Internal Validation and Comparative Analysis of the Promega PowerPlex® Fusion and the Applied Biosystems® GlobalFiler™ Express Amplification Kits for Direct Amplification

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Abstract

In 2010, the FBI CODIS Core Loci Working Group identified reasons for expanding the CODIS Core Loci in the United States. Among the reasons is a need to increase international data compatibility and the power of discrimination in missing person cases (1).

Both the Promega PowerPlex® Fusion and the Applied Biosystems® GlobalFiler™ Express PCR systems have adapted to the extension of CODIS Core Loci by increasing their multiplex reactions to 24 loci. The PowerPlex® Fusion amplification kit is a five-dye system that allows for the amplification and fluorescent detection of the 13 core CODIS (US) loci and the 12 core European Standard Set loci (2). The GlobalFiler™ Express amplification kit is a six-dye assay that targets 21 autosomal STR loci (including all CODIS US loci and the 12 core European Standard Set loci), one Y STR locus, and one Y insertion/deletion locus (3). Both the PowerPlex® Fusion and the GlobalFiler™ Express amplification kits have been approved for NDIS. Therefore, the evaluation of both kits is important to determine the best fit for each individual laboratory as these are implemented for DNA testing.

Sensitivity, precision, concordance, reproducibility and contamination studies were completed as part of the internal validation of the PowerPlex® Fusion and the GlobalFiler™ Express amplification kits using the Applied Biosystems® 3500 Genetic Analyzer (4). In addition, sample preparation, injection time, analytical threshold, and stochastic threshold were determined to optimize the protocol for analysis of blood and saliva on FTA cards and buccal samples. The goal of the validation was not only to ensure that both kits produce reliable and robust results, but to also optimize a single thermal cycling parameter and one optimal injection time for all three types of samples.

The internal validation determined that blood and saliva on FTA cards, as well as buccal samples, can be amplified using 26 cycles and 12 second injections for the PowerPlex® Fusion Amplification Kit. Alternatively, for the GlobalFiler™ Express amplification kit, these three types of samples can be amplified using 25 cycles and 15 second injections. The ability to amplify and inject blood and saliva from FTA cards and buccal reference samples on the same 96-well reaction plate will assist the laboratory with manual sample throughput until a fully automated workflow can be established.

Section 1: Introduction

Direct amplification is one of the most recent developments in forensic DNA analysis. The improvement of DNA polymerases and reaction components have allowed PCR amplification without DNA extraction. Previous amplification kits such as the PowerPlex® 16 HS and Identifiler® Plus Direct enable reference samples to be processed more rapidly (5). According to Standard 9.4 of the FBI's Quality Assurance Standards for Forensic DNA Testing, the amount of human DNA must be quantified in all forensic samples prior to nuclear DNA amplification, however, casework reference samples are listed as an exception if a validated method is used (9).

Direct amplification eliminates the need to perform DNA extraction and DNA quantification of reference samples. Two of the most novel direct PCR kits are the PowerPlex® Fusion and the GlobalFiler™ Express amplification kits, which were developed as part of the effort to extend the Combined DNA Index System (CODIS) core loci. In 2010, the FBI CODIS Core Loci Working Group identified reasons for expanding the CODIS Core Loci in the United States and began exploring the possibility to include the European Standard Set (5). The expansion allows an increase in international data compatibility and discrimination power to aid

in missing person cases. In addition, it reduces the likelihood of adventitious matches due to the rapid increase in the number of profiles stored in the National DNA Index System (NDIS) (1).

The PowerPlex® Fusion System is a 24-locus multiplex that uses a five-dye chemistry allowing co-amplification and fluorescent detection of the 13 core CODIS loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11), the 12 core European Standard Set loci (TH01, vWA, FGA, D21S11, D3S1358, D8S1179, D18S51, D10S1248, D22S1045, D2S441, D1S1656 and D12S391), Amelogenin, Penta D and Penta E. In addition, D2S1338, D19S433 and a male-specific locus, DYS391, were added to increase the power of discrimination (2). The GlobalFiler™ Express amplification kit is a six-dye chemistry, short tandem repeat (STR) assay that amplifies 21 autosomal STR loci encompassing both the 13 US core loci and the 12 core European Standard Set loci (D3S1358, vWA, D16S539, CS1FPO, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, D2S1338). In addition, GlobalFiler™ Express includes Amelogenin, DYS391, and 1 Y insertion/deletion (Y INDEL) locus (3). The evaluation of both kits is important to determine the best fit for each individual laboratory as these are implemented for DNA testing.

Both of the kits have been previously validated to ensure that they produce robust and reliable results (7, 8). However, as outlined by FBI's Quality Assurance Standards for Forensic DNA Testing Laboratories (9), "A laboratory shall use validated methodologies for DNA analyses." The internal validation of both the PowerPlex® Fusion and the GlobalFiler™ Express amplification kits was necessary before implementing a procedure for forensic applications at the Department of Forensic Sciences in the District of Columbia. The side-by-side validation would

allow the laboratory to properly evaluate the advantages and disadvantages of both kits and choose the one better suited for their needs.

As part of the SWGDAM Validation Guidelines for DNA Analysis Methods (4), sensitivity, precision, concordance, reproducibility and contamination studies were completed as part of the internal validation of the PowerPlex® Fusion and the GlobalFiler™ Express amplification kits using the Applied Biosystems® 3500 Genetic Analyzer (10). The guidelines also recommend a mixture study, however, this study was deemed unnecessary due to the single-source nature of reference samples. Nevertheless, sample preparation, injection time, analytical threshold, and stochastic threshold were determined to optimize the protocol for analysis of blood and saliva on FTA cards and buccal samples. The goal of the validation was not only to ensure that both kits produce reliable and robust results, but to also optimize a single thermal cycling parameter and one optimal injection time for all three types of samples.

Section 2: Methods

2.1 Precision Studies

Eight replicates of the PowerPlex® Fusion allelic ladder were loaded on a 96 well plate. Another eight replicates of the GlobalFiler™ Express allelic ladder were loaded on a separate 96 well plate. Each replicate was injected 12 times for a total of 96 allelic ladder samples for each amplification kit. By injecting each replicate 12 times, a total of 96 samples were used to demonstrate the precision of base pair sizing across a full plate for each kit. For PowerPlex® Fusion, each allelic ladder well contained 1.0 µL of Fusion Ladder, 10 µL of Hi-Di Formamide and 1.0 µL of CC5 Internal Lane Standard (ILS) as outlined by the PowerPlex® Fusion System Technical Manual. For GlobalFiler™ Express, each allelic ladder well contained 1.0 µL of GlobalFiler Ladder, 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal

size standard as outlined by the GlobalFiler™ Express PCR Amplification Kit User Guide. Both plates were run using the 3500 Genetic Analyzer.

The standard deviation of the base pairs was calculated for all injections across the plate and for six of the separate injections to confirm precision within an injection. The base pair averages, minimums, maximums and standard deviations were also calculated for each allele at all loci.

2.2 Sensitivity Studies

Sensitivity studies were conducted for FTA blood, FTA saliva and buccal samples. The goal of each sensitivity study was to assess amplification and instrument performance, assess any artifacts, determine an analytical threshold, determine a stochastic threshold, establish a minimum peak height ratio and determine the optimal number of amplification cycles for the PowerPlex® Fusion and GlobalFiler™ Express amplification kits. A total of nine samples (three FTA blood, three FTA saliva and three buccal swabs) were amplified using 25, 26 and 27 cycles.

Both PowerPlex® Fusion and GlobalFiler™ Express sensitivity studies involved the incubation of swab samples. Three buccal swab heads were detached from the swab shaft by cutting the stick before cell lysis. For Fusion, the three swab heads were placed in individual 2 mL tubes with 1 mL of SwabSolution™ (11). The swabs were incubated at 70^0C for 30 minutes. For GlobalFiler™ Express, the three swab heads were placed in individual 2 mL tubes with 400 µL of Prep-n-Go™ Buffer (3). The swabs were incubated at room temperature for 20 minutes. No substrates were removed from the extract after incubation. Promega Fusion recommends using 2 µL of extract. Life Technologies recommends using 3 µL of extract for GlobalFiler™ Express. A reagent blank consisting of 1 mL of SwabSolution™ was also incubated for the

Fusion sensitivity studies. A reagent blank consisting of 400 µL of Prep-n-Go™ Buffer was also incubated for the GlobalFiler™ Express sensitivity studies. No incubation was needed for saliva or blood samples on FTA cards.

All PowerPlex® Fusion samples and controls were amplified with 5 µL of Master Mix, 5 µL of the Primer Set and either 13 µL or 15 µL of Amplification Grade Water (13 µL for buccal swab samples and 15 µL for FTA samples). Buccal swab samples consisted of an additional 2 µL of extract (or reagent blank) while FTA samples (both blood and saliva) were simply amplified using one 1.2mm punch of sample. The buccal swab and FTA sample amplification negative controls for PowerPlex® Fusion consisted of with 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL Amplification Grade Water. The amplification positive (DNA Control 2800M) preparation was the same for all cycle numbers but different for FTA and buccal swab samples. The FTA amplification positive consisted of 5 µL of Master Mix, 5 µL of the Primer Set, 15 µL Amplification Grade Water (13 µL for positive control) and 1 µL of 2800M. On the other hand, the buccal swab amplification positive required 2 µL of diluted 2800M DNA Control. The dilution was completed using 2 µL of 2800M DNA control and 2 µL of Amplification Grade Water. The total volume for PowerPlex® Fusion for both FTA and buccal swab samples and their corresponding controls was 25 µL.

All of the GlobalFiler™ Express samples and controls were amplified with 6 µL of Master Mix and 6 µL of the Primer Set. However, buccal swab samples consisted of an additional 3 µL of extract (or reagent blank) while FTA samples (both blood and saliva) were amplified using one 1.2mm punch of sample plus 3 µL of TE buffer. The buccal swab amplification negative control for GlobalFiler™ Express consisted of 3 µL of Prep-n-Go™ Buffer and 3 µL of TE buffer for FTA samples. The amplification positive (DNA Control 007)

preparation was the same for all type of samples. However, the amplification positive differed for 25/26 and 27 cycles. For 25 and 26 cycles, the amplification positive consisted of 3 µL of DNA control 007. For 27 cycles, the amplification positive consisted of 2 µL of DNA control 007 and 1 µL of Prep-n-Go™ Buffer. The total volume for GlobalFiler™ Express for both FTA and buccal swab samples and their corresponding controls was 15 µL.

The GeneAmp® PCR System 9700 was used and set in Max ramping mode for amplification of all samples for both PowerPlex® Fusion and GlobalFiler™ Express (2,3). The thermal cycling protocols for PowerPlex® Fusion and GlobalFiler™ Express are outlined in Appendix A. The estimated total cycle time for PowerPlex® Fusion is 1.5 hours (2) while the estimated total cycle time for GlobalFiler™ Express is 30 minutes (3).

The consumables used for the 3500 Genetic Analyzer were the same for both PowerPlex® Fusion and GlobalFiler™ Express sensitivity studies. The cathode and anode buffers were replaced weekly and POP-4 Polymer was replaced every other week. Capillary electrophoresis of PowerPlex® Fusion consisted of 9.2 µL of Hi-Di Formamide and 0.8 µL of CC5 Internal Lane Standard (ILS) and a 24-second injection (2). These volumes were adjusted from manual recommendations to allow the use of a 10 µL multi-channel pipette. Capillary electrophoresis of GlobalFiler™ Express contained 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal size standard and a 15-second injection (3).

The DNA profiles and corresponding controls from this study were evaluated for concordance, allelic drop-out, peak height, peak height ratios and potential artifacts using GeneMapper® ID-X version 1.4. The results helped establish optimal thermal cycling parameters and interpretation guidelines for the direct amplification of known samples.

2.3 Reproducibility Studies

Reproducibility studies were conducted for FTA blood, FTA saliva and buccal samples. The goal of the reproducibility studies was to ensure that the peak heights and peak height ratios in 25 and 26 cycles for both PowerPlex® Fusion and GlobalFiler™ Express amplification kits fell within an acceptable number of standard deviations of the data obtained in sensitivity studies. A total of 30 samples (10 FTA blood, 10 FTA saliva and 10 buccal swabs) were amplified for each thermal cycle parameter assessed in reproducibility.

Both PowerPlex® Fusion and GlobalFiler™ Express reproducibility studies involved the incubation of swab samples. Ten buccal swab heads were detached from the swab shaft by cutting the stick before cell lysis. For PowerPlex® Fusion, the ten swab heads were placed in individual 2 mL tubes with 1 mL of SwabSolution™ (11). The swabs were incubated at 70^0C for 30 minutes. For GlobalFiler™ Express, ten swab heads were placed in individual 2 mL tubes with 400 µL of Prep-n-Go™ Buffer (3). The swabs were incubated at room temperature for 20 minutes. The substrate was not removed from the extract after incubation. Promega's amplification recommendations of using 2 µL of extract were followed for PowerPlex® Fusion. Life Technologies' recommendations of using 3 µL of extract were followed for GlobalFiler™ Express. A reagent blank, consisting of 1 mL of SwabSolution™, was also incubated for Fusion reproducibility studies. A reagent blank, consisting of 400 µL of Prep-n-Go™ Buffer, was also incubated for the GlobalFiler™ Express reproducibility studies. No incubation was needed for saliva or blood samples on FTA cards. Ten FTA saliva and ten FTA blood samples were used in reproducibility studies for both PowerPlex® Fusion and GlobalFiler™ Express amplification kits.

