Comparison of Direct Amplification vs. Extraction, Quantitation, and Amplification using GlobalFiler™ or Fusion Kits™ using 3500 Genetic Analyzers

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Abstract

With the recent purchase of Applied Biosystems® 3500 Genetic Analyzers, the Tennessee Bureau of Investigation (TBI) is looking to optimize these instruments for their CODIS division. The purpose of this comparison study was to compare two methods and also two separate kits for obtaining the profiles for input into CODIS. The traditional method consisted of performing the more time consuming process of extraction, quantitation, and amplification of buccal swabs before performing capillary electrophoresis. This would be performed using either the Applied Biosystems® GlobalFiler™ DNA Amplification kit or the Promega PowerPlex® Fusion™ kit using 7 different reference samples for multiple runs. The direct method utilized direct amplification of buccal swabs before performing capillary electrophoresis using either the GlobalFiler™ Express Kit or the Fusion™ Direct kit. This was performed using the same 7 reference samples in the first study for multiple runs as well. For the direct method, three different cycle numbers were also compared to find an optimization of cycle number within each separate kit. For both methods, traditional and direct, the test was performed with multiple runs to ensure quality across all runs and ensure reproducibility of results.

Once all samples were ran and analyzed, statistical comparison was performed between GlobalFiler™ and Fusion™ and between the traditional and direct methods themselves. The comparisons were performed by looking at each individual run within the specific kit and averaging the runs for each sample and then between the two kits for each individual's sample. Between the two methods, sensitivity and averages were compared to view if similar results could be obtained for each.

Overall, this comparison showed that reportable results were able to be obtained between the two methods and better results for the direct method in some cases. When comparing the kits

of GlobalFiler™ Express and Fusion™ Direct, it was found that Fusion™ Direct produced statistically better results especially in terms of peak height ratio. It was suggested that future studies, and eventually validation, be performed with the Fusion™ kit for use in the CODIS unit at TBI. This will be needed due to the FBI's Combined DNA Index System (CODIS) Core Loci Working Group recommendations are changing from 13 core CODIS loci to 20 required and 3 recommended loci (15). This would be possible with either kit that TBI would decide to continue with for validation.

Introduction

The world of DNA and DNA technology is ever changing. Significant advancements have been made in the past decade in the way DNA evidence is being tested and analyzed with the most common now being Short Tandem Repeat (STR) Analysis (1). These advancements include techniques, kits, and instruments utilized throughout the testing process. For any DNA lab, whether for law enforcement or research, the choice of what instruments and kits to choose can be a time consuming and costly decision. Many kits and instruments have the same methodology, target loci, and cost the same price to run a sample, but one might be better suited for the methods and needs for a particular lab. At the Tennessee Bureau of Investigation (TBI) Applied Biosystems® 3500 Genetic Analyzers have been recently purchased for their Combined DNA Index System (CODIS) division. This is a database of DNA profiles maintained at the local, state, and national level. The purpose of the database is to assist law enforcement by linking perpetrators to biological evidence (1). Currently, TBI is outsourcing their samples for the database where it can take up to a month to receive results. With the purchase of the new 3500s, TBI is hoping slowly to start bringing CODIS in-house.

At of the start of this study, TBI was using the Applied Biosystems® AmpFLSTR® Identifiler® PCR Amplification Kit (Life Technologies™, Foster City, CA). With the purchase of new instruments and new kits, TBI is testing to see if direct amplification would be possible with the same level of results as the traditional analysis method. With the number of backlogs that every lab has today (6), more cost effective method with a faster turnaround time is needed. Single-source samples, which were the only sample type used for this study, are of good quality and do not need to be reamplified or rerun to produce a full profile. With the abundance of reference samples and the lack of need to quantitate them, per Standard 9.4 of the FBI's Quality Assurance Standards (7), a decrease in time and cost for the GlobalFiler™ Express Amplification Kit or Fusion™ Direct Kit is optimal for reference samples.

There are two main differences between the two individual kits. The first difference is that GlobalFiler™ and GlobalFiler™ Express utilizes a 6-dye chemistry while the PowerPlex® Fusion™ and Fusion™ Direct utilizes a 5-dye kit. While one kit is a 6-dye and one kit is a 5-dye, the same number (24) loci are called. The second difference is that the GlobalFiler™ and GlobalFiler™ Express kit have more sex determining loci called (13).

The Fusion™ system is also an upgrade from the PowerPlex® 16 System in that 36 new alleles have been added to the allelic ladder and the maximum discriminatory power increased from 2.82E-19 for PowerPlex® 16 to 1.36E-28 for Fusion™. This is compared to the GlobalFiler™ power of discrimination of 1.60E-27 (13).

Materials and Methods

DNA Extraction

For this study and comparison, buccal cotton swabs were used for the known reference samples. The single-source swabs were collected from 7 individuals working in the lab and whose DNA profile was previously analyzed and placed in the employee DNA database. For the first half of the comparison study, samples were extracted using a Qiagen BioRobot EZ1 Workstation and the EZ1 DNA Investigator Kit. From the swab, 1/3 of the cotton tip was cut and placed into a 2 mL extraction tube. 10µL of proteinase K was added to each sample. A dilute Buffer G2 of 1:1 with ultrapure water for n+1 samples was created and 190µL was added to each sample. The sample was then vortexed for 10s and centrifuged briefly. Samples were incubated at 56°C for a minimum for 15 minutes. After incubation, any solid material present was removed and disposed of. Using the EZ1 BioRobot, "Start" was pressed to display the "Protocols" menu. Normalization protocol was performed; TE buffer was chosen as the elution buffer, and eluted in 50µL. Reagent cartridges were placed in the instrument making sure to flick the cartridge to mix the magnetic particles. Samples were added to the EZ1 BioRobot with sample tubes being placed in the 4^{th} row, nothing in the 3^{rd} row, tips and tip holders in the 2^{nd} and elution tubes in the 1^{st} row. After loading of cartridges, tubes, and tips, "Start" was pressed and allowed to run. After the run, all tubes and cartridges were discarded except for the elution tubes containing the purified sample (2).

