Internal Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit and Comparison to Identifiler® for the Boston Police Department Crime Laboratory

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ABSTRACT

Amplification is an important step in forensic DNA analysis, and the amplification kit used can affect the quantity and quality of results obtained. An internal validation of the AmpFℓSTR® Identifiler® Plus PCR amplification kit was performed at the Boston Police Department Crime Laboratory. An optimal protocol for DNA target input, cycle number, and injection time was established. This protocol produced reliable, reproducible, and robust results through numerous validation studies including precision, reproducibility, and concordance studies. The kit demonstrated improved performance over the Identifiler® amplification kit on degraded and mixture samples, as well as greater sensitivity. As a result of the validation studies, Identifiler® Plus will be implemented. Continuing studies should be performed to ensure the validated protocol is working well.

INTRODUCTION

The amplification step in forensic DNA analysis allows quantities as low as a few cells to generate millions to billions of copies so that the sample DNA can be detected. Because quantity and quality of results obtained from a sample can be affected by the amplification kit used, the amplification kit best-suited for a laboratory should be evaluated. The Boston Police Department Crime Laboratory currently has only one amplification kit in use for casework, the AmpFℓSTR® Identifiler® PCR Amplification Kit (Life Technologies, Foster City, CA). The majority of cases processed for DNA at the Boston Police Department Crime Laboratory are breaking and entering (property crimes) and sexual assault cases, so a more sensitive kit would be beneficial for processing their casework because of the low amount of DNA recovered from some of their evidence items. Therefore, they wanted to begin using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies, Foster City, CA) in casework as well. The Crime

Laboratory was interested in Identifiler® Plus due to improved interlocus and intralocus balance, faster amplification time, and increased sensitivity in comparison to Identifiler® (Applied Biosystems 2010). Therefore, an internal validation of the amplification kit was performed according to Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines (SWGDAM 2004).

Life Technologies released the Identifiler® Plus PCR amplification kit as an improvement over the Identifiler® amplification chemistry. Identifiler® and Identifiler® Plus amplify the same loci with the same primer sequences in the same concentrations, although the Identifiler® Plus amplification kit has demonstrated improved performance on severely inhibited samples, greater sensitivity, improved performance on mixture samples, a cleaner baseline, and a reduced number of artifacts due to an improved primer manufacturing process (Applied Biosystems 2010, Wang and others 2012). Identifiler® Plus' improvement in typing inhibited samples is due to an enhanced buffer formulation containing a carrier protein that helps provide better tolerance to PCR inhibition. Identifiler® Plus PCR cycling protocols are also optimized for increased sensitivity and higher efficiency and quality of the PCR reaction. The final extension step is decreased from 60 minutes in the Identifiler® protocol down to 10 minutes in the Identifiler® Plus protocol. In addition, Identifiler® Plus combines the annealing and extension steps, decreasing the ramp time needed to get from one temperature to another. Taken together, these improvements provide a thermal cycling protocol that is one hour faster with the Identifiler® Plus kit. The Identifiler® Plus User's Guide also allows for an increased sensitivity option at a higher cycle number (Applied Biosystems 2012). Previous studies have shown that when tested on aged bone samples, the higher cycle number used with the Identifiler® Plus kit (29 cycles) showed improved results compared to the Identifiler® amplification kit at normal cycle number

(28 cycles) and sometimes even the increased cycle number of 34 cycles with Identifiler® (Romanini and others 2011). This data supports Identifiler® Plus' increased success in amplification of degraded samples as well as its ability to overcome PCR inhibition.

A developmental validation according to FBI/National Standards and Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines was performed on the Identifiler® Plus amplification kit (Wang and others 2012). The authors demonstrated the enhanced sensitivity and performance of Identifiler® Plus for human identity and parentage testing. Their validation also showed increased peak heights of 1.7-fold when using Identifiler® Plus over Identifiler®, as well as a vast improvement in inhibitor tolerance. Intralocus peak height ratios and intracolor peak height balances also were improved compared to Identifiler® results.

An internal validation performed at the Boston Police Department Crime Laboratory was necessary to implement the use of the Identifiler® Plus amplification kit to obtain improved results in less time. The lab-specific target, PCR cycle number, and injection time were chosen to generate the best DNA profiles on their instrumentation. Various mixture, precision, reproducibility, reinjection, degraded samples, touch DNA samples, and contamination studies were performed to test the performance and limitations of the kit. Previous studies documented that higher injection settings on the genetic analyzer such as increased injection time and/or injection voltage had led to higher sensitivity when generating a DNA profile (Westen and others 2009). Therefore, it was important to test to determine whether an increased injection time would be beneficial for normal target and/or low level samples. It was also determined whether denaturing and snap cooling prior to running samples on the 3130*xl* was necessary for correct typing. Once the analytical and stochastic thresholds for analysis were determined and the

optimized protocol established, the subsequent validation studies demonstrated that the Identifiler® Plus amplification kit was reliable, reproducible, and robust.