It is important to note that blood and saliva on FTA cards, as well as buccal samples, were amplified in the same 96-well plate during reproducibility studies. Therefore, two replicates of the same plate were set-up and amplified using 25 and 26 cycles. All of PowerPlex® Fusion samples and controls were amplified with 5 µL of Master Mix, 5 µL of the Primer Set and either 13 µL or 15 µL of Amplification Grade Water (13 µL for buccal swab samples and 15 µL for FTA samples). Buccal swab samples consisted of an additional 2 µL of extract (or reagent blank) while FTA samples (both blood and saliva) were simply amplified using one 1.2mm punch of each sample. The buccal swab and FTA sample amplification negative controls for PowerPlex® Fusion consisted of 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL of Amplification Grade Water. Because all three types of samples were amplified in the same 96-well plate, two amplification positives (DNA Control 2800M) were included in each thermal cycling parameter. An amplification positive consisting of 5 µL of Master Mix, 5 µL of the Primer Set, 15 µL Amplification Grade Water and 1 µL of 2800M was used as recommended by the Fusion System Technical Manual for amplification of FTA samples. In addition, a positive control consisting of 2 µL of diluted 2800M DNA Control was set-up in the same 96-well amplification plate as recommended by the Fusion System Technical Manual for amplification for buccal swab samples. The dilution was completed using 2 µL of 2800M DNA control and 2 µL of Amplification Grade Water. Both 2800M DNA amplification positive target was 1 ng. The total volume for PowerPlex® Fusion for both FTA and buccal swab samples and their corresponding controls was 25 µL.

As with PowerPlex® Fusion, blood and saliva on FTA cards, as well as buccal samples, were amplified using GlobalFiler™ Express in the same 96-well plate during reproducibility studies. Therefore, two replicates of the same plate were set-up and amplified using 25 and 26

cycles. All of the GlobalFiler™ Express samples and controls were amplified with 6 µL of Master Mix and 6 µL of the Primer Set. Buccal swab samples consisted of an additional 3 µL of extract (or reagent blank) while FTA samples (both blood and saliva) were amplified using one 1.2mm punch of sample plus 3 µL of TE buffer. The buccal swab amplification negative control for GlobalFiler™ Express consisted of 3 µL of Prep-n-Go™ Buffer and 3 µL of TE buffer for FTA samples. The amplification positive (DNA Control 007) preparation was the same for all types of samples using 25 and 26 cycles. The amplification positive consisted of 3 µL of DNA control 007. The total volume for GlobalFiler™ Express for both FTA and buccal swab samples and their corresponding controls was 15 µL.

The GeneAmp® PCR System 9700 was used and set in max ramping mode for amplification of all samples for both PowerPlex® Fusion and GlobalFiler™ Express (2,3). The thermal cycling protocols for PowerPlex® Fusion and GlobalFiler™ Express are outlined in Appendix A. The estimated total cycle time for PowerPlex® Fusion is 1.5 hours (2) while the estimated total cycle time for GlobalFiler™ Express is 30 minutes (3).

The consumables used for the 3500 Genetic Analyzer were the same for both PowerPlex® Fusion and GlobalFiler™ Express reproducibility studies. The cathode and anode buffers were replaced weekly and POP-4 Polymer was replaced every other week. Capillary electrophoresis of PowerPlex® Fusion consisted of 9.1 µL of Hi-Di Formamide and 0.9 µL of CC5 Internal Lane Standard (ILS) and a 24-second injection (2). These volumes were adjusted from manual recommendations to allow the use of a 10 µL multi-channel pipette. Capillary electrophoresis of GlobalFiler™ Express contained 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal size standard and a 15-second injection (3).

The DNA profiles and corresponding controls from this study were evaluated for concordance, allele drop-out, peak height, peak height ratios and potential artifacts using GeneMapper® ID-X 1.4. The results assisted in ensuring that both the PowerPlex® Fusion and GlobalFiler™ Express amplification kits produce robust, reproducible and reliable results.

2.4 Injection Time Studies

For PowerPlex® Fusion, ten FTA blood, ten FTA saliva and ten buccal swab samples were amplified using 26 cycles and were then subjected to a 24-second injection time during capillary electrophoresis. The same 96-well plate set-up for capillary electrophoresis during reproducibility studies was re-injected using a 12-second injection. No re-set up was completed. The consumables used for the 3500 Genetic Analyzer were also the same as the ones used in reproducibility studies. Each capillary electrophoresis well contained 9.1 µL of Hi-Di Formamide and 0.9 µL of CC5 Internal Lane Standard (ILS). The goal of a decreased injection time was to validate a procedure that would allow the amplification of all sample types while decreasing the number of artifacts observed due to off-scale data. In addition, low-level samples with allelic drop-out could be injected using a 24 second injection to potentially obtain a full DNA profile.

For GlobalFiler™ Express, ten FTA blood, ten FTA saliva and ten buccal swab samples previously amplified using 26 cycles were used. The same amplicons produced during reproducibility studies were used to re-setup a 96-well plate for a 15-second injection during capillary electrophoresis. Each capillary electrophoresis well contained 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal size standard. Just as with PowerPlex® Fusion, the goal of validating a second injection time was to allow the amplification of all sample types using one thermal cycling parameter. Additionally, a second injection would

provide an opportunity to potentially obtain a full DNA profile from low-level samples with allelic drop-out.

2.5 Contamination Studies

The goal of the contamination study was to ensure that punching a blank in between FTA samples would eliminate contamination during sampling. This contamination study consisted of the amplification of blank punches. In addition, contamination was assessed for all known samples and controls in precision, sensitivity, reproducibility and injection studies using both the PowerPlex® Fusion and GlobalFiler™ Express amplification systems.

The three samples that had the highest observed peak height averages during reproducibility studies were used in the contamination studies. Choosing high yield samples ensured that the contamination studies resembled samples in which contamination would be most likely to occur. After each sample was punched, two 1.2mm blank punches followed. All samples and blanks were amplified, set-up for capillary electrophoresis and analyzed using GeneMapper® ID-X version 1.4.

For PowerPlex® Fusion, all FTA punches (1.2mm) were amplified using 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL of Amplification Grade Water. The amplification negative controls also consisted of 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL of Amplification Grade Water. An amplification positive consisting of 5 µL of Master Mix, 5 µL of the Primer Set, 15 µL Amplification Grade Water and 1 µL of 2800M was also used as recommended by the Fusion System Technical Manual for amplification of FTA samples.

For GlobalFiler™ Express, all FTA punches and the amplification negative control were amplified with 6 µL of Master Mix, 6 µL of the Primer Set and 3 µL of TE buffer. The

amplification positive consisted of 6 µL of Master Mix, 6 µL of the Primer Set and 3 µL of DNA control 007 as recommended by the GlobalFiler™ Express PCR Amplification Kit User Guide.

The GeneAmp® PCR System 9700 was used and set in Max ramping mode for amplification during contamination studies for both PowerPlex® Fusion and GlobalFiler™ Express (2,3). The thermal cycling protocols for PowerPlex® Fusion and GlobalFiler™ Express are outlined in Appendix A. The estimated total cycle time for PowerPlex® Fusion is 1.5 hours (2) while the estimated total cycle time for GlobalFiler™ Express is 30 minutes (3).

The consumables used for the 3500 Genetic Analyzer were the same for both PowerPlex® Fusion and GlobalFiler™ Express contamination studies. The cathode and anode buffers were replaced weekly and POP-4 Polymer was replaced every other week. Capillary electrophoresis of PowerPlex® Fusion consisted of 9.1 µL of Hi-Di Formamide and 0.9 µL of CC5 Internal Lane Standard (ILS). The contamination assessment for PowerPlex® Fusion was completed using a 12-second and a 24-second injection without re-setup. Capillary electrophoresis of GlobalFiler™ Express contained 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal size standard. The contamination assessment for GlobalFiler™ Express was completed using a 15- and 30-second injection without re-setup.

The DNA profiles and corresponding controls from this study were evaluated for concordance and contamination using GeneMapper® ID-X version 1.4.

2.6 NIST Studies

According to FBI Quality Assurance Standard 9.5.5, a laboratory must check its DNA procedures against an appropriate and available NIST standard reference material (12). NIST Standard Reference Material 2391c Component F was amplified using both the PowerPlex® Fusion and GlobalFiler™ Express amplification systems to ensure concordant results.

For PowerPlex® Fusion, the NIST FTA punch (1.2mm) was amplified using 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL of Amplification Grade Water. The amplification negative controls also consisted of 5 µL of Master Mix, 5 µL of the Primer Set and 15 µL of Amplification Grade Water. An amplification positive consisting of 5 µL of Master Mix, 5 µL of the Primer Set, 15 µL Amplification Grade Water and 1 µL of 2800M was also used as recommended by the Fusion System Technical Manual for amplification of FTA samples.

For GlobalFiler™ Express, the NIST FTA punch (1.2mm) and the amplification negative control were amplified with 6 µL of Master Mix, 6 µL of the Primer Set and 3 µL of low TE buffer. The amplification positive consisted of 6 µL of Master Mix, 6 µL of the Primer Set and 3 µL of DNA control 007 as recommended by the GlobalFiler™ Express PCR Amplification Kit User Guide.

The GeneAmp® PCR System 9700 was used and set in Max ramping mode for amplification during contamination studies for both PowerPlex® Fusion and GlobalFiler™ Express (2,3). The thermal cycling protocols for PowerPlex® Fusion and GlobalFiler™ Express are outlined in Appendix A. The estimated total cycle time for PowerPlex® Fusion is 1.5 hours (2) while the estimated total cycle time for GlobalFiler™ Express is 30 minutes (3).

The consumables used for the 3500 Genetic Analyzer were the same for both PowerPlex® Fusion and GlobalFiler™ Express NIST studies. The cathode and anode buffers were replaced weekly and POP-4 Polymer was replaced every other week. Capillary electrophoresis of PowerPlex® Fusion consisted of 9.1 µL of Hi-Di Formamide and 0.9 µL of CC5 Internal Lane Standard (ILS). The NIST study for PowerPlex® Fusion was completed using a 12- and 24-second injection without re-setup. Capillary electrophoresis of GlobalFiler™ Express contained 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan™ 600 LIZ® internal

size standard. The NIST study for GlobalFiler™ Express was completed using a 15- and 30-second injection without re-setup.

The DNA profiles and corresponding controls from this study were evaluated for concordance and contamination using GeneMapper® ID-X version 1.4.

2.7 Analytical Threshold

There are two generally accepted ways to calculate the analytical threshold. Equation 1 shown below is suggested by the Scientific Working Group on DNA Analysis Methods (SWGDAM) in section 1.1. of the Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories (13).

Equation 1:

$$Analytical\ Threshold\ = 2(Maximum\ peak\ height - Minimum\ peak\ height)$$

Equation 2 was developed by the International Union of Pure & Applied Chemists (IUPAC). This equation is believed to result in an analytical threshold with 89-99.86% confidence that noise will be below this value. However, in DNA Analysis, this equation is often used to determine the limit of detection (14):

Equation 2:

$$Limit\ of\ Detection = Average\ peak\ height + (3 \times Standard\ Deviation\ peak\ height)$$

Another important calculation is the Limit of Quantitation (LOQ). The LOQ is the estimated limit in which the peak height is unreliable and may not be an accurate indicator of mixture ratio (15).

$$Limit\ of\ Quantitation = Average\ peak\ height + (10 \times Standard\ Deviation\ peak\ height)$$

Equations 1 and 2 were used to calculate an analytical threshold for each dye in sensitivity, reproducibility and injection studies separately. The results were then compared and evaluated

within each kit. The established analytical threshold assisted in determining which detected peaks could be distinguished from background noise using both the PowerPlex® Fusion and GlobalFiler™ Express.

2.8 Stochastic Threshold

The stochastic threshold is the limit in which there is certainty that a sister allele has not dropped out. The stochastic threshold will always be higher than the limit of quantitation (16). The stochastic threshold was calculated for sensitivity, reproducibility and injection studies separately. This threshold was calculated by taking the calculated analytical threshold and dividing it by the minimum peak height observed (16). In addition, the peak heights and peak height ratios for samples in each validation study were plotted to illustrate the relationship of peak height ratios over corresponding peak heights. The plot helped establish an expected peak height ratio for both PowerPlex® Fusion and GlobalFiler™ Express.

Section 3: Results

3.1 Precision Studies

The PowerPlex® Fusion and GlobalFiler™ Express precision studies consisted of the standard deviation calculation of the base pair sizes for all injections across the plate and for six of the separate injections to confirm precision within injection. The base pair averages, minimums, maximums and standard deviations were calculated for each allele at all loci for each amplification kit.

The PowerPlex® Fusion and GlobalFiler™ Express precision results are illustrated in Table 3.1.1 and Table 3.1.2, respectively. These tables include the average, minimum and maximum standard deviations in 6 independent injections and across all injections. All standard deviations observed are lower than 0.15 base pairs. For the PowerPlex® Fusion amplification kit,

the highest standard deviation observed within injections is 0.104 base pairs and 0.072 base pairs across all injections. For the GlobalFiler™ Express amplification kit, the highest standard deviation observed within injections is 0.082 base pairs and 0.061 base pairs across all injections.

Table 3.1.1 Average, minimum and maximum standard deviation calculated per injection and across all injections for PowerPlex® Fusion amplification kit.