Quantification

Quantification was performed using ABI 7500 and the Quantifiler Human DNA Quantitation Kit. Eight standards were used ranging in 8 different concentrations of 50.000 ng/µL down to 0.023 ng/µL. These standards were created previously for lab use. Using the Quantifiler Human Primer Mix and the Quantifiler PCR Reaction Mix, a master mix was created in a 1.5 mL microcentrifuge tube, using n+2 where n is the number of samples and 2 accounts for

loss, consisting of 10.5µL of the Human Primer Mix and 12.5µL of the PCR Reaction Mix for each sample. Once created, the PCR master mix was vortexed and then centrifuged. 23µL of this master mix was added into each reaction well along with 2µL of sample, standard, or control into appropriate wells. The standards were added in duplicate in the first two columns as well as having TE/glycogen blanks in the wells after the sample wells. The plate was sealed with an Optical Adhesive Cover and each well was scored to ensure a proper seal. Once sealed, the plate was centrifuged for 20 seconds to ensure no bubbles were present in the wells. The plate was then loaded onto the ABI 7500 and ran using the 7500 System Software. Each well was defined using the sample name and any wells not used were left blank. The plate was saved and run was started.

Once the run was finished, the plate was analyzed omitting any wells that did not contain sample. The "Analyze" arrow on the taskbar was chosen. Looking at the "Results" tab, the "Standard Curve" was chosen and reviewed for any inconsistencies. An R^2 value of ≥ 0.99 was needed to be present as well as a slope between -2.9 and -3.32. Also under the "Results" tab, "Report" was chosen in order to view the report. The report was then printed and then used for amplification calculations. The amount of DNA present for each sample was used in the equation 0.75/sample DNA quantity to determine the amount of DNA to pipette out and add to TE to give a final volume of 15µL for use during the amplification process for the Fusion kit (3). For the GlobalFiler kit, the amount of DNA that was shown to be present was divided by .1 to indicate the amount of TE to add to 1µL of the DNA sample. From this TE and DNA mixture, 15 µL was pipetted out for PCR amplification as discussed in the next section in order to obtain a concentration of .1 ng/µL of sample DNA.

Traditional PCR Amplification

Amplification was performed using GeneAmp PCR 9700 thermal cycler and the two kits of comparison interest. The first kit tested was the AB® GlobalFiler™ PCR Amplification Kit. First, a master mix consisting of 7.5µL of GlobalFiler™ Master Mix and 2.5µL of GlobalFiler™ Primer Set for n+2 samples was created also accounting for a negative control and a positive control. 10µL of the master mix was added into each appropriate well on a 96 well plate. For each sample or control a final volume of 25µL was obtained. For the negative control, this was done by adding 15µL of low TE buffer to the reaction mix. For the positive control, 10µL of the control DNA (0.1 ng/µL) with 5µL of low-TE buffer was added to the reaction mix. For each sample, 15µL of the TE and DNA mixture created during the quantitation step was added to their appropriate well. Once all samples and controls had been added to the plate, the plate was capped and centrifuged at 3000 rpm for about 1 minute. The plate was then amplified using the GeneAmp® PCR System 9700 for 29 cycles (10).

For the PowerPlex® Fusion™ System, a master mix was also created using 5 µL of the PowerPlex® Fusion 5X Master Mix, 5.0µL of the PowerPlex® Fusion 5X Primer Pair Mix, and 10µL of amplification grade water for n+2 samples and also accounting for a negative and positive control. Into each well on a 96 well plate, 20µL was added along with 5µL of the DNA and TE mixture that was created the same as with GlobalFiler™ for each sample, negative control, and positive control giving a total reaction volume of 25µL. Once all samples and controls had been added to the plate, the tubes were capped, centrifuged, and placed on the GeneAmp® PCR System 9700 for 30 cycles (11).

Capillary Electrophoresis and Data Analysis

Capillary electrophoresis was performed using Applied Biosystems® 3500 Genetic Analyzer (Life Technologies™, Foster City, CA). For the GlobalFiler kit, a master mix was created using 9.5µL of deionized formamide and 0.5µL of GeneScan LIZ 600v.2 size standard for n+4, where n is the number of samples and 4 accounts for loss. After vortexing and centrifuging of the master mix, 10.0µL was added into each well used on the 96-well plate. Into each designated well on the plate, 1.0µL of amplified DNA was added to the sample wells and 1.0µL of ladder into the designated ladder wells. Once all samples were added, a 96-well septum was added and the plate was briefly centrifuged in order to make the sure sample was at the bottom of the plate and no bubbles were present. The plate was then denatured at 95°C on a 9700 thermal cycler, using the 95°C temperature program, for five minutes and then snap-chilled in the freezer for three minutes. Once snap-chilled, the plate was placed back into the plate base and covered with the white plate retainer for analysis.

For the Fusion™ kit, a master mix containing 1.0µL CC5 Internal Lane Standard, ILS, 500 and 10.0µL Hi-Di™ formamide was created for n+2 samples. 11µL of this master mix was pipetted into each well. 1.0 µL of amplified sampled was added to the samples wells and 1.0 µL of PowerPlex® Fusion Allelic Ladder mix into the appropriate ladder well(s) before covering the plate with a plate septa and centrifuging the plate. The plate was then denatured at 95°C for 3 minutes and then immediately chilled for 3 minutes to snap-cool (7).

In the 3500 software, the same method was used for both the GlobalFiler™ and Fusion™ kits with the exception of GlobalFiler™ being a 6-dye system and Fusion™ being a 5-dye. Also, it was determined that a 15 second injection time was optimal for the Fusion™ kit even though the recommendation was 25 seconds. A 15 second time is also the sample injection time for GlobalFiler™. First, the oven was started by clicking “Start Pre-heat” on the dashboard screen.

A new plate was created naming the plate appropriately and choosing Number of Wells=96, Plate Type=HID, Capillary Length=36 cm, and Polymer=POP4. Under "Assign Plate Contents", the designated wells were named using the sample name. Allelic ladders, as well as positive and negative controls, were defined as such under Sample Type. From the Library, the appropriate Assay, File Name Convention, and Results Group were chosen for the specific kit being tested before saving the plate. The plate was then loaded onto the 3500 instrument, linked, and ran (4).

