*l*STR® Identifiler® PCR Amplification Kit were used in the Concordance Study and Reproducibility Study to show that the final results obtained in casework were still obtained using the Yfiler® Amplification Kit.

National Institute of Standards and Technology (NIST) Standard Reference Material (SRM)

NIST SRMs are utilized during a validation to demonstrate that the profile obtained matches the certified profile from NIST, demonstrating quality assurance [7]. The certificate of analysis for 2391b Genomic 1 does not include any Y-STR profile, Yfiler™ or any other Y chromosome specific amplification kit [1]. SRM 2391c has superseded 2391b, and does have a published Y-STR profile for Yfiler™[1]. Another SRM that has a Y-STR profile based on Yfiler™ is the SRM 2395, the designated the Human Y-Chromosome DNA Profiling Standard. Prince George's County Police Department Serology/DNA Laboratory only had NIST SRM 2391b, so in the future either SRM2391c or SRM 2395 should be utilized in a Y-filer™ validation.

Microvariants and Off Ladder (OL) Alleles

Microvariants are produced when a repeat unit is incomplete and an OL allele occurs when an allele falls outside of the calling bins of the Allelic Ladder. One microvariant and two OL alleles were discovered in this validation. The microvariant and OL alleles were observed by other laboratories and detailed on Short Tandem Repeat DNA Internet Database [10].

Conclusions

The internal validation has shown that the AmpF*l*STR® Yfiler™ PCR Amplification Kit has successfully been validated for use in the Prince George's County Police Department Serology/DNA Laboratory, as outlined in SWGDAM's revised validation guidelines. Together the validation studies performed placed the analytical threshold at 150 RFU for a 5 or 10 second injection or 200 RFU for a 15 second injection and demonstrated that the Yfiler™ kit is precise, reproducible, and concordant. The studies also showed that the minor male contributor's full Y-STR profile can be determined in a 1:5 male-male mixture, while it can be determined in a 1:1 male-female mixture. A stutter study was performed at the end of the validation with the results

being compared to the published stutter values. Ultimately the published values, the more conservative of the two, were reasoned to be used primarily while referencing the values from the stutter study. The results demonstrated that the Yfiler™ kit successfully amplified evidence samples from adjudicated casework, is male specific in overwhelming concentrations of female DNA, and is reliably types. The successful validation reinforces the manufacturer's developmental validation performed prior to Yfiler™ being placed on the market. One future action that should be taken is that a NIST SRM with a Y-STR profile, such as SRM 2395, should be analyzed to ensure quality assurance. Due to the successful validation, analysts will need to undergo proficiency testing before implementing the Yfiler™ kit into casework.

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