Injection	Average Standard Deviation (bp)	Minimum Standard Deviation (bp)	Maximum Standard Deviation (bp)
1	0.036	0.0	0.079
2	0.037	0.0	0.076
3	0.050	0.0	0.104
4	0.038	0.0	0.064
5	0.041	0.0	0.075
6	0.040	0.0	0.074
All	0.045	0.024	0.072

Table 3.1.2 Average, minimum and maximum standard deviation calculated per injection and across all injections for GlobalFiler™ Express amplification kit.

Injection	Average Standard Deviation (bp)	Minimum Standard Deviation (bp)	Maximum Standard Deviation (bp)
1	0.033	0.0	0.061
2	0.036	0.0	0.066
3	0.033	0.0	0.071
4	0.033	0.0	0.071
5	0.034	0.0	0.082
6	0.032	0.0	0.065
All	0.037	0.016	0.061

The standard deviation was plotted over the average allele size to determine whether there was a relationship between standard deviation and locus size. Figures 3.1.1 and 3.1.2 illustrate an observed trend across injections observed for The PowerPlex® Fusion and GlobalFiler™ Express, respectively. As the base pair size increases, the standard deviation increases. The following loci have the highest standard deviations in the PowerPlex® Fusion amplification kit: D22S1045, DYS391, Penta E and Penta D. The following loci have the highest standard deviations in the GlobalFiler™ Express amplification kit: SE33, DYS391 and TPOX. The observation can be attributed to the large base pair sizes at these loci.

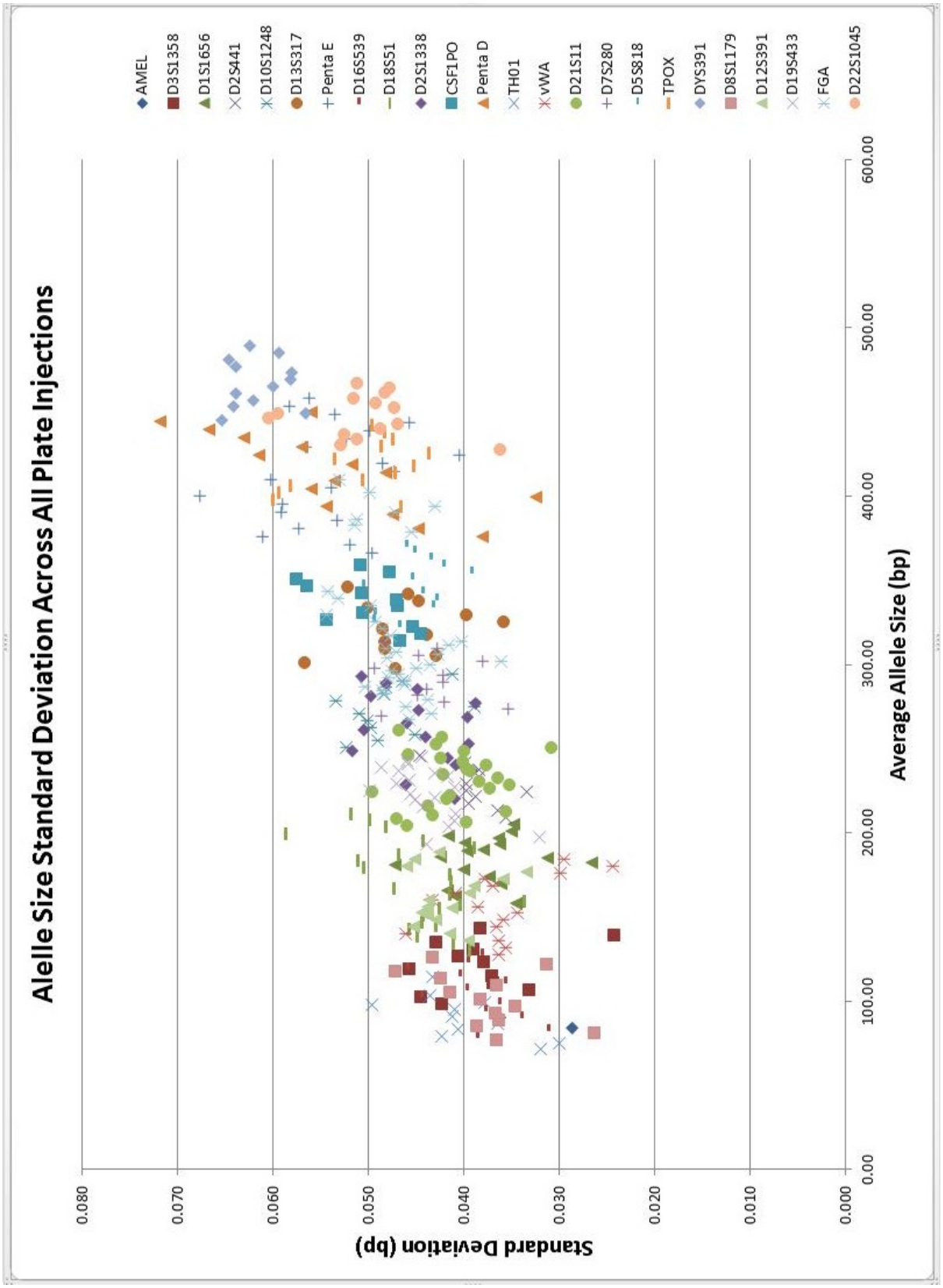


Figure 3.1.1 The standard deviation in allele size (bp), by average allele size, for each ladder allele across all plate injections in PowerPlex® Fusion amplification kit.

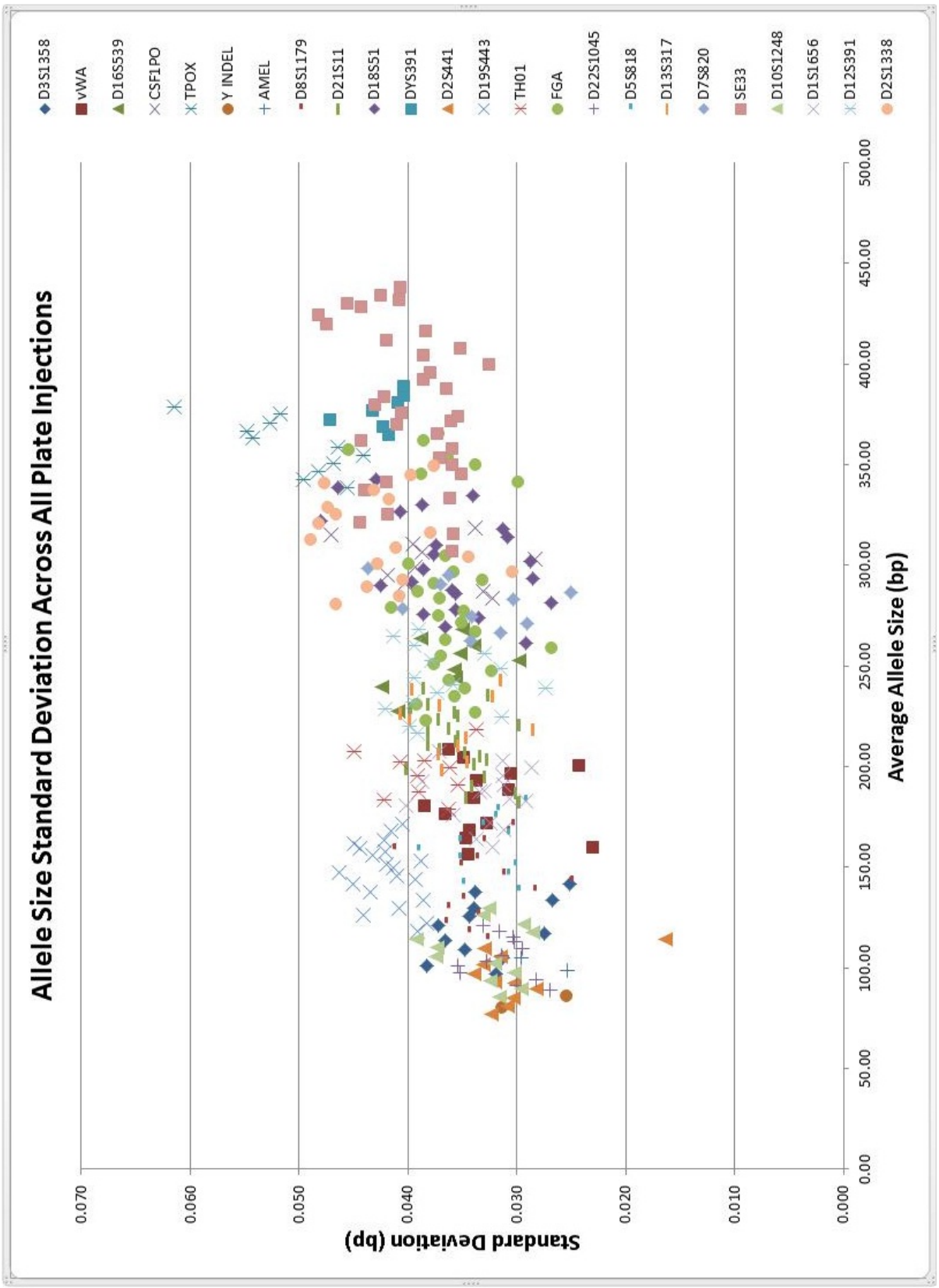


Figure 3.1.2 The standard deviation in allele size (bp), by average allele size, for each ladder allele across all plate injections in GlobalFiler™ Express amplification kit.

3.2 Sensitivity Studies

FTA Blood Samples

Table 3.2.1 illustrates the calculated peak heights, peak height ratios and their corresponding averages for FTA blood samples using PowerPlex® Fusion and GlobalFiler™ Express. For PowerPlex® Fusion, the average peak heights were 5357, 10629 and 10608 RFUs for 25, 26 and 27 cycles respectively. The average peak height ratio is relatively the same for all cycles at approximately 93%. However, the lowest peak height ratio of 68.5% was observed at 27 cycles. The peak height standard deviation increases with an increasing cycle. In addition, the highest heterozygous peak heights of 27737 and 24423 were observed at 26 and 27 cycles respectively.

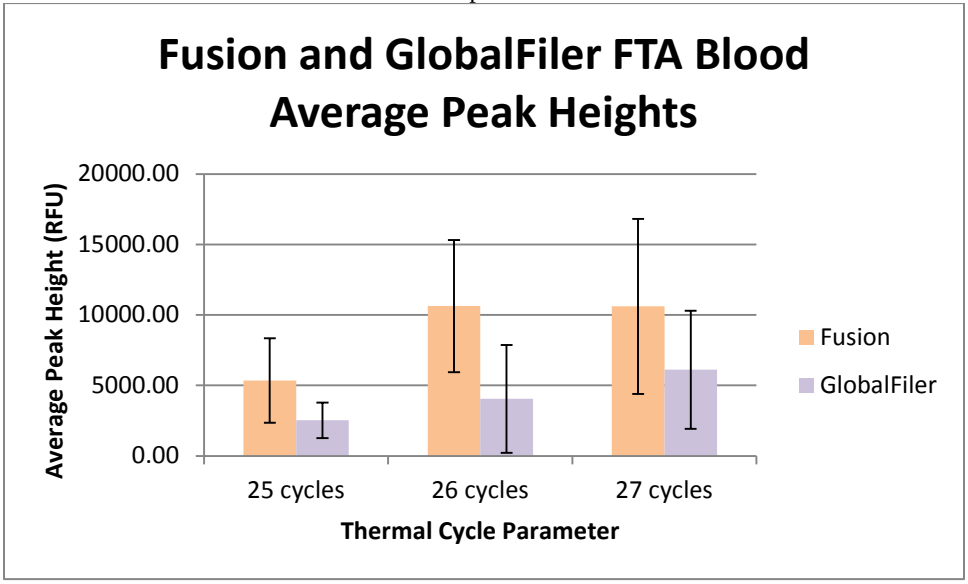
For GlobalFiler™ Express, the average peak heights were 2522, 4043 and 6110 RFUs for 25, 26 and 27 cycles respectively (Table 3.2.1). The average peak height ratio is relatively the same for all cycles at approximately 91%. However, the lowest peak height ratio of 65.8% was observed at 27 cycles. The peak height standard deviation increases as cycle number increases. Notably, the peak height standard deviation of 26 cycles is almost three times higher than that of 25 cycles.

Table 3.2.1 Fusion and GlobalFiler sensitivity summary for blood samples on FTA cards (one 1.2mm punch).

	25 cycles		26 cycles		27 cycles	
	Fusion	GlobalFiler	Fusion	GlobalFiler	Fusion	GlobalFiler
Min PH (RFU)	1,272	786	3,022.5	475	1,808.5	1,453
Max PH (RFU)	16,124	6,551	27,737	16,031	24,423	19,059
Avg. PH (RFU)	**5,357**	**2,522**	**10,629**	**4,043**	**10,608**	**6,110**
PH Std. Dev. (RFU)	2,995	1,263	4,685	3,816	6,207	4,198
Min PHR	0.765	0.770	0.757	0.730	0.685	0.658
Max PHR	0.999	0.997	1.000	0.998	0.988	0.998
Avg PHR	0.935	0.917	0.930	0.915	0.917	0.907
PHR Std. Dev.	0.045	0.047	0.052	0.060	0.054	0.065

As observed on Figure 3.2.1, PowerPlex® Fusion produced higher peak heights than GlobalFiler™ Express when amplifying blood on FTA cards. In addition, the standard deviation of both amplification kits increases with increasing cycles.