Once the run is completed, the analysis was performed using GeneMapper® *ID-X* Analysis Software Version 1.4. A new project was created and samples previously ran on the 3500 were added. Once samples were imported, analysis method, panel, size standard, and sample type columns were filled in for the appropriate kit. Once thresholds were chosen, the green arrow on the toolbar was pressed in order to analyze the samples and also to name the project. Once analyzed, each sample was viewed individually using the icon with multiple colored peaks from the Samples Tab and then printed for further analysis (5).

Direct Amplification

Direct amplification was used for the second half of this study to determine if the same quality of results could be produced and reproduced at the same level as the traditional method of extraction, quantitation, and amplification.

For the GlobalFiler™ Express PCR Amplification Kit, the sample lysates were first prepared. This was done by adding 400µL of the Prep-n-Go™ Buffer to a 96 deep well plate before adding the entire head of each swab to the appropriate well. For the negative control, 200µL of the Prep-n-Go™ Buffer was added to a well with no swab present. The plate was then left to sit for 20 minutes at room temperature to lyse the sample. After 20 minutes, the sample

lysates were transferred out of the plate into new tubes (8). After this transfer, a master mix consisting of 6.0µL of the Master Mix and 6.0µL of Primer Set was combined for n+2 samples. 12µL of this created reaction mix was pipetted into appropriate wells on a 96-well plate. Samples and controls were then added to the appropriate wells with 3µL of the sample lysate added to their appropriate wells and 3µL of the DNA Control 007 added to the positive control for 25 and 26 cycles, 2µL of the DNA Control 007 for 27 cycles, and 1µL for 28 cycles. The plate was then capped, and then centrifuged at 3000 rpm for about 1 minute. The plate was then amplified on the GeneAmp® PCR System 9700 for the optimum cycle number (9).

For the PowerPlex® Fusion™ System, sample lysates were first prepared using the SwabSolution™ Kit. This was performed by first placing the buccal swab heads in the appropriate 1.5 mL tubes and adding 1 mL of the SwabSolution™ Reagent to each tube. The tubes were then placed in a heat block at 70°C for 30 minutes (11). A master mix consisting of 13µL of amplification grade water, 5.0µL of PowerPlex® Fusion 5X Master Mix, and 5.0µL of PowerPlex® Fusion 5X Primer Pair Mix was created using n+2, where n is the number of samples to be amplified, including positive and negative control and 2 accounts for loss of product. After created, 23µL of this master mix was pipetted into each MicroAmp® reaction tube. For each sample, 2.0µL of swab extract was pipetted into each appropriate tube. For the positive control, 2.0µL of the 2800M Control was added to the PCR amplification mix. For the negative control, 2.0µL of amplification grade water or TE buffer was added to the PCR amplification mix. After all samples and controls were added the tubes were capped and briefly centrifuged. The tubes were then placed in the GeneAmp® PCR System 9700 thermal cycler for the amplification process (12).

Capillary Electrophoresis and Data Analysis

Capillary electrophoresis and data analysis was performed using the same method for the direct amplification kits as was used with the extraction and quantitation kits.

Results and Discussion

Cycle Times

For GlobalFiler™ Express, 26, 27, and 28 cycle times for amplification were tested to determine the optimum number of cycles for the instruments in the lab. These cycle times were recommended by the kit for testing and comparison. It was determined that 26 and 27 produced better results than 28 cycles due to the overloading and stutter present in the results for 28 cycles. 26 and 27 cycles were then ran again using new samples and compared. 27 cycles was shown to produce better results than 26 due to the fewer artifacts present and the peak height ratios calculated. Figure 1 shows an example of a color channel of a profile and why 27 cycles were chosen. 26 cycles are present in the first row, 27 in the second, and 28 in the last. 26 and 28 cycles both had the same loci flagged red while 27 cycles are all green.