Figure 3.2.1 illustrates a visual average peak heights comparison between PowerPlex® Fusion and GlobalFiler™ Express.



FTA Saliva Samples

Table 3.2.2 illustrates the calculated peak heights, peak height ratios and their corresponding averages for FTA saliva samples using PowerPlex® Fusion and GlobalFiler™ Express. For PowerPlex® Fusion, the average peak heights were 3909, 9305 and 16101 RFUs for 25, 26 and 27 cycles respectively. The average peak height ratio is relatively the same for all cycles at approximately 93%. For all samples analyzed, the peak height ratios were greater than 74.8%. It is important to note that the standard deviation is almost 3 times higher in 26 cycles than it is using 25 cycles. In addition, the highest heterozygous peak heights of 26687 and 32094 were observed at 26 and 27 cycles respectively.

For GlobalFiler™ Express, The average peak heights were 5098, 7011 and 11890 RFUs for 25, 26 and 27 cycles respectively. The average peak height ratio average is highest at 25

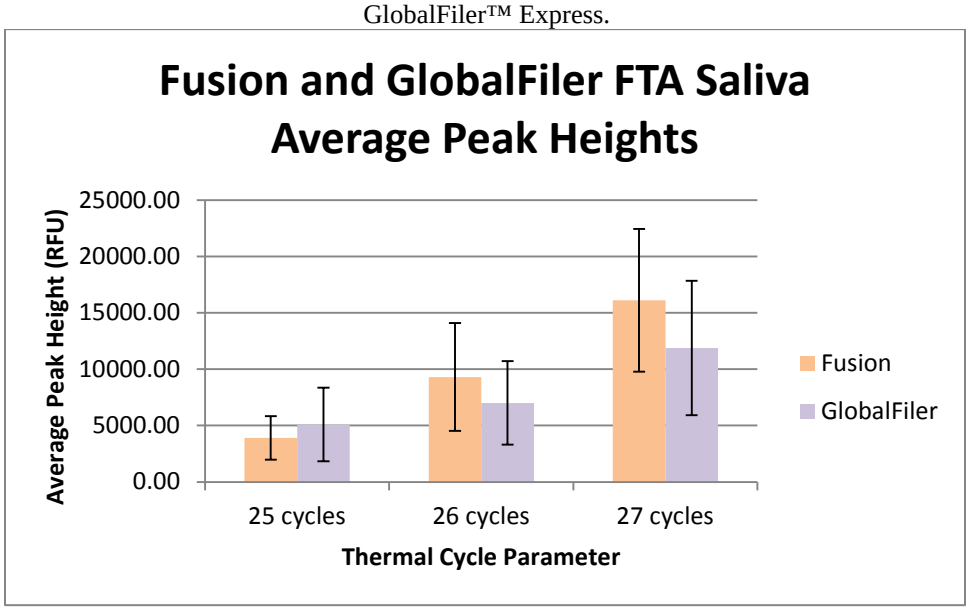
cycles (92.2%) and lowest at 27 cycles (89.6%). However, at 27 cycles the lowest peak height ratio was 55.8%. The observed low peak height ratio is located at SE33 and can be attributed to the distance between alleles 13 and 32.2.

Table 3.2.2 Fusion and GlobalFiler sensitivity summary for saliva samples on FTA cards (one 1.2mm punch).

	25 cycles		26 cycles		27 cycles	
	Fusion	GlobalFiler	Fusion	GlobalFiler	Fusion	GlobalFiler
Min PH (RFU)	696	938.5	1,424	876.5	3,233	1,934
Max PH (RFU)	11,864	15,662	26,687	15,251	32,094	29,504
Avg. PH (RFU)	**3,910**	**5,098**	**9,305**	**7,011**	**16,101**	**11,890**
PH Std. Dev. (RFU)	1,930	3,254	4,781	3,699	6,325	5,960
Min PHR	0.748	0.718	0.768	0.697	0.761	0.558
Max PHR	0.999	0.999	1.000	0.993	0.999	0.998
Avg PHR	0.933	0.922	0.928	0.919	0.927	0.896
PHR Std. Dev.	0.049	0.057	0.046	0.054	0.050	0.075

As observed on Figure 3.2.2, PowerPlex® Fusion produced higher peak heights than GlobalFiler™ Express during 26 and 27 cycles when amplifying saliva on FTA cards. However, GlobalFiler™ Express produced higher peak heights during the 25 thermal cycle parameter.

Figure 3.2.2 illustrates a visual average peak heights comparison between PowerPlex® Fusion and GlobalFiler™ Express.



Buccal Swab Samples

Table 3.2.3 illustrates the calculated peak heights, peak height ratios and their corresponding averages for FTA saliva samples using PowerPlex® Fusion and GlobalFiler™ Express. For PowerPlex® Fusion, the average peak heights were 4160, 8650 and 16083 RFUs for 25, 26 and 27 cycles respectively. The average peak height ratio is relatively the same for all cycles at approximately 92%. The 25 cycle amplification offers the most robust set of data (smallest standard deviation). The peak height ratio average decreases with an increasing cycle number. The lowest peak height ratio of 73.4% was observed during 26 cycles. In addition, the highest heterozygous peak heights of 22476 and 31965 were observed at 26 and 27 cycles respectively.

For GlobalFiler™ Express, the average peak heights were 5644, 7761 and 10874 RFUs for 25, 26 and 27 cycles respectively. The average peak height ratio is relatively the same for all cycles at approximately 87%. However, the lowest peak height ratios of 41.3% and 42.1% are observed at 26 and 27 cycles, respectively. The observed low peak height ratios are located at SE33 due to heterozygote alleles 13 and 32.2. Based on the base pair size difference among these alleles, it is not unexpected to have imbalance. In addition, the peak height standard deviation increases as cycle number increases.

Table 3.2.3 Fusion and GlobalFiler sensitivity summary for buccal swab samples.

	25 cycles		26 cycles		27 cycles	
	Fusion	GlobalFiler	Fusion	GlobalFiler	Fusion	GlobalFiler
Min PH (RFU)	740	740	2,062	1,972	4,934	2,402
Max PH (RFU)	11,109	21,341	22,476	29,584	31,965	31,993
Avg. PH (RFU)	**4,160**	**5,644**	**8,650**	**7,761**	**16,083**	**10,874**
PH Std. Dev. (RFU)	2,131	3,984	4,340	4,288	5,838	5,574
Min PHR	0.767	0.566	0.734	0.413	0.783	0.421
Max PHR	0.998	0.998	1.000	0.995	0.998	0.997
Avg PHR	0.934	0.885	0.929	0.878	0.919	0.863
PHR Std. Dev.	0.046	0.093	0.051	0.097	0.051	0.093

As observed on Figure 3.2.3, PowerPlex® Fusion produced higher peak heights than GlobalFiler™ Express using 26 and 27 cycles when amplifying buccal swab samples. However, GlobalFiler™ Express produced higher peak heights usingthe 25 thermal cycle parameter.

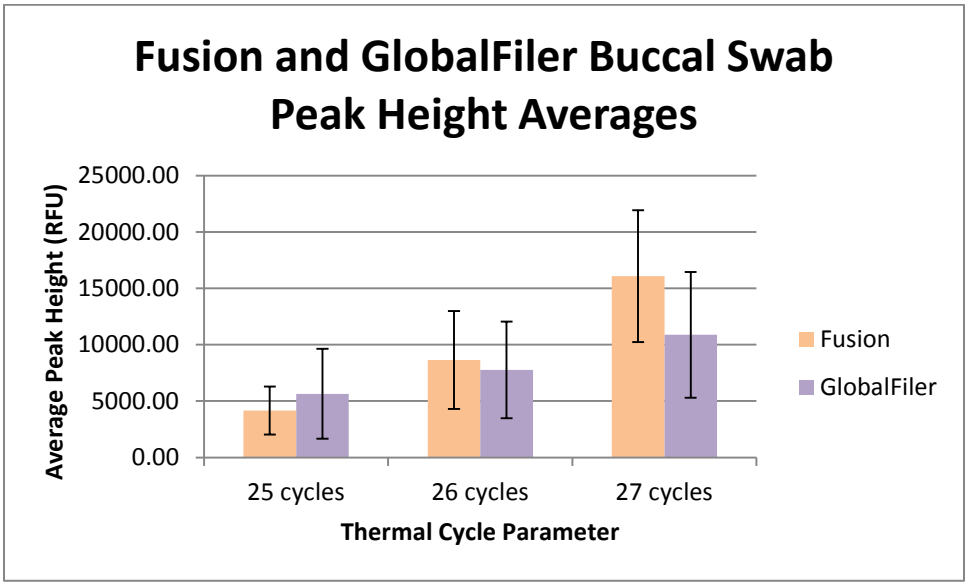


Figure 3.2.3 illustrates a visual average peak heights comparison between PowerPlex® Fusion and GlobalFiler™ Express.

3.3 Reproducibility Studies

FTA Blood Samples

Table 3.3.1 illustrates a comparison of the FTA blood amplification results obtained during sensitivity and reproducibility studies for PowerPlex® Fusion (26 cycles). The average peak height increased from 10629 RFUs to 11237 RFUs. However, the increase falls within 1 standard deviation. The minimum and average peak height ratios are moderately the same. The main difference during reproducibility studies is the increase in maximum peak height from 27737 to 32188 RFUs.

Table 3.3.1 Illustrates a comparison of the results from sensitivity and reproducibility studies for one 1.2mm punch of blood on FTA cards using 26 cycles.

PowerPlex® Fusion	Sensitivity	Reproducibility
Min PH	3022.5	2325.5
Max PH	27737	32188
Avg. PH	10629.5	11237
PH Std. Dev.	4685	5826
Min PHR	0.757	0.745
Max PHR	1.000	0.999
Avg PHR	0.930	0.934
PHR Std. Dev.	0.052	0.045

Table 3.3.2 illustrates a comparison of the FTA blood amplification results obtained during sensitivity and reproducibility studies for GlobalFiler™ Express (25 cycles). The peak height average increased from 2522 RFUs to 4489 RFUs. However, the increase falls within 2 standard deviations. The average peak height ratio in reproducibility falls within 1 standard deviation of the average peak height ratio calculated in sensitivity. One of the main differences is the decrease of the minimum peak height ratio in the reproducibility studies. A minimum peak height ratio of 60.6% in reproducibility was observed in a blood sample that had a peak height ratio of 78.7% in sensitivity studies. Another difference is the increased maximum peak height observed in reproducibility (12799 RFUs). This increased peak height is close to the published optimum peak heights of 3,000-12,000 RFUs (10).

Table 3.3.2 Illustrates a comparison of the results from sensitivity and reproducibility studies for one 1.2mm punch of blood on FTA cards using 25 cycles.

GlobalFiler™ Express	Sensitivity	Reproducibility
Min PH	786	980
Max PH	6551	12799
Avg. PH	2522	4489
PH Std. Dev.	1263	1972
Min PHR	0.770	0.606
Max PHR	0.997	0.998
Avg PHR	0.917	0.908
PHR Std. Dev.	0.047	0.060

FTA Saliva Samples

Table 3.3.3 illustrates a comparison of the FTA saliva amplification results obtained during sensitivity and reproducibility studies for PowerPlex® Fusion (26 cycles). The average peak height decreased from 9305 to 7969 RFUs. Nonetheless, the decrease is within 1 standard deviation. The maximum peak height ratio, minimum peak height ratio and average peak height ratio are relatively the same and within 1 standard deviation. One main difference is the decrease in the minimum peak height observed, from 1424 to 289 RFUs. However, the low peak height was observed in a sample which had an average peak height more than 1 standard deviation from all other samples. This sample may be an outlier. In addition, drop-out was observed in one sample. The sample with observed drop-out was omitted from all calculations.

Table 3.3.3 Illustrates a comparison of the results from sensitivity and reproducibility studies for one 1.2mm punch of saliva on FTA cards using 26 cycles.

PowerPlex® Fusion	Sensitivity	Reproducibility
Min PH	1424	289
Max PH	26687	32130
Avg. PH	9305	7969
PH Std. Dev.	4781	6218
Min PHR	0.768	0.74
Max PHR	1.000	1.00
Avg PHR	0.928	0.93
PHR Std. Dev.	0.046	0.05

Table 3.3.4 illustrates a comparison of the FTA saliva amplification results obtained during sensitivity and reproducibility studies for GlobalFiler Express (25 cycles). The peak height average increased from 5098 to 6084 RFUs. However, the increase falls within 1 standard deviation. The average peak height ratio of reproducibility studies falls within 1 standard deviation of the average peak height ratio calculated for sensitivity studies. Another difference is the increased maximum peak height observed in reproducibility (19071 RFUs).

Table 3.3.4 illustrates a comparison of the results from sensitivity and reproducibility studies for one 1.2mm punch of saliva on FTA cards using 25 cycles.

GlobalFiler™ Express	Sensitivity	Reproducibility
Min PH	938.5	548
Max PH	15662	19071
Avg. PH	5098	6084
PH Std. Dev.	3254	4560
Min PHR	0.718	0.736
Max PHR	0.999	0.999
Avg PHR	0.922	0.918
PHR Std. Dev.	0.057	0.051

Buccal Swab Samples

Table 3.3.5 illustrates a comparison of the FTA buccal swab results obtained during sensitivity and reproducibility studies for PowerPlex® Fusion (26 cycles). The average peak height decreased from 8650 to 7877 RFUs. However, the decrease is within 1 standard deviation. The minimum and maximum peak height ratios, along with the average peak height ratio are also within 1 standard deviation. The main difference in reproducibility is the decrease of minimum peak height from 2062 to 1415 RFUs. The decrease is also within 1 standard deviation.

Table 3.3.5 illustrates a comparison of the results from sensitivity and reproducibility studies for buccal samples using 26 cycles.

PowerPlex® Fusion	Sensitivity	Reproducibility
Min PH	2062	1415
Max PH	22476	24096
Avg. PH	8650	7877
PH Std. Dev.	4340	4524

Min PHR	0.734	0.744
Max PHR	1.000	1.000
Avg PHR	0.929	0.933
PHR Std. Dev.	0.051	0.046

Table 3.3.6 illustrates a comparison of the buccal swab amplification results obtained during sensitivity and reproducibility studies for GlobalFiler™ Express (25 cycles). The peak height average decreased from 5644 to 4051 RFUs. However, the decrease falls within 1 standard deviation. The average peak height ratio of reproducibility is within 1 standard deviation of the average peak height ratio calculated in sensitivity. One of the main differences is the increase of the minimum peak height ratio in the reproducibility studies. Another difference is the increased maximum peak height observed in reproducibility (27,098 RFUs). This increased peak height is above the optimum peak heights of 3,000-12,000 RFUs (10).

Table 3.3.6 Illustrates a comparison of the results from sensitivity and reproducibility studies for buccal samples using 25 cycles.

GlobalFiler™ Express	Sensitivity	Reproducibility
Min PH	740	293
Max PH	21341	27098
Avg. PH	5644	4051
PH Std. Dev.	3984	3693
Min PHR	0.566	0.698
Max PHR	0.998	0.999
Avg PHR	0.885	0.900
PHR Std. Dev.	0.093	0.063

3.4 Injection Time Studies

FTA Blood Samples

Table 3.4.1 compares the results obtained in PowerPlex® Fusion reproducibility and the results from the same FTA blood amplicons injected for 12 seconds. Decreasing the injection time led to a decreased peak height average of 5215 RFUs. The minimum and maximum peak

heights also decreased to 984 and 14424 RFUs. However, the minimum and average peak height ratios remained the same.

Table 3.4.1 Illustrates a comparison between reproducibility (24 second injection) and the decreased injection study (12 second injection) at 26 cycles for FTA blood samples.

PowerPlex® Fusion	24 Second Injection	12 Second Injection
Min PH	2325.5	984.5
Max PH	32188	14424.5
Avg. PH	11237	5215
PH Std. Dev.	5826	2709
Min PHR	0.745	0.73
Max PHR	0.999	1.00
Avg PHR	0.934	0.935
PHR Std. Dev.	0.045	0.047

Table 3.4.2 compares the results obtained in GlobalFiler™ Express reproducibility and the results from the same FTA blood amplicons injected for 30 seconds. Doubling the injection time led to an increased peak height average of 9542 RFUs. The minimum peak height also increased from 980 to 1534 RFUs. However, the minimum and average peak height ratios remained the same.

Table 3.4.2. Illustrates a comparison between reproducibility (15 second injection) and the increased injection study (30 second injection) at 25 cycles for FTA blood samples.

GlobalFiler™ Express	15 Second Injection	30 Second Injection
Min PH	980	1534.5
Max PH	12799	30071
Avg. PH	4488.70	9542
PH Std. Dev.	1972	1534.5
Min PHR	0.606	0.603
Max PHR	0.998	0.998
Avg PHR	0.908	0.904
PHR Std. Dev.	0.060	0.060

FTA Saliva Samples

Table 3.4.3 compares the results obtained in PowerPlex® Fusion reproducibility and the results from the same FTA saliva amplicons injected for 12 seconds. The decreased injection

time led to a decreased peak height average of 3292 RFUs. The average peak height ratio remained the same at 93%. Additionally, the minimum peak height ratio remained at 74%. Allele drop-out was observed in one sample. This sample has been omitted from all calculations.

Table 3.4.3 Illustrates a comparison between reproducibility (24 second injection) and the decreased injection time study (12 second injection) at 26 cycles for FTA saliva samples.

PowerPlex® Fusion	24 seconds	12 Seconds
Min PH	289	110
Max PH	32130	15459
Avg. PH	7968.7	3292
PH Std. Dev.	6218.1	2874.545
Min PHR	0.74	0.743
Max PHR	1.00	0.999
Avg PHR	0.93	0.934
PHR Std. Dev.	0.05	0.047

Table 3.4.4 compares the results obtained in GlobalFiler™ Express reproducibility and the results from the same FTA saliva amplicons injected for 30 seconds. Doubling the injection time led to an increased peak height average of 9567 RFUs. One of the noticeable differences in the increased injection time study is the decrease of minimum peak height ratio from 73.6% to 51.6% due to the inclusion of a sample which dropped out in the 15 second injection and was not included in reproducibility calculations. In addition, the peak height standard deviation increased from 4560 to 9179 RFUs. Nonetheless, the average peak height ratio remained the same at 91%.

Table 3.4.4 Illustrates a comparison between reproducibility (15 second injection) and the increased injection study (30 second injection) at 25 cycles for FTA saliva samples.

GlobalFiler™ Express	15 Seconds	30 Seconds
Min PH	548	201
Max PH	19071	31675
Avg. PH	6084	9567
PH Std. Dev.	4560	9179
Min PHR	0.736	0.516
Max PHR	0.999	0.997
Avg PHR	0.918	0.912
PHR Std. Dev.	0.051	0.071

Buccal Swab Samples

Table 3.4.5 compares the results obtained in PowerPlex® Fusion reproducibility and the results from the same buccal swab amplicons injected for 12 seconds. Decreasing the injection time led to a lower peak height average of 3247 RFUs. There was also a decrease in the peak height standard deviation. The minimum and average peak height ratio remained within 1 standard deviation at 94%.

Table 3.4.5 Illustrates a comparison between reproducibility (24 second injection) and decreased injection time study (12 second injection) at 26 cycles for buccal swab samples.

PowerPlex® Fusion	24 Seconds	12 Seconds
Min PH	1415	564
Max PH	24096	13137.5
Avg. PH	7877	3247
PH Std. Dev.	4525	2046
Min PHR	0.744	0.73
Max PHR	1.000	1.00
Avg PHR	0.933	0.94
PHR Std. Dev.	0.046	0.05

Table 3.4.6 compares the results obtained in GlobalFiler™ Express reproducibility and the results from the same buccal swab amplicons injected for 30 seconds. Doubling the injection time led to an approximate doubling of peak height average and minimum peak height. However, there was also an increase in the peak height standard deviation. The average peak height ratio remained the same at 90%. There was a decrease in minimum peak height ratio from 69.8% to 64.3%; however, the decrease is within 1 standard deviation.

Table 3.4.6 Illustrates a comparison between reproducibility (15 second injection) and increased injection studies (30 second injection) at 25 cycles for buccal swab samples.

GlobalFiler™ Express	15 Seconds	30 Seconds
Min PH	293	676
Max PH	27098	31849
Avg. PH	4051	8215
PH Std. Dev.	3694	6849
Min PHR	0.698	0.643

Max PHR	0.999	1.000
Avg PHR	0.900	0.902
PHR Std. Dev.	0.063	0.067

3.5 Analytical Threshold

PowerPlex® Fusion

The analytical threshold was calculated for each dye separately in sensitivity, reproducibility and injection studies for both PowerPlex® Fusion and GlobalFiler™ Express amplification kits. As observed in Table 3.5.1, during the PowerPlex® Fusion sensitivity studies, the highest analytical threshold was observed in the red dye channel (188 RFUs) at 26 cycles. For simplicity, this value was rounded up to 200 RFUs and was recommended as the analytical threshold for 26 cycles with a 24 second injection. To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) were re-analyzed using the recommended 200 RFUs. No drop-out was observed using 200 RFUs as the analytical threshold. However, a total of 4 artifacts were observed. All artifacts were easily identified as pull up.

Table 3.5.1 PowerPlex® Fusion Analytical threshold, limit of detection and quantitation for all negative samples amplified using 26 cycles during sensitivity studies.

PowerPlex® Fusion Sensitivity 26 cycles – 24 second injection							
Dye	**Average PH (RFU)**	**St. Dev. PH (RFU)**	**Max PH (RFU)**	**Min PH (RFU)**	**Analytical Threshold: 2*(Max-Min)**	**Limit of Detection**	**Limit of Quantitation**
Blue	7.90	6.72	62	1	*122*	*28.06*	*75.11*
Green	6.58	2.95	39	1	*76*	*15.44*	*36.10*
Red	6.80	4.39	95	1	*188*	*19.96*	*50.68*
Yellow	5.62	3.34	62	1	*122*	*15.64*	*39.02*

Table 3.5.2 illustrates the analytical threshold calculated for the PowerPlex® Fusion reproducibility studies. The highest analytical threshold was observed in the blue dye channel (184 RFUs). This value resembles the results obtained in sensitivity from the red dye channel. The highest analytical threshold observed was rounded up to 200 RFUs for simplicity and was

recommended as the analytical threshold. To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) were re-analyzed using 200 RFUs. Allelic drop-out was observed in one FTA saliva sample using the recommended analytical threshold. However, this sample may be a possible outlier due to improper collection. Multiple artifacts including background or pull-up were observed in samples amplified for 26 cycles. A total of 16 artifacts due to off-scale data were observed in blood samples. A total of 5 artifacts due to off-scale data were observed in buccal swab and FTA saliva samples.

Table 3.5.2 PowerPlex® Fusion Analytical threshold, limit of detection and quantitation for all negative samples amplified using 26 cycles in reproducibility studies.

PowerPlex® Fusion Reproducibility 26 cycles – 24 second injection							
Dye	**Average PH (RFU)**	**St. Dev. PH (RFU)**	**Max PH (RFU)**	**Min PH (RFU)**	**Analytical Threshold: 2*(Max-Min)**	**Limit of Detection**	**Limit of Quantitation**
Blue	9.02	7.43	94	2	*184*	*31.31*	*83.31*
Green	7.05	3.11	22	1	*42*	*16.37*	*38.12*
Red	6.56	2.51	21	2	*38*	*14.08*	*31.63*
Yellow	5.86	2.56	20	1	*38*	*13.54*	*31.45*

Table 3.5.3 illustrates the analytical threshold calculated for PowerPlex® Fusion using 26 thermal cycles and a 12-second injection. The highest analytical threshold of 64 RFUs was observed in the blue dye channel. Due to the similar threshold values, the recommended analytical threshold was rounded up to 100 RFUs for all dyes. The recommended analytical threshold for a 12 second injection is lower than the recommended analytical threshold for a 24 second injection in sensitivity and reproducibility studies. To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) were re-analyzed using 100 RFUs. Allelic drop-out was observed in one FTA saliva sample using the recommended analytical threshold. As mentioned before, this sample may be a possible outlier due to improper collection. There were no artifacts caused by elevated background or pull-up in samples

amplified at 26 cycles and injected for 12 seconds. There were no samples flagged with off-scale data.

Table 3.5.3 PowerPlex® Fusion Analytical Threshold, Limit of Detection and Limit of Quantitation calculated for each dye using 26 thermal cycles and a 12 second injection.

PowerPlex® Fusion 26 cycles – 12 second injection							
Dye	**Average PH (RFU)**	**St. Dev. PH (RFU)**	**Max PH (RFU)**	**Min PH (RFU)**	**Analytical Threshold: 2*(Max-Min)**	**Limit of Detection**	**Limit of Quantitation**
Blue	7.35	3.42	34	2	*64*	*17.60*	*41.53*
Green	6.45	2.18	18	2	*32*	*12.99*	*28.24*
Red	6.55	2.18	19	1	*36*	*13.09*	*28.33*
Yellow	5.57	2.03	2	1	*42*	*11.67*	*25.90*

GlobalFiler™ Express

Table 3.5.4 illustrates the analytical threshold calculated for GlobalFiler™ Express sensitivity studies. The highest analytical threshold was observed in the purple dye channel (56 RFUs). For simplicity, this value was rounded up to 70 RFUs and was recommended as the analytical threshold for 25 cycles with a 15 second injection. To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) were re-analyzed using the recommended 70 RFUs. All samples (blood and saliva on FTA cards, and buccal swabs) for 25 cycles were analyzed using 70 RFUs. No drop-out was observed, and only 5 total artifacts were noted. All of the artifacts observed were background noise due to off-scale data.

Table 3.5.4 Analytical threshold, limit of detection and quantitation for all negative samples amplified using 25 cycles.

GlobalFiler™ Express Sensitivity 25 cycles – 15 second injection							
Dye	**Average PH (RFU)**	**St. Dev. PH (RFU)**	**Max PH (RFU)**	**Min PH (RFU)**	**Analytical Threshold: 2*(Max-Min)**	**Limit of Detection**	**Limit of Quantitation**
Blue	4.92	2.15	21	1	*40*	*11.36*	*26.39*
Green	8.68	3.11	23	2	*42*	*18.02*	*39.80*
Purple	7.49	3.01	30	2	*56*	*16.51*	*37.56*
Red	7.26	2.45	24	2	*44*	*14.61*	*31.78*
Yellow	3.86	1.55	13	1	*24*	*8.51*	*19.38*

Table 3.5.5 illustrates the analytical threshold calculated for GlobalFiler™ Express reproducibility studies. The highest analytical threshold was observed in the green dye channel (40 RFUs). To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) for all cycles were analyzed using the previously recommended analytical threshold 70 RFUs during sensitivity studies. Allele drop-out was observed in one FTA saliva sample. However, this sample may be a possible outlier due to improper collection. For consistency, the analytical threshold for 25 cycles with a 15-second injection remained 70 RFUs. Only two artifacts were observed on one buccal sample. The artifacts were caused by background noise due to off scale data.