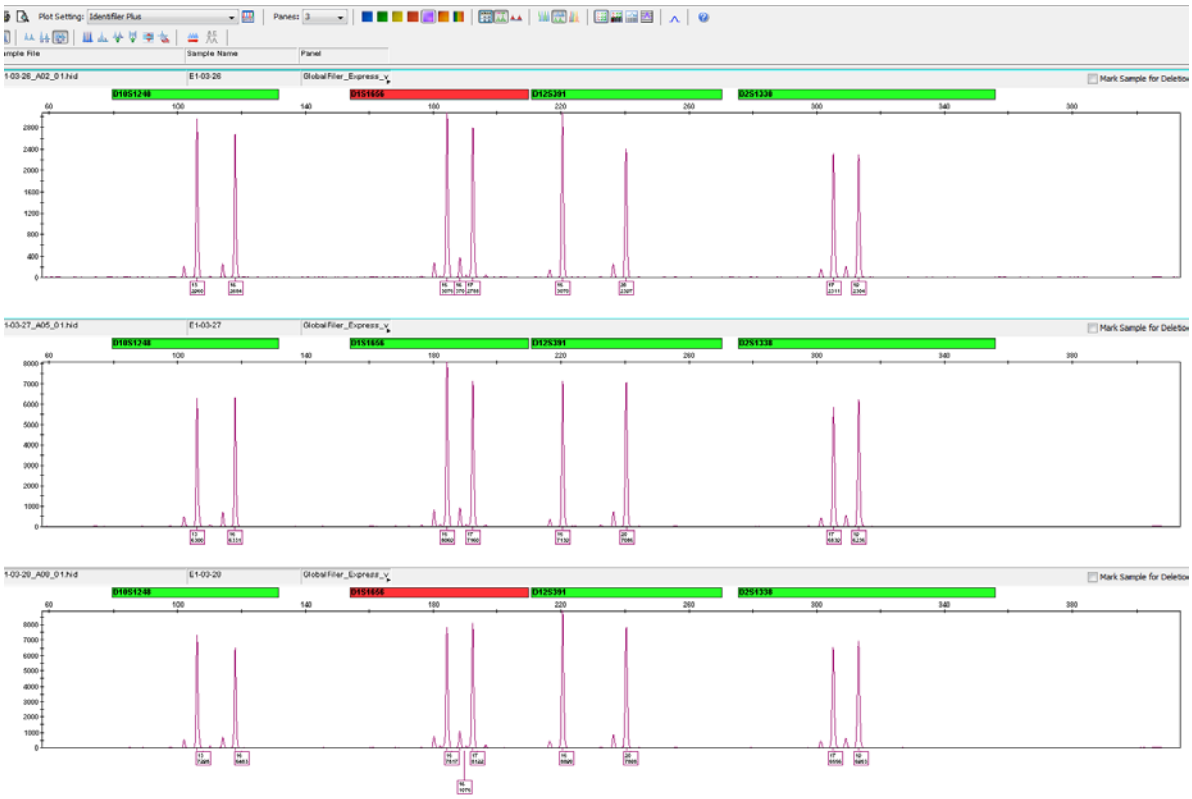

Figure 1: GlobalFiler™ Express Cycle Test for Amplification

For Fusion™ Direct, 27 cycle times was suggested by the manufacturer but in keeping with the cycle study for Express, 26 and 28 cycles were also tested. 28 cycles was discarded before even analyzing the profiles due to the fact that every sample was flagged in the software for overloading of DNA. 26 cycles was determined to be the best time due to the amount of fewer artifacts that were present when compared to 27 cycles. Figure 2 shows an example of a color channel of a profile and why 26 cycles were chosen. 26 cycles are present in the second row, 27 in the first, and 28 in the last. 27 and 28 cycles both had loci flagged red due to pull up, while 26 cycles are all green meaning no artifacts and good peak heights and peak height ratios.

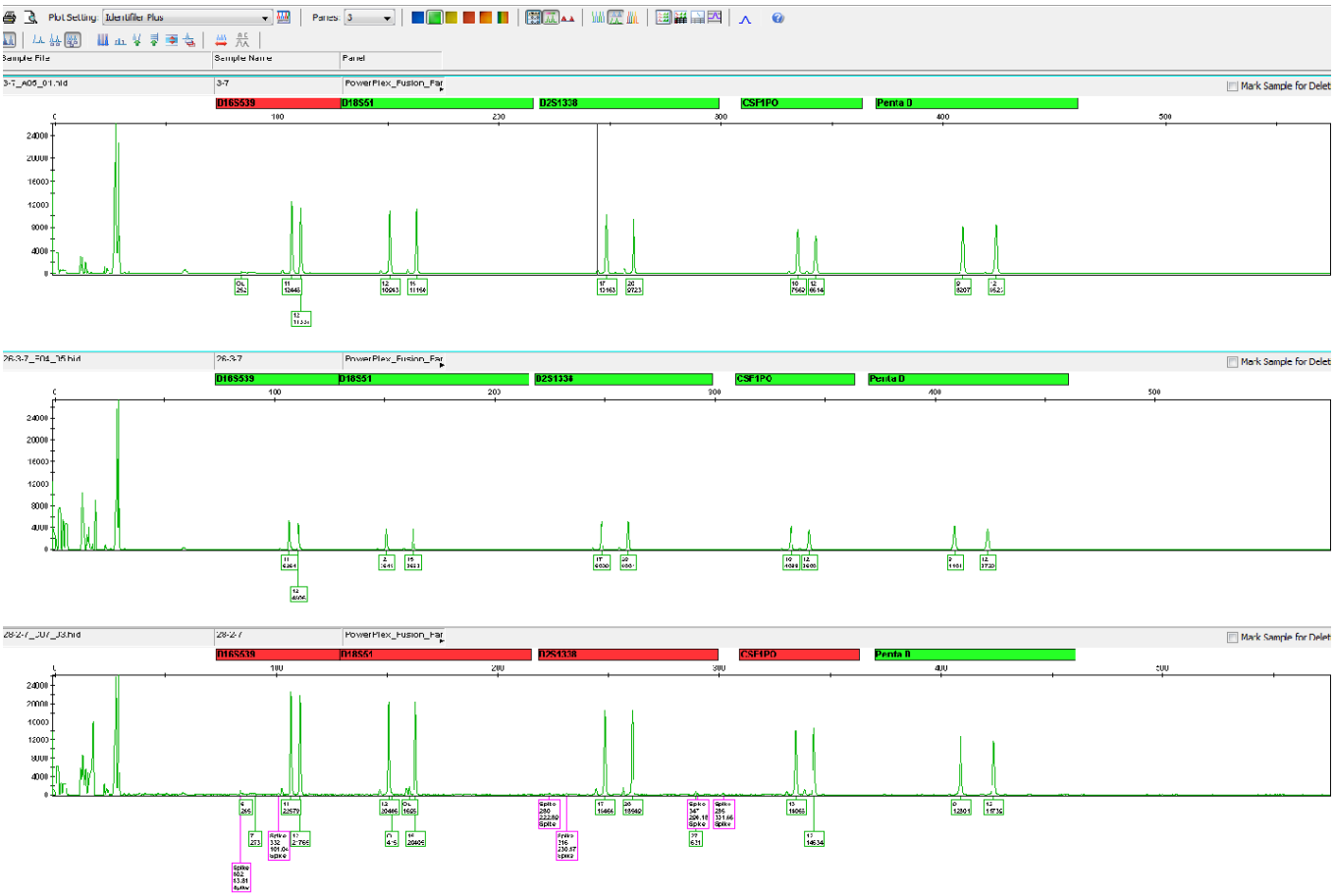

Figure 2: Fusion™ Direct Cycle Test for Amplification

GlobalFiler™ and GlobalFiler™ Express Peak Heights and Peak Height Ratios

The averages of the peak height for homozygotes and the peak height ratio for heterozygotes at the different markers was determined across 6 sets of the 7 samples for GlobalFiler™ and across 9 sets of the 7 samples for GlobalFiler™ Express. These averages are included in Table 1 with GlobalFiler™ being in the top row for each marker and Express being in the bottom row for each marker. Colored spaces indicate female samples where no results could be obtained for that

particular male loci marker. Markers italicized indicate markers present in GlobalFiler™ and not present in Fusion™.

Table 1: GlobalFiler™ and GlobalFiler™ Express Peak Height and Peak Height Ratios Averages

Marker	Sample Number						
	1	2	3	4	5	6	7
D3S1358	0.9085	4406	0.89402	7615.5	0.85693	0.87076	0.8591
	0.775178	2912	0.927544	9844	0.869022	0.849222	0.883556
vWA	5698	0.89595	0.87032	0.881283	0.846217	0.9158	0.91994
	4960	0.863656	0.8917	0.882411	0.860656	0.826511	0.929133
D16S539	0.83364	0.9282	0.85408	4038	0.905183	0.91836	0.88696
	0.76541	0.78262	0.88562	8955.67	0.85366	0.84491	0.86503
CSF1PO	0.84884	0.70386	0.84168	0.89685	0.862667	0.90508	0.88626
	0.79788	0.78441	0.88041	0.90117	0.84533	0.85311	0.86714
TPOX	0.82426	0.79938	0.81152	0.849283	0.934683	4649.2	0.90644
	0.81705	0.78087	0.87402	0.88225	0.81761	4626	0.85832
Yindel	5069.2		5558.2			3012.4	
	5333.44		2274.89			3663.78	
AMEL	0.8201	4601.8	0.90042	10206.83	9514.33	0.9109	14122
	0.90906	3833.33	0.92178	13715.3	8438	0.87276	10658.1
D8S1179	0.87308	2350.6	0.918925	0.878283	0.911283	4262.6	0.87474
	0.87001	1138.889	0.91053	0.91284	0.8804	3351.33	0.91756
D21S11	0.90738	0.86574	7795.6	0.904483	0.896683	0.90774	0.85378
	0.8267	0.89319	9310	0.89324	0.93439	0.88456	0.88965
D18S51	0.81672	0.82276	0.912	0.82325	0.775617	0.92632	0.90158
	0.80859	0.85094	0.90922	0.86038	0.88018	0.86647	0.9333
DYS391	2489.6		3964.4			3295.8	
	2005		3137.67			3794.22	
D2S441	0.86054	0.88614	0.855517	0.888817	0.88575	0.86374	0.938016
	0.88801	0.88482	0.87493	0.91024	0.81652	0.9243	0.90826
D19S433	0.9316	4656.2	9255.167	0.930117	0.903433	0.89182	0.91666
	0.90516	2459.67	3903.78	0.9145	0.8636	0.89859	0.93537
TH01	0.91648	0.84986	0.914	0.847883	0.903233	0.93064	0.86856
	0.8945	0.84311	0.93827	0.92403	0.90424	0.93306	0.93744
FGA	0.86152	0.88014	11309	0.884533	0.884083	0.96364	0.86206

	0.82903	0.86939	4442.33	0.83717	0.87899	0.92453	0.92082
D22S1045	0.93622	0.76474	7031.2	0.91445	7108.833	0.92262	0.91214
	0.7369	0.88014	6703	0.85446	11812.3	0.84966	0.89134
D5S818	0.79008	3800.5	0.73822	6525.833	0.8779833	5872.2	0.91422
	0.89731	3385.67	0.89132	12201.8	0.68168	8856.67	0.93996
D13S317	7602.4	0.7114	0.91452	0.893133	0.90915	0.8731	9455.75
	5994.67	0.87934	0.92753	0.86199	0.85913	0.8604	10336.3
D7S820	7219.6	0.80148	0.91948	0.83745	0.845333	0.88578	10856.2
	5813.78	0.85469	0.95611	0.85418	0.88913	0.83873	10808.3
SE33	0.88764	0.80488	0.88096	0.8642	0.79155	0.83998	11670.2
	0.74454	0.8709	0.81673	0.87048	0.68397	0.77347	13533.2
D10S1248	0.90054	0.82586	0.82062	0.823617	6839.5	0.83636	0.9376
	0.83366	0.87373	0.89499	0.89958	8663.89	0.9006	0.90969
D1S1656	7214.6	0.80742	0.8991	5036.333	0.845217	0.92204	0.89348
	7820.78	0.89546	0.89568	13999.6	0.87647	0.77092	0.89241
D12S391	0.94954	0.75806	0.84188	0.825417	0.846167	0.89134	8036.4
	0.83073	0.8738	0.88536	0.89516	0.89492	0.86619	11062.8
D2S1338	0.77978	0.78784	0.905033	0.8992	0.907217	0.88928	0.87678
	0.78396	0.79962	0.84901	0.85317	0.78983	0.71142	0.84519

The average peak height across all homozygotes for GlobalFiler™ was determined to be 6467.157 with a standard deviation of 2845.86. The average peak height ratio for heterozygotes was determined to be 0.8715 with a standard deviation of 0.04907. For GlobalFiler™ Express, the average peak height for homozygotes was determined to be 7048.39 with a standard deviation of 3722.612. The average peak height ratio for heterozygotes was determined to be 0.8655 with a standard deviation of 0.052139.

The average of the difference between the peak height or peak height ratio between the two methods was also calculated across all samples. Out of the 159 samples calculated for, 123 were heterozygotes with 51.2% of the average peak height ratio being better for GlobalFiler™ at an average of 0.05433 difference in the peak height ratios between the two methods. There was an

0.045981 average difference in the peak hieght ratios of the two methods when GlobalFiler™ Express had a better peak height ratio at a particular loci and sample. For the remaining 36 homozygote samples, GlobalFiler™ had a greater peak height average than GlobalFiler™ Express 55.56% of the time with an average difference between the two methods of 1782.674. While fewer samples had a greater peak height average for Express, the average for the difference was almost double GlobalFiler™ at 2756.924.

Fusion™ and Fusion™ Direct Peak Heights and Peak Height ratios

The same methods of analysis used for comparison of the GlobalFiler™ kits was performed for the Fusion™ kits. The averages of the peak height for homozygotes and the peak height ratio for heterozygotes at the different markers was determined across 6 sets of samples for Fusion™ and across 9 sets of samples for Fusion™ Express. These averages are included in Table 2 with Fusion™ being in the top row for each marker and Direct being in the bottom row for each marker. Colored spaces indicate female samples where no results could be obtained for that particular male loci marker. Markers italicized indicate markers present in Fusion™ and not present in GlobalFiler™.