Table 3.5.5 Analytical threshold, limit of detection and quantitation for all negative samples amplified using 25 cycles in reproducibility studies.

GlobalFiler™ Express Reproducibility 25 cycles – 15 second injection							
Dye	**Average PH (RFU)**	**St. Dev. PH (RFU)**	**Max PH (RFU)**	**Min PH (RFU)**	**Analytical Threshold: 2*(Max-Min)**	**Limit of Detection**	**Limit of Quantitation**
Blue	5.14	1.89	16	2	*28*	*10.80*	*24.00*
Green	8.72	2.87	22	2	*40*	*17.32*	*37.40*
Purple	7.65	2.39	18	3	*30*	*14.83*	*31.58*
Red	7.50	2.37	20	3	*34*	*14.61*	*31.17*
Yellow	3.93	1.32	11	2	*18*	*7.88*	*17.10*

Table 3.5.6 illustrates the analytical threshold calculated for the GlobalFiler™ Express increased injection study. The highest analytical threshold was observed in the blue dye channel (66 RFUs). For simplicity, the analytical threshold recommended for 25 cycles with a 30-second injection was 100 RFUs. To assess the established analytical threshold, all samples (blood and saliva on FTA cards, and buccal swabs) were analyzed at 100 RFUs. There was no allelic drop-out observed. However, multiple artifacts including background and pull-up were caused by the off scale data observed in samples amplified at 25 cycles with a 30 second injection. A total of 29 artifacts due to off-scale data were observed across 6 samples.

Table 3.5.6 Analytical Threshold, Limit of Detection and Limit of Quantitation calculated for each dye using 25 thermal cycles and a 30 second injection.

GlobalFiler™ Express 25 cycles – 30 second injection							
Dye	Average PH (RFU)	St. Dev. PH (RFU)	Max PH (RFU)	Min PH (RFU)	Analytical Threshold: 2*(Max-Min)	Limit of Detection	Limit of Quantitation
Blue	7.14	3.68	36	3	*66*	*18.17*	*43.89*
Green	10.21	4.20	31	4	*54*	*22.82*	*52.26*
Purple	8.84	3.13	25	3	*44*	*18.22*	*40.12*
Red	9.52	4.97	43	4	*78*	*24.43*	*59.24*
Yellow	4.69	1.98	19	2	*34*	*10.63*	*24.48*

3.6 Stochastic Threshold

PowerPlex® Fusion

The stochastic threshold was separately calculated for sensitivity, reproducibility and injection studies for both the PowerPlex® Fusion and GlobalFiler™ Express amplification kits. During PowerPlex® Fusion sensitivity studies, most samples have a peak height ratio between 80-100% regardless of the peak height average. The lowest peak height ratio observed at 25 cycles is 74.8% in locus D12S39 (Figure 3.6.1). Based on the minimum peak height ratio observed, the recommended stochastic threshold was 275 RFUs for all samples amplified at 25 and 26 cycles with a 24 second injection.

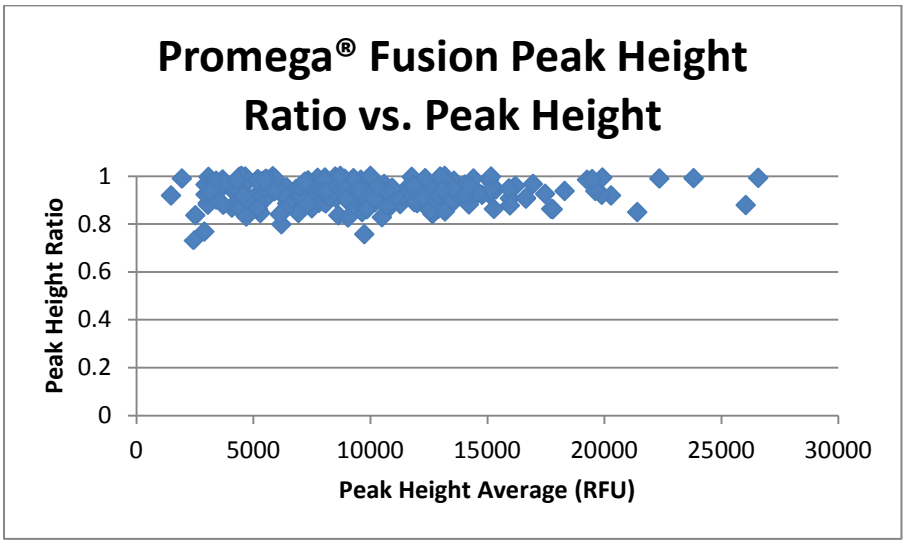


Figure 3.6.1 Peak height ratio over corresponding average peak heights of all samples processed at 26 cycles with a 24 second injection.

As observed during PowerPlex® Fusion sensitivity studies, most samples had a peak height ratio between 80-100% regardless of the peak height average during reproducibility studies (Figure 3.6.2). The lowest peak height ratio observed at 26 cycles was 74.3% in locus D12S39. Based on the minimum peak height ratio observed, the recommended stochastic threshold remained 275 RFUs for all samples amplified at 26 cycles with a 24 second injection.

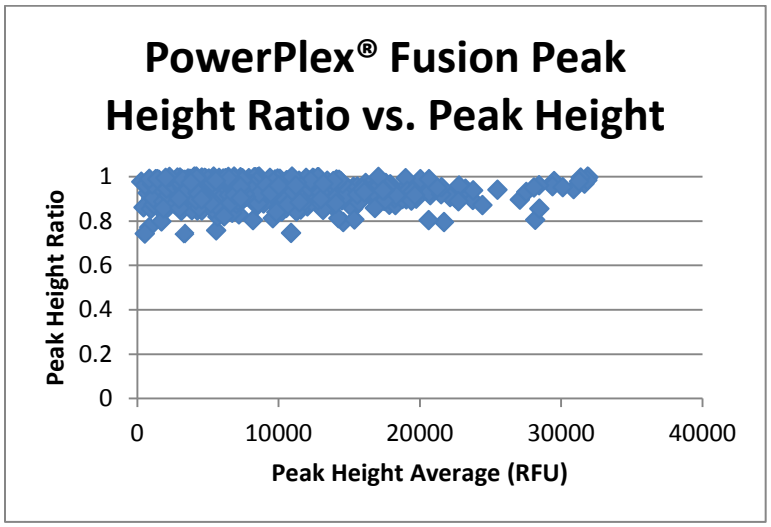


Figure 3.6.2 Peak height ratio over corresponding average peak heights of all samples processed at 26 cycles with a 24 second injection (reproducibility studies).

A stochastic threshold was also established for samples amplified at 26 cycles with a 12-second injection. Most samples had a peak height ratio between 70-100% regardless of the peak height average. The lowest peak height ratio of 73% was observed in locus D12S391 (Figure 3.6.3). Based on the minimum peak height ratio observed, a stochastic threshold of 150 RFUs for all samples amplified at 26 cycles with a 12-second injection was recommended.

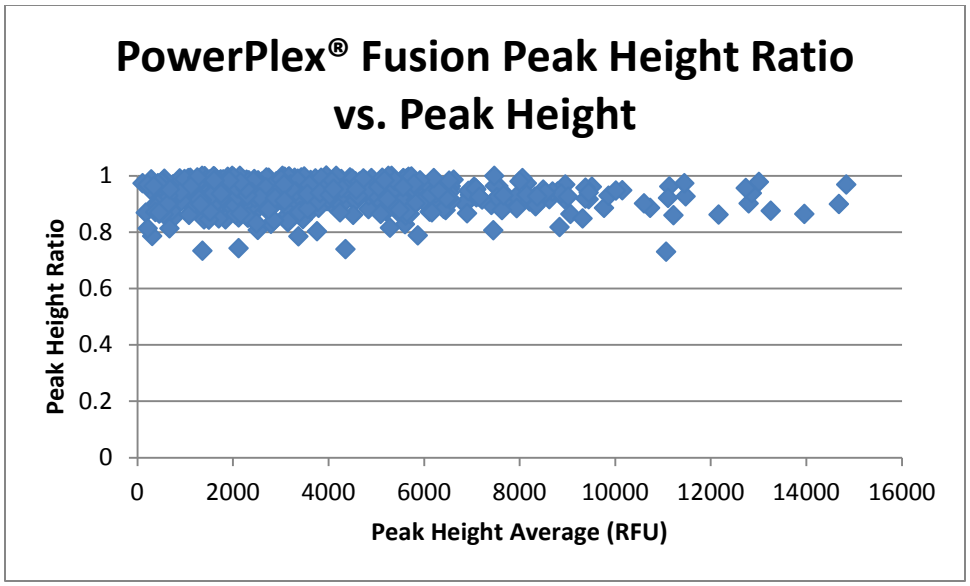


Figure 3.6.3 Peak height ratio over corresponding average peak heights of all samples processed at 26 cycles with a 12 second injection.

GlobalFiler™ Express

During GlobalFiler™ Express sensitivity studies, the lowest peak height ratio at 25 cycles observed was 56.6% at locus SE33 with allele calls 13 and 32.2 (Figure 3.6.4). Based on the minimum peak height ratio observed, a stochastic threshold of 130 RFUs for all samples amplified at 25 cycles with a 15-second injection was recommended.

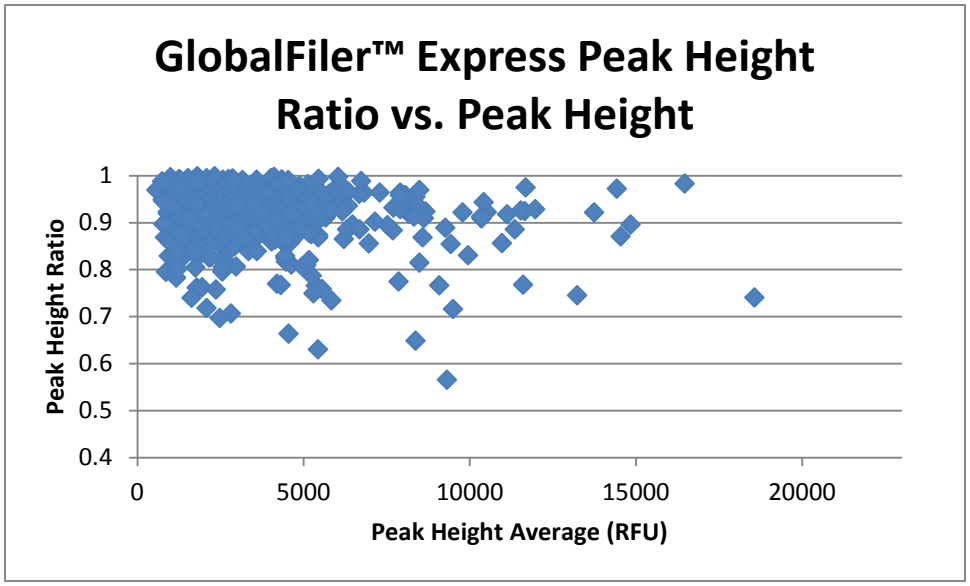


Figure 3.6.4 Peak height ratio over corresponding average peak heights of all samples processed at 25 cycles with a 15 second injection.

During GlobalFiler™ Express reproducibility studies, the lowest peak height ratio at 25 cycles observed was 60.6% at locus SE33 with allele calls 15 and 28.2 (Figure 3.6.5). Based on the minimum peak height ratio observed, the recommended stochastic threshold of 130 RFUs for all samples amplified at 25 cycles with a 15-second injection established in sensitivity studies remained the same.

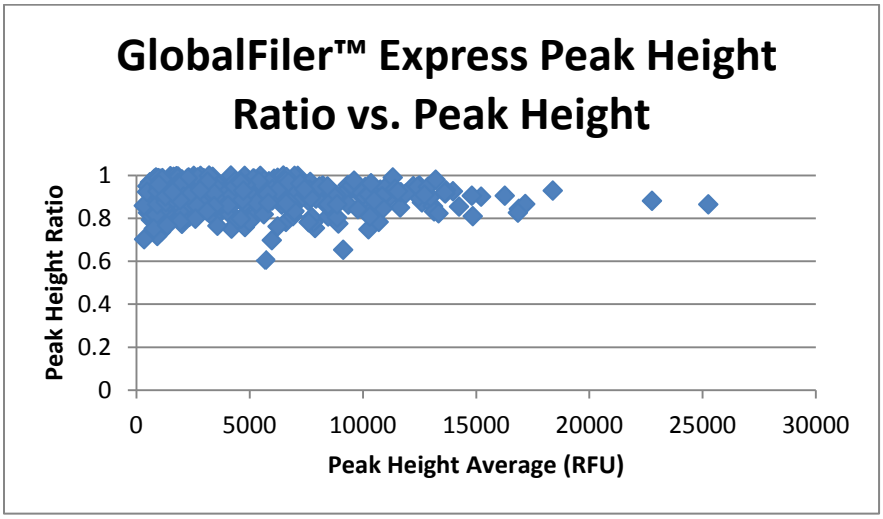


Figure 3.6.5 Peak height ratio over corresponding average peak heights of all samples processed at 25 cycles with a 15 second injection.

A stochastic threshold was also established for samples amplified at 25 cycles with a 30-second injection. The lowest peak height ratio of 51.6% was observed at locus D12S391 (Figure 3.6.6). Based on the minimum peak height ratio observed, a stochastic threshold of 200 RFUs for all samples amplified at 25 cycles with a 30-second injection was recommended.

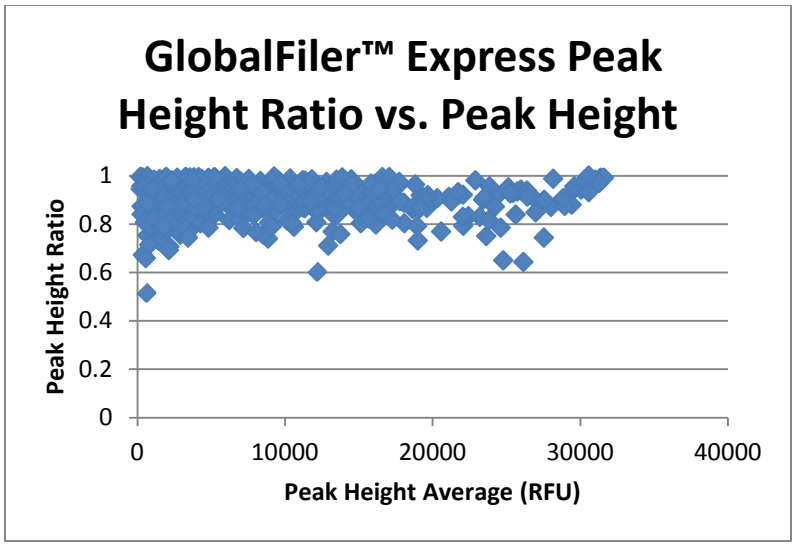


Figure 3.6.6 Peak height ratio over corresponding average peak heights of all samples processed at 25 cycles with a 30 second injection.

3.7 Contamination Studies

PowerPlex® Fusion studies showed no contamination in either blank punch 1 or blank punch 2 using 26 cycles with a 12- or 24-second injection using the recommended analysis parameters. In addition, no contamination was observed in any sample or control during the entirety of the validation.

GlobalFiler™ Express studies showed no contamination in either blank punch 1 or blank punch 2 using 25 cycles with a 15- or 30-second injection using the recommended analysis parameters. However, contamination was observed in one negative control amplified using 27 cycles during sensitivity studies. Contamination during sensitivity studies was attributed to static issues addressed in subsequent studies.

3.8 Concordance Studies

Concordance was checked using profiles from Identifiler® Plus, PowerPlex® Fusion, and GlobalFiler™ Express at all loci. Concordant results were obtained for all samples used in all internal validation studies. All non-matching and off-ladder alleles can be attributed to pull-up or background due to off-scale data.

3.9 NIST Studies

PowerPlex® Fusion

For a 12 second injection, the average peak height of amplified component F was 9300 RFUs. The average peak height ratio was 92.5%. The average peak height falls within 2 standard deviations of the results obtained in decreased injection time study. The peak height ratio falls within 1 standard deviation of the results obtained in decreased injection time study. For a 24 second injection, the average peak height of amplified component F was 11132 RFUs. The average peak height ratio was 92.4%. Both the average peak height and peak height ratios fall within 1 standard deviation of the results obtained in reproducibility studies. Concordant results were obtained (Table 3.9.1).

Table 3.9.1 DNA profiles obtained from amplified component F using PowerPlex® Fusion with a 12 and 24 second injection compared with NIST Reference.

Marker	NIST Reference Material	Fusion Component F (12 seconds)	Fusion Component F (24 seconds)
AMEL	X,Y	X,Y	X,Y
D3S1358	16,17	16,17	16,17
D1S1656	17.3,17.3	17.3,17.3	17.3,17.3
D2S441	14,14	14,14	14,14
D10S1248	14,15	14,15	14,15
D13S317	8,11	8,11	8,11
Penta E	11,15	11,15	11,15
D16S539	9,11	9,11	9,11
D18S51	17,22	17,22	17,22
D2S1338	17,17	17,17	17,17
CSF1PO	10,11	10,11	10,11
Penta D	9,10	9,10	9,10
TH01	7,9.3	7,9.3	7,9.3
vWA	16,18	16,18	16,18
D21S11	29,32.2	29,32.2	29,32.2
D7S820	8,12	8,12	8,12
D5S818	11,13	11,13	11,13
TPOX	8,8	8,8	8,8
DYS391	12	12	12
D8S1179	10,13	10,13	10,13
D12S391	18,19	18,19	18,19
D19S433	13,14	13,14	13,14

FGA	21,25	21,25	21,25
D22S1045	11,15	11,15	11,15

GlobalFiler™ Express

For a 15 second injection, the average peak height of amplified component F was 4783 RFUs, the average peak height ratio was 91%. Both the average peak height and peak height ratios fall within 1 standard deviation of the results obtained in reproducibility studies. For a 30 second injection, the average peak height of amplified component F was 8986 RFUs and the average peak height ratio was 91%. Both the average peak height and peak height ratios fall within 1 standard deviation of the results obtained in the increased injection time study. Concordant results were obtained (Table 3.9.2).

Table 3.9.2 DNA profiles obtained from amplified component F using a 15 and 30 second injection compared with NIST Reference.

Marker	NIST Reference Material	GlobalFiler Component F (15 seconds)	GlobalFiler Component F (30 seconds)
D3S1358	16,17	16,17	16,17
vWA	16,18	16,18	16,18
D16S539	9,11	9,11	9,11
CSF1PO	10,11	10,11	10,11
TPOX	8,8	8,8	8,8
Yindel	N/A	2	2
AMEL	X,Y	X,Y	X,Y
D8S1179	10,13	10,13	10,13
D21S11	29,32.2	29,32.2	29,32.2
D18S51	17,22	17,22	17,22
DYS391	12	12	12
D2S441	14,14	14,14	14,14
D19S433	13,14	13,14	13,14
TH01	7,9.3	7,9.3	7,9.3
FGA	21,25	21,25	21,25
D22S1045	11,15	11,15	11,15
D5S818	11,13	11,13	11,13
D13S317	8,11	8,11	8,11
D7S820	8,12	8,12	8,12
SE33	12,21	12,21	12,21
D10S1248	14,15	14,15	14,15
D1S1656	17.3,17.3	17.3,17.3	17.3,17.3
D12S391	18,19	18,19	18,19

D2S1338	17,17	17,17	17,17

Section 4: Discussion

4.1 Precision Studies

The degree of precision was assessed by analyzing the standard deviation of each allele base pair sized within an injection and across injections for both PowerPlex® Fusion and GlobalFiler™ Express amplification kits. In order for alleles to be called correctly, they must fall within the +/- 0.5 base pair allelic ladder window set by the GMID-X software. There may be occasions in which a sample allele may fall outside the acceptable base pair window due to measurement error. This measurement error is rare in systems having a standard deviation of 0.15 base pairs or less.

The data indicated that no markers and/or alleles within injections or across injections exceed a standard deviation of 0.15 base pairs. Therefore, both amplification kits are precise and only 1 allelic ladder is required per 3 injections during capillary electrophoresis setup (10). Within injections, PowerPlex® Fusion had a standard deviation of 0.104 basepairs compared to 0.081 in GlobalFiler™ Express. In addition, across all injections, PowerPlex® Fusion had a standard deviation of 0.072 compared to 0.061 in GlobalFiler™ Express. Regardless of the variations among both amplification kits, both PowerPlex® Fusion and GlobalFiler™ Express produce precise base pair sizing using the 3500 Genetic Analyzer.

4.2 Sensitivity Studies

PowerPlex® Fusion

Overall, the 27 cycle amplification parameter was unsuitable because it produced off-scale data and significant amounts of elevated baseline, pull-up, elevated stutter and background. At 26 cycles, pull-up was observed in at least one FTA blood, FTA saliva and buccal sample.

The most robust results were obtained from samples amplified at 25 cycles. The peak height averages of all types of samples at 25 cycles fall within the optimal peak height range of 3,000 – 12,000 RFUs (10). Further evaluation of using 25 and 26 cycles was completed in reproducibility studies.

Based on the data obtained from this study, the analytical threshold was set at 200 RFUs and the stochastic threshold was set at 275 RFUs for all samples amplified at 25 and 26 cycles and run with a 24 second injection. The lowest peak height for all samples in both 25 and 26 cycles was 696 RFUs. The lowest peak height ratio observed was 73.3% (26 cycles). Therefore, it can be expected that for both 25 and 26 cycles, peak height ratios should be at least 70% for all samples.

Using 9.2 µL of Formamide and 0.8 µL of CC5 Internal Lane Standard (ILS) produced peak heights between 400-1200 RFUs for all samples in sensitivity studies. These lower than usual peak heights did not affect base pair sizing. In subsequent studies, the ILS and Hi-Di Formamide volumes were further adjusted to 9.1 µL of Formamide and 0.9 µL of CC5 ILS to increase peak heights.

GlobalFiler™ Express

Overall, using a 27 cycle amplification parameter was determined unsuitable due to off-scale data and significant amounts of pull-up, elevated stutter and background. The most robust results were obtained from samples amplified at 25 cycles. With the exception of FTA blood samples, peak height averages at 25 cycles fell within the optimal peak height range of 3,000 – 12,000 RFUs (10). However, due to the significant increase of peak height standard deviation, and a lower than ideal minimum peak height ratio at 26 cycles, all samples were only assessed at 25 cycles in reproducibility.

One of the key observations during GlobalFiler™ Express sensitivity studies was the documentation of an unknown artifact (Figure 4.2.1). The artifact was observed in most FTA blood samples but was not observed in buccal or FTA saliva samples. In conjunction with the artifact at D18S51, an artifact that closely resembles "pull-up" at locus FGA was also observed. However, any artifacts at FGA were filtered out using a 20% global filter. The artifact was made known to Life Technologies but the issue has not been resolved. It is important to note that the artifact is easily distinguishable from true alleles and stutter. These loci were closely evaluated in all validation studies.

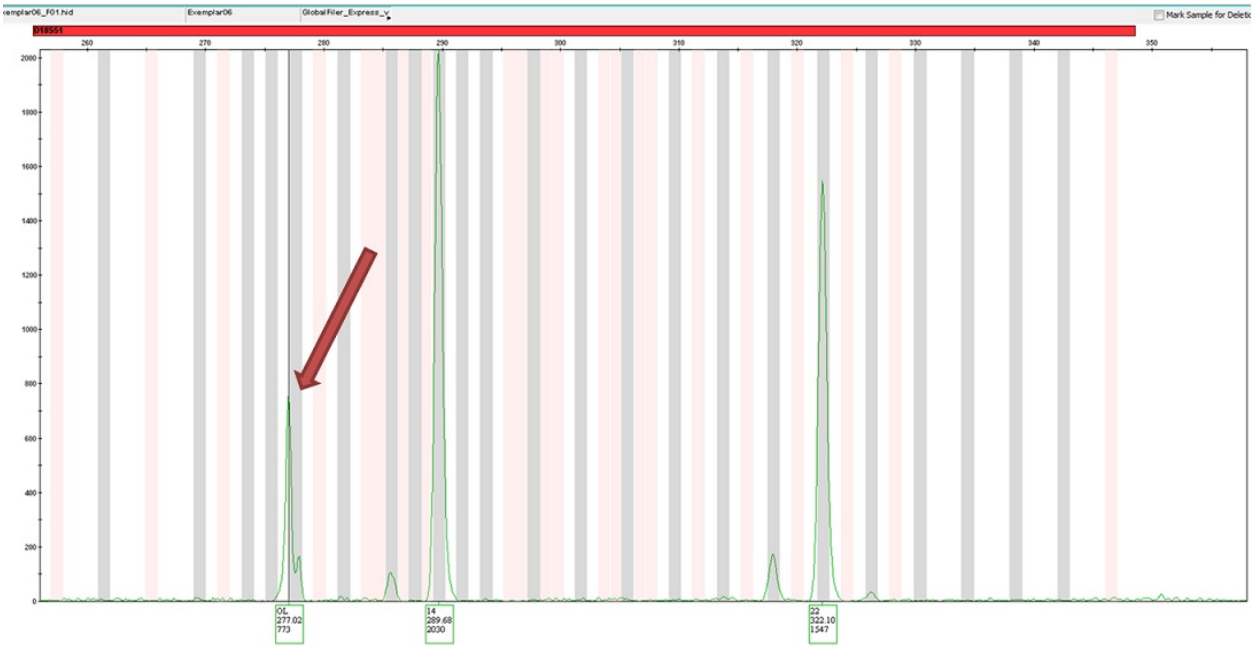


Figure 4.2.1 Example of unknown artifact at locus D18S51 on FTA blood samples.

Based on the data obtained from this study, the analytical threshold was set at 70 RFUs and the stochastic threshold was set at 140 RFUs for all samples amplified at 25 cycles with a 15 second injection. The lowest peak height for all samples using these parameters was 740 RFUs. These values were further evaluated in reproducibility studies.

4.3 Reproducibility

PowerPlex® Fusion

In reproducibility studies, the average peak heights for FTA blood, FTA saliva and buccal samples amplified for 26 cycles were 11236, 7968 and 7876 RFUs, respectively. Overall, 26 thermal cycles for all samples yielded averages that better fit the optimum peak heights recommended by the 3500 Genetic Analyzer manual. However, 26 cycles produced some samples with off scale data. A proposed solution to the observed off-scale data in 26 cycles was to decrease the injection time from 24 to 12 seconds. An evaluation of all samples amplified at 26 cycles with a 12 second injection was completed in the injection studies.