Table 2: Fusion™ and Fusion™ Direct Peak Height and Peak Height Ratios Averages

Marker	Sample Number						
	1	2	3	4	5	6	7
D3S1358	0.884967	7925.5	0.81154	8323.33	0.90146	0.83475	0.84175
	0.907367	3784.667	0.960656	8013.889	0.915311	0.940167	0.918367
vWA	8914.167	0.7425	0.74272	0.897317	0.88784	0.733733	0.83135
	3520.444	0.941889	0.913111	0.945222	0.949256	0.918211	0.921522
D16S539	0.820167	0.830417	0.88702	14620.17	0.81858	0.872583	0.908017
	0.891778	0.936433	0.91978	12080.78	0.955489	0.944289	0.910978
CSF1PO	0.936067	0.7934	0.83544	0.77385	0.88696	0.776783	0.869317

	0.884911	0.920244	0.919967	0.9294	0.892478	0.8957	0.919133
TPOX	0.72955	0.780633	0.88732	0.827683	0.79394	11929.83	0.74865
	0.873389	0.903011	0.974467	0.902422	0.927956	8919.667	0.955489
Penta D	0.79655	0.82175	0.88012	8241.333	0.67602	10874.67	0.676683
	0.886033	0.921422	0.951322	7632.222	0.949311	9079.556	0.928389
Penta E	0.8457	0.7574	0.83458	0.791483	0.87948	0.858217	0.67085
	0.904656	0.9019	0.940433	0.945711	0.926767	0.912556	0.931578
AMEL	0.785017	13737.5	0.76006	14600.5	11351	0.8296	11937.5
	0.877644	5957.778	0.909167	12100.89	12070.67	0.936144	9228.778
D8S1179	0.8573	23982.17	0.84324	0.7924	0.82232	25225.83	0.895867
	0.933822	10704.67	0.924567	0.962444	0.953744	18780.78	0.919644
D21S11	0.8288	0.8514	8823.8	0.890067	0.86248	0.838417	0.88418
	0.896822	0.941433	17324	0.931011	0.934678	0.933478	0.924533
D18S51	0.820583	0.7889	0.91058	0.82345	0.83274	0.85175	0.830933
	0.893544	0.911778	0.940878	0.895578	0.934867	0.936167	0.964133
DYS391	3246.167		3987.2			5944.5	
	1148.556		8426.556			4086.444	
D2S441	0.75615	0.72585	0.77648	0.7457	0.83316	0.893833	0.869517
	0.8758	0.911356	0.936367	0.938056	0.908133	0.952978	0.9385
D19S433	0.8007	11308	15209.8	0.845317	0.80834	0.84905	0.835733
	0.913667	5847.667	23903	0.941978	0.945611	0.9182	0.959011
TH01	0.841483	0.8222	0.86972	0.864083	0.91654	0.842967	0.863467
	0.947256	0.9548	0.969589	0.955556	0.957	0.949378	0.951422
FGA	0.87945	0.781317	14970	0.88745	0.78462	0.875783	0.812617
	0.869956	0.916822	23375.89	0.921156	0.942822	0.917089	0.921189
D22S1045	0.832783	0.755783	10987.6	0.7728	7755.4	0.768	0.678783
	0.900444	0.915956	15713.22	0.895889	8149.556	0.925656	0.936867
D5S818	0.835033	8144	0.85638	8192	0.79822	10533.33	0.76252
	0.8953	4156.667	0.953144	8697.778	0.937389	8065.62	0.9322
D13S317	4958.167	0.902033	0.88146	0.76865	0.82726	0.823267	5114.667
	2063	0.940378	0.946044	0.920022	0.937567	0.927122	5129.778
D7S820	6774.667	0.810317	0.77032	0.825317	0.82918	0.873667	7238.333
	2928.556	0.896256	0.9666	0.949878	0.952811	0.948278	7678.778
D10S1248	0.7196	0.7756	0.83376	0.80335	5339	0.769317	0.868067
	0.901178	0.917056	0.954689	0.884389	6346.111	0.876122	0.925711
D1S1656	5576.83	0.8853	0.87936	8012.83	0.88868	0.80835	0.83884
	2122.333	0.937422	0.966244	7298.222	0.9262	0.956578	0.897667
D12S391	0.827983	0.822133	0.84072	0.8507	0.81548	0.864717	8761.333
	0.857313	0.919022	0.911522	0.916533	0.931956	0.906422	4975.222

D2S1338	0.765517	0.791117	0.88282	0.797333	0.78474	0.804783	0.800467
	0.900378	0.907256	0.948489	0.9416	0.911922	0.903889	0.931067

The average peak height across all homozygotes for Fusion™ was determined to be 10077.00 with a standard deviation of 5028.165. The average peak height ratio for heterozygotes was determined to be 0.82326 with a standard deviation of 0.05362. For Fusion™ Direct, the average peak height for homozygotes was determined to be 8435.407 with a standard deviation of 5687.885. The average peak height ratio for heterozygotes was determined to be 0.925695 with a standard deviation of 0.023819.

The average of the difference between the peak height or peak height ratio between the two methods was also calculated across all samples. Out of the 164 samples calculated for, 131 were heterozygotes. Only two of those peak height ratios were better balanced for Fusion™ at an average of 0.030325 difference in the ratios between the two kits. Fusion™ Direct was more balanced in the other 129 samples at an average of 0.104852 average difference in the two methods at a particular loci and sample. For the remaining 33 homozygote samples, Fusion™ had a greater peak height average than Fusion™ Direct 63.6% of the time with an average difference between the two kits of 3826.728. For the other 36.4% of the time, Direct was greater at an average of 3213.429.

Comparison Between GlobalFiler™/Express and Fusion™/Direct

After comparison was made between the two methods for each kit, both kits were compared to each other. The averages for peak heights, peak height ratios, and standard deviations were used and placed into Table 3 for comparison.

Table 3: Comparison of Averages Between Kits and Methods

	Peak Height average	Peak Height standard deviation	Peak Height Ratio Average	Peak Height Ratio Standard Deviation
GlobalFiler™	6465.157	2845.86	0.8715	0.04907
GlobalFiler™ Express	7048.39	3722.612	0.8655	0.052139
Fusion™	10077	5028.165	0.82326	0.05362
Fusion™ Direct	8435.407	5687.885	0.925695	0.023819

Next, GlobalFiler™ and Fusion™ were compared in the same fashion as was done previous between the two methods within an individual kit. Numbers were placed into Table 4 with GlobalFiler™ being in the first row for each marker and Fusion™ being in the second. Markers that were only present for an individual kit, such a Yindel, were not used in comparison. Colored spaces indicate female samples where no results could be obtained for that particular male loci marker.

Table 4: GlobalFiler™ and Fusion™ Peak Height and Peak Height Ratios Averages

Marker	Sample Number						
	1	2	3	4	5	6	7
D3S1358	0.9085	4406	0.89402	7615.5	0.85693	0.87076	0.8591
	0.884967	7925.5	0.81154	823.333	0.90146	0.83475	0.84175
vWA	5698	0.89595	0.87032	0.881283	0.846217	0.9158	0.91994
	8914.167	0.7425	0.74272	0.897317	0.88784	0.733733	0.83135
D16S539	0.83364	0.9282	0.85408	4038	0.905183	0.91836	0.88696
	0.820167	0.830471	0.88702	14620.17	0.81858	0.872583	0.908017
CSF1PO	0.84884	0.70386	0.84168	0.89685	0.862667	0.90508	0.88626
	0.936067	0.7934	0.83544	0.77385	0.88696	0.776783	0.869317
TPOX	0.82426	0.79938	0.81152	0.849283	0.934683	4649.2	0.90644

	0.72955	0.780633	0.88732	0.827683	0.79394	11929.83	0.74865
AMEL	0.8201	4601.8	0.90042	10206.83	9514.33	0.9109	14122
	0.785017	13737.5	0.76006	14600.5	11351	0.8296	11937.5
D8S1179	0.87308	2350.6	0.918925	0.878283	0.911283	4262.6	0.87474
	0.8573	23982.17	0.84324	0.7924	0.82232	25225.83	0.895867
D21S11	0.90738	0.86574	7795.6	0.904483	0.896683	0.90774	0.85378
	0.8288	0.8514	8823.8	0.890067	0.86248	0.838417	0.88418
D18S51	0.81672	0.82276	0.912	0.82325	0.775617	0.92632	0.90158
	0.820583	0.7889	0.91058	0.82345	0.83274	0.85175	0.830933
DYS391	2489.6		3964.4			3295.8	
	3246.167		3987.2			5944.5	
D2S441	0.86054	0.88614	0.855517	0.888817	0.88575	0.86374	0.938016
	0.75615	0.72585	0.77648	0.7457	0.83316	0.893833	0.869517
D19S433	0.9316	4656.2	9255.167	0.930117	0.903433	0.89182	0.91666
	0.8007	11308	15209.8	0.845317	0.80834	0.84905	0.835733
TH01	0.91648	0.84986	0.914	0.847883	0.903233	0.93064	0.86856
	0.841783	0.8222	0.86972	0.864083	0.91654	0.842967	0.863467
FGA	0.86152	0.88014	11309	0.884533	0.884083	0.96364	0.86206
	0.87945	0.781317	14970	0.88745	0.78462	0.875783	0.812617
D22S1045	0.93622	0.76474	7031.2	0.91445	7108.833	0.92262	0.91214
	0.832783	0.755783	10987.6	0.7728	7755.4	0.768	0.678783
D5S818	0.79008	3800.5	0.73822	6525.833	0.8779833	5872.2	0.91422
	0.835033	8144	0.85638	8192	0.79822	10533.33	0.76252
D13S317	7602.4	0.7114	0.91452	0.893133	0.90915	0.8731	9455.75
	4958.167	0.902033	0.88146	0.76865	0.82726	0.823267	5114.667
D7S820	7219.6	0.80148	0.91948	0.83745	0.845333	0.88578	10856.2
	6774.667	0.810317	0.77032	0.825317	0.82918	0.873667	7238.333
D10S1248	0.90054	0.82586	0.82062	0.823617	6839.5	0.83636	0.9376
	0.7196	0.7756	0.83376	0.80335	5339	0.769317	0.868067
D1S1656	7214.6	0.80742	0.8991	5036.333	0.845217	0.92204	0.89348
	5576.833	0.8853	0.87936	8012.833	0.88868	0.80835	0.83884
D12S391	0.94954	0.75806	0.84188	0.825417	0.846167	0.89134	8036.4
	0.827983	0.822133	0.84072	0.8507	0.81548	0.864717	8761.333
D2S1338	0.77978	0.78784	0.905033	0.8992	0.907217	0.88928	0.87678
	0.765517	0.791117	0.88282	0.797333	0.78474	0.804783	0.800467

148 samples were calculated for the GlobalFiler™ and Fusion™ kits with 117 being heterozygotes and the remaining 31 being homozygotes. GlobalFiler™ had a better peak height ratio for 90 of the 117 heterozygote samples with an average difference of 0.074718. Fusion™ showed a better peak height ratio for 27 out of the 117 heterozygote samples with an average of 0.042894.

For the 31 homozygotes, Fusion™ had a higher peak height at 24 of those with an average of 5123.586 between the two kits. For the remaining 7 homozygotes where GlobalFiler™ was better, there was an average difference of 2338.698.