Additionally, this validation supports the ability to amplify blood and saliva samples from FTA cards, as well as buccal swabs, in the same 96-well plate using a single cycle number (26 cycles) and a default injection time (12 seconds) and the ability to re-inject low-level samples using a 24 second injection.

Based on the data obtained from this study, the analytical and stochastic threshold remained at 200 RFUs and 275 RFUs for all samples amplified at 26 cycles and run with a 24 second injection. The lowest peak height ratio observed was 74.3%. Therefore, it can be expected that for 26 cycles, peak height ratios should be at least 70% for all samples. These values matched those observed in sensitivity studies.

Using 9.1 µL of Formamide and 0.9 µL of CC5 Internal Lane Standard (ILS) produced peak heights between 800-1600 RFUs for all samples. These peak heights are higher than the ones observed in sensitivity studies with 9.2 µL of Formamide and 0.8 µL of ILS. In subsequent studies, 9.1 µL of Formamide and 0.9 µL of ILS were used to allow the use of a 10 µL multi-channel pipette.

GlobalFiler™ Express

FTA blood samples were assessed using a 26 cycle parameter in reproducibility in an attempt to raise the average peak heights observed at 25 cycles during sensitivity to the optimal range as described by the manual. While 26 cycles did increase the average peak heights to 6158 RFUs, this cycling parameter was not recommended for use. Even though using 26 cycles was reproducible, 25 cycles produced a more robust set of data (lower standard deviation).

In reproducibility, amplifying one 1.2mm punch of blood using 25 cycles resulted in an average of 4488 RFUs (within the optimal peak height range). For this reason, this validation supported the ability to amplify blood and saliva samples from FTA cards, as well as buccal swabs, in the same 96-well plate using a single cycle number (25 cycles) and a default injection time (15 seconds).

A minimum peak height ratio of 60.6% was observed in one blood sample. However, in sensitivity, the minimum peak height ratio of 56.6% was observed in one buccal sample. Both minimums were observed at locus SE33. Therefore, it can be expected that for 25 cycles, all samples must have at least a 56% peak height ratio. Due to the high variability at this locus, a minimum peak height ratio of 50% was recommended for interpretation parameters. This recommendation raised the stochastic threshold for GlobalFiler™ Express to 140 RFUs after completing reproducibility studies.

Using these parameters, drop out was observed in only one sample. To provide an additional option for re-processing, a 30 second injection time was investigated in an injection study of this validation. In this manner, low level samples amplified at 25 cycles may be re-injected using 30 seconds without a need to re-amplify or obtain a new sample.

4.4 Injection Time Studies

PowerPlex® Fusion

Decreasing the injection time from 24 to 12 seconds had the same overall results for all types of samples. There was a decrease in peak height averages for FTA blood, FTA saliva and buccal samples to 5214, 3291 and 3246 RFUs, respectively. All peak heights are less than half of the corresponding average peak heights at 24 second injections. Regardless, all peak height averages fell within the 3,000-12,000 optimum range (10).

Even though there was a decrease of peak height averages, the minimum and average peak height ratios remained the same. The average peak height ratio for all samples was higher than 93%. In addition, the minimum peak height ratio observed was 73%. Therefore, it is expected that all samples amplified at 26 cycles and injected for 12 seconds will have a peak height ratio of at least 73%. The recommended peak height ratio was set for 70% (matching the recommended peak height ratio during reproducibility). One of the advantages of using a 12 second injection is the reliability and precision of the results. The decrease in peak height standard deviations indicates that the range of peak heights is smaller, creating a more robust set of peak heights for all samples.

The limit of detection and quantitation, and analytical threshold were calculated for samples injected for 12 seconds. The highest analytical threshold of 64 RFUs was observed in the blue dye channel. For simplicity and to be conservative, it was determined to round up the analytical threshold to 100 RFUs for all dye channels. Using this analytical threshold, the stochastic threshold was calculated as 150 RFUs for all samples. To assess these thresholds, all samples were re-analyzed. No homozygous alleles fell below the stochastic threshold and no pull-up or noise artifacts were observed. However, only one FTA saliva sample had observed

allelic drop-out. As previously discussed, this FTA saliva sample is a possible outlier due to improper collection. It is important to note that the analytical and stochastic thresholds for samples injected at 12 seconds were lower than the thresholds set using a 24 second injection. The difference can be explained by a lower signal-to-noise ratio.

Overall, decreasing the injection time to 12 seconds is a reliable method to eliminate artifacts caused by off-scale data without affecting peak height ratios across all samples. After the injection study, it was recommended that all samples be amplified using 26 cycles and injected for 12 seconds. If drop-out or single peaks below the stochastic threshold are observed, these samples may be improved by re-injecting for 24 seconds.

GlobalFiler™ Express

Increasing the injection time from 15 to 30 seconds for FTA blood samples does not affect the minimum recommended peak height ratio. Additionally, increased peak heights are observed. The peak height average for FTA blood samples injected for 30 seconds was 9542 RFUs and falls within the 3,000-12,000 optimum range. This observed increase is desired for low level samples which did not produce sufficient peak heights at a 15 second injection.

For FTA saliva samples, the average peak height ratio remained the same at 91%. There was an increase in peak height averages from 6083 to 9567 RFUs. One of the noticeable differences in the increased injection time study of FTA saliva samples is the decrease of minimum peak height ratio from 73.6% to 51.6%. The lowest peak height ratio was observed in an FTA saliva that had dropped out in reproducibility studies (not included in reproducibility study calculations). The recommended peak height ratio remained 50% as discussed in reproducibility studies. This low peak height ratio was recommended to account for the potential distance of alleles observed at locus SE33.

The addition of the FTA saliva sample that previously dropped out explains the decrease in minimum peak height ratio and also contributed to the noticeable increase in peak height standard deviation (from 4559 to 9178 RFUs). Even though there was an observed minimum peak height ratio decrease, it is important to be able to interpret low-level samples without the need for re-amplification or re-collection. This particular FTA saliva sample had a peak height ratio above the recommended minimum peak height ratio in reproducibility studies.

For buccal swab samples, doubling the injection time led to an approximate doubling of peak height average and minimum peak height. The average peak height ratio remained the same at 90%. There was a decrease in minimum peak height ratio from 69.8% to 64.3%; however, the decrease is within 1 standard deviation.

The limit of detection and quantitation, and analytical threshold were calculated for samples injected for 30 seconds. The highest analytical threshold of 78 RFUs was observed in the red dye channel. For simplicity and to be conservative, it has been determined to round up the analytical threshold to 100 RFUs. Using this analytical threshold, the stochastic threshold was set to 200 RFUs for all samples amplified at 25 cycles with a 30 second injection. Both the analytical threshold and stochastic thresholds are higher than the thresholds that were observed during sensitivity studies using 25 cycles with a 15 second injection.

Overall, increasing the injection time to 30 seconds is a reliable method to increase peak heights without affecting peak height ratios across all samples. Furthermore, the increase in peak heights supports the ability to analyze samples with observed drop-out at a 15 second injection.

4.5 Contamination

No contamination was observed in any sample or control during the entirety of the PowerPlex® Fusion internal validation.

In all GlobalFiler™ Express internal validation studies, the only instance of observed contamination was during sensitivity studies. Contamination was observed in the FTA blood negative control amplified at 27 cycles. However, during sensitivity studies, static issues were encountered. Static issues with punches often lead to contamination due to the movement of these punches to neighboring wells. In studies following sensitivity, static issues were eliminated by placing a dryer sheet under the 96-well plate prior to adding punches. No contamination was observed in any samples or controls once static issues were resolved. In addition, the 27 cycle amplification was not recommended for used after the sensitivity study.

Lastly, based on PowerPlex® Fusion and GlobalFiler™ Express contamination assessments, it was determined that punching one blank in between samples during amplification set-up is sufficient to eliminate contamination. Using a dryer sheet to diminish static issues is critical to replicate these contamination free results.

4.6 Concordance & NIST

Concordance was checked using profiles from Identifiler® Plus, PowerPlex® Fusion, and GlobalFiler™ Express at all loci. Concordant results were obtained for all samples used in reproducibility studies. All non-matching and off-ladder alleles were attributed to pull-up or background due to off-scale data. A confirmation of genotypes was completed by a second analyst. Concordant results for NIST Standard Reference Material 2391c Component F were obtained using the PowerPlex® Fusion and GlobalFiler™ Express amplification systems.

4.7 Protocol Observations

A disadvantage of using PowerPlex® Fusion is the inability to incubate buccal swabs at room temperature. Buccal swabs must be incubated at 70^0C for 30 minutes. All PowerPlex® Fusion reagents are also stored between -15^0C and -25^0C, therefore, all reagents must be thawed

out for at least 5 minutes before use. An advantage of using GlobalFiler™ Express is the ability to incubate buccal swabs at room temperature and that reagents do not need to be thawed because they are stored at 2 - 8°C.

A disadvantage of GlobalFiler™ Express is the need to add TE buffer to all FTA samples except the amplification positive. The inconsistency of the reaction mix among the amplification positive and the FTA samples creates an inconvenience when preparing the reaction mix. This problem is not encountered in PowerPlex® Fusion because a uniform reaction mix can be used for all FTA sample reaction wells, including the amplification negative and positive.

In PowerPlex® Fusion, the FTA amplification positive does not require a 2800M DNA control dilution, while a dilution of 2800M is needed for buccal samples. Additionally, purchase of the SwabSolution™ Kit is needed for the incubation of buccal swab samples. On the other hand, GlobalFiler™ Express requires the additional purchase of TE Buffer for amplification of FTA samples and Prep-n-Go™ Buffer for incubation of buccal swab samples.

Conclusion

Using both the Promega PowerPlex® Fusion PCR System and Applied Biosystems® GlobalFiler™ Express kit with the 3500 Genetic Analyzer will produce concordant, reliable and robust results.

The 3500 Genetic Analyzer is precise when using both amplification kits. No markers and/or alleles with high standard deviation values (exceeding 0.15 base pairs) within injections or across the plate were observed. Therefore, only one allelic ladder is required per three injections.

The following amplification parameters were recommended for PowerPlex® Fusion: 26 cycles with a 12 second injection for blood and saliva on FTA cards, as well as buccal swab

samples. Amplification will involve one 1.2mm punch of saliva or blood on FTA cards and 2 µL of swab extract. If drop-out or single peaks below the stochastic threshold are observed, these samples may be improved with a 24 second injection. The recommended analytical and stochastic thresholds for the amplification of samples at 26 cycles with a 12-second injection using PowerPlex® Fusion are 100 and 150 RFUs, respectively. However, both the analytical and stochastic thresholds are different for samples injected for 24 seconds. With a 24-second injection, the analytical and stochastic thresholds are 200 and 275 RFUs respectively. Additionally, a minimum peak height of 70% is expected using a 12- and 24-second injection.

The following amplification parameters are recommended for GlobalFiler™ Express: 25 cycles with a 15 second injection for blood and saliva on FTA cards, as well as buccal swab samples. Amplification will involve one 1.2mm punch of saliva or blood on FTA cards and 3 µL of swab extract. If drop-out or single peaks below the stochastic threshold are observed, these samples may be improved with a 30 second injection. The recommended analytical and stochastic thresholds for the amplification of samples at 25 cycles and a 15-second injection using GlobalFiler™ Express are 70 and 140 RFUs, respectively. However, both the analytical and stochastic thresholds are different for samples injected for 30 seconds. With a 30-second injection, the analytical and stochastic thresholds are 100 and 200 RFUs respectively. Additionally, a minimum peak height of 50% is expected using a 15- and 30-second injection.

All known samples amplified in either Promega PowerPlex® Fusion PCR System and Applied Biosystems® GlobalFiler™ Express kit can be analyzed using a 20% global filter. A 20% filter will eliminate the majority of artifacts including background, pull-up, spikes, stutter and minus A. However, occasional pull-up or background due to off-scale data may be observed.

In regard to contamination, placing a dyer sheet under the 96-well plate eliminated static issues for both amplification kits. Adding this step to the procedure will also allow samples to be punched in the well before or after adding the reaction mix. Punching one blank in between samples during amplification set-up is sufficient to eliminate contamination.

The evaluation of both Promega PowerPlex® Fusion PCR System and Applied Biosystems® GlobalFiler™ Express was important to determine the best fit for the Department of Forensic Sciences. The optimization of a single thermal cycling parameter and one optimal injection time for all three types of samples will allow the amplification and injection of blood and saliva samples on FTA cards and buccal swabs on the same 96-well reaction.

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Disclaimer

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Appendix A: Thermal Cycling Parameters

Table 1. Promega® Fusion thermal cycling parameters used for all internal validation studies.

Initial Incubation Step	Cycle (25, 26 or 27)			Final Extension	Final Hold
	Denature	Anneal	Extend		
HOLD	CYCLE			HOLD	HOLD
96 ºC 1 min	94 ºC 10 sec	59º C 1 min	72º C 30 sec	60º C 20 min	4 ºC ∞

Table 2. GlobalFiler™ Express thermal cycling parameters used for all internal validation studies.

Initial Incubation Step	Cycle (25, 26 or 27)		Final Extension	Final Hold
	Denature	Anneal/Extend		
HOLD	CYCLE		HOLD	HOLD
95 ºC 1 min	94 ºC 3 sec	60º C 30 sec	60º C 8 min	4 ºC ∞