GlobalFiler™ Express and Fusion™ Direct were compared as well. Numbers were placed into Table 5 with GlobalFiler™ Express being in the first row for each marker and Direct in the second. As with GlobalFiler™ and Fusion™ comparison, markers not present in both kits were not compared. Colored spaces indicate female samples where no results could be obtained for that particular male loci marker

Table 5: GlobalFiler™ Express and Fusion™ Direct Peak Height and Peak Height Ratios Averages

Marker	Sample Number						
	1	2	3	4	5	6	7
D3S1358	0.775178	2912	0.927544	9844	0.869022	0.849222	0.883556
	0.907367	3784.667	0.960656	8013.889	0.915311	0.940167	0.918367
vWA	4960	0.863656	0.8917	0.882411	0.860656	0.826511	0.929133
	3520.444	0.941889	0.913111	0.945222	0.949256	0.918211	0.921522
D16S539	0.765411	0.782622	0.885622	8955.667	0.853656	0.844911	0.865033
	0.891778	0.936433	0.91978	12080.78	0.955489	0.944289	0.910978
CSF1PO	0.797878	0.784411	0.880411	0.901167	0.845333	0.853111	0.867144
	0.884911	0.920244	0.919967	0.9294	0.892478	0.8957	0.919133
TPOX	0.81705	0.780867	0.874022	0.88225	0.817611	4626	0.858322
	0.873389	0.903011	0.974467	0.902422	0.927956	8919.667	0.955489

AMEL	0.909056	3833.333	0.921778	13715.33	8438	0.872756	10658.11
	0.877644	5957.778	0.909167	12100.89	12070.67	0.936144	9228.778
D8S1179	0.870011	1138.889	0.910533	0.912844	0.8804	3351.333	0.917556
	0.933822	10704.67	0.924567	0.962444	0.953744	18780.78	0.919644
D21S11	0.8267	0.893189	9310	0.893244	0.934389	0.884556	0.889556
	0.896822	0.941433	17324	0.931011	0.934678	0.933478	0.924533
D18S51	0.808589	0.850944	0.909222	0.860378	0.880178	0.866467	0.9333
	0.893544	0.911778	0.940878	0.895578	0.934867	0.936167	0.964133
DYS391	2005		3137.667			3794.222	
	1148.556		8426.556			4086.444	
D2S441	0.888011	0.884822	0.874933	0.910244	0.816522	0.9243	0.908256
	0.8758	0.911356	0.936367	0.938056	0.908133	0.952978	0.9385
D19S433	0.905156	5459.667	3903.778	0.9145	0.8636	0.898589	0.935367
	0.913667	5847.667	23903	0.941978	0.945611	0.9182	0.959011
TH01	0.8945	0.843111	0.938267	0.924025	0.904244	0.933063	0.937444
	0.947256	0.9548	0.969589	0.955556	0.957	0.949378	0.951422
FGA	0.829033	0.869389	4442.333	0.837167	0.878989	0.924533	0.920822
	0.869956	0.916822	23375.89	0.921156	0.942822	0.917089	0.921189
D22S1045	0.7369	0.880144	6703	0.854456	11812.33	0.849656	0.891344
	0.900444	0.915956	15713.22	0.895889	8149.556	0.925656	0.936867
D5S818	0.897311	3385.667	0.891322	12001.78	0.681678	8856.667	0.939956
	0.8953	4156.667	0.953144	8697.778	0.937389	8065.62	0.9322
D13S317	5994.667	0.879344	0.927533	0.861989	0.859133	0.8604	10336.33
	2063	0.940378	0.946044	0.920022	0.937567	0.927122	5129.778
D7S820	5813.778	0.854689	0.956111	0.854178	0.889133	0.838733	10808.33
	2928.556	0.896256	0.9666	0.949878	0.952811	0.948278	7678.778
D10S1248	0.833656	0.873733	0.894989	0.899578	8663.889	0.9006	0.909689
	0.901178	0.917056	0.954689	0.884389	6346.111	0.876122	0.925711
D1S1656	7820.778	0.895456	0.895678	13999.56	0.876467	0.770922	0.892411
	2122.333	0.937422	0.966244	7298.222	0.9262	0.956578	0.897667
D12S391	0.830733	0.8738	0.885356	0.895156	0.894922	0.866189	11062.78
	0.857313	0.919022	0.911522	0.916533	0.931956	0.906422	4975.222
D2S1338	0.783956	0.799622	0.849011	0.853167	0.789833	0.711422	0.845189
	0.900378	0.907256	0.948489	0.9416	0.911922	0.903889	0.931067

149 samples were calculated for GlobalFiler™ Express and Fusion™ Direct with 118 being heterozygotes and the remaining 31 being homozygotes. For the heterozygotes, Fusion™ Direct

had a more balanced peak height ratio at 109 of the 118 heterozygote samples with an average difference of 0.062838. For the remaining 9 samples where GlobalFiler™ Express produced a better peak height ratio, the average difference was 0.013414. For the homozygotes, there was more of a balance between the two kits and their peak heights. GlobalFiler™ Express exhibited higher peak heights at 16 of the 31 with an average of 2992.86. Fusion™ Direct exhibited a much higher difference of 6782.725 for the remaining 15 homozygote samples.

Conclusions

The two questions that were needing to be answered during this comparison study was first, can direct amplification produce the same level of results as the traditional method of extraction, quantitation, and amplification and second, which kit produces superior results as compared to the other kit. With GlobalFiler™, almost equal results were obtained for it and GlobalFiler™ Express. With PowerPlex® Fusion™ and Fusion™ Direct, Direct was shown to have preferred results over the traditional method using extraction. Comparing the two kits of the direct amplification method, PowerPlex® Fusion™ Direct was shown to be a choice kit in terms of allele calling when compared to GlobalFiler™ Express due to the peak height ratios and peak heights obtained when using the Fusion™ Direct kit. Fewer artifacts were also shown to be present with the Fusion™ Direct kit.

With all of this information and data compiled and with questions answered, it was recommended to the CODIS unit of TBI to continue future studies and validation with the PowerPlex® Fusion™ kit, and in particular, the Fusion™ Direct kit. One of the positives in going with the Direct kit is the fact that all the other components of the kit are the same for the Fusion™ kit except the Direct kit uses a swab solution. This would be helpful if an analyst was

to come across a tricky sample in the future that would require extraction. It was also suggested to perform a future study seeing if comparable peak heights and reproducibility could be provided across all analysts in the unit.

One other study that was not able to be performed in the time allotted was a volume study. It was recommended to perform that particular study at a later date using half volume, and even quarter volume, reactions and compare those results to full volume reactions. This study had also been done previously at Marshall University (14). If same results were able to be obtained, this would save the TBI money in the long run with being able to perform more reactions with a single kit.

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