MATERIALS AND METHODS

DNA Samples

Known control blood samples previously extracted and quantitated were provided by the Boston Police Department Crime Laboratory. Control DNA 9947A was included in the Identifiler® Plus amplification kit. Touch DNA samples were collected by DNA analysts from various items found within the laboratory and within their homes. Samples were extracted using the EZ1 DNA Investigator Kit on the EZ1 Advanced (Qiagen, Hilden, Germany). The samples were then quantitated using Quantifiler® Duo (Applied Biosystems, Foster City, CA).

PCR Amplification

The protocols in the Identifiler® Plus Kit User's Guide were followed unless otherwise stated (Applied Biosystems 2012). Samples were amplified with 10 µL of Master Mix and 5 µL Primer Set from the Identifiler® Plus amplification kit, with up to 10 µL of DNA added to meet the target DNA input. If less than 10 µL of DNA was needed to achieve the target load, TE buffer was added to give a 10 µL total volume added. For amplification negatives, 10 µL of TE buffer was used. For a positive control, the 9947A provided with the Identifiler® Plus kit was amplified. The 9947A input study ultimately determined that the addition of 5 µL of 9947A was optimal and that amount was amplified for all subsequent studies. The samples were amplified on the GeneAmp® PCR system 9700 in the 9600 emulation mode with a high temperature hold at 95 °C for 11 minutes, denaturation at 94 °C for 20 seconds, and a combined anneal/extend step at 59 °C for 3 minutes. The denature/anneal/extend 2-step process was repeated for 28 or 29

cycles. This cycling was followed by a final extension period at 60 °C for 10 minutes and then a final hold at 4 °C.

Capillary Electrophoresis and Data Analysis

Amplified products were separated and detected using an Applied Biosystems 3130*xl* Genetic Analyzer. 1 µL of amplified product or allelic ladder was added to 9 µL of formamide/size standard mix (8.7 µL of deionized Hi-Di™ formamide with 0.3 µL GeneScan™ 500 LIZ® size standard; Applied Biosystems). Samples were denatured at 95°C for 3 minutes and snap cooled on ice for 3 minutes prior to electrophoresis. Samples were injected at 3kV for 10 seconds and electrophoresed at a run temperature of 60 °C in Performance Optimized Polymer-4 (POP-4™ polymer; Applied Biosystems) for 1500 seconds at 15kV. Data was analyzed using GeneMapper® *ID* v3.2.1 (Applied Biosystems) with an analytical/calling threshold of 50 RFU for all dyes unless otherwise stated.

DNA Target Input/Cycle Number Study

Three control blood samples were chosen due to the large number of heterozygous loci that would be helpful in peak height ratio calculations. Targets for the study were 2.0 ng, 1.0 ng, 0.75 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.0625 ng, and 0.03125 ng, amplified at both 28 and 29 cycles. Peak height averages and peak height ratios were analyzed to determine the optimal target and cycle number combination.

9947A Input Study

The Boston Police Department Crime Laboratory currently uses 5 µL (approximately 0.5 ng) of 9947A with the Identifiler® amplification kit. The Identifiler® Plus User's Guide

recommends adding 10 µL (1.0 ng) of 9947A for an Identifiler® Plus amplification (Applied Biosystems 2012). Both 1.0 ng and 0.5 ng 9947A targets were amplified at 28 and 29 cycles as part of the target/cycle number study. Peak height averages and peak height ratios were analyzed to determine which 9947A target is better for the cycle number chosen.

Injection Time Study

Once a cycle number and target DNA load was chosen, an injection time study was performed. One sample previously amplified for 28 cycles at a target of 0.75 ng was injected at 10 seconds, 15 seconds, and 20 seconds at a 3kV injection voltage. Samples previously amplified targeting 0.125 ng and 0.0625 ng were also injected at 10 seconds, 15 seconds, and 20 seconds. Peak height averages and peak height ratios were calculated to determine the optimal injection time that produced the best data.

Denature/Snap Cooling Study

Samples previously amplified for 28 cycles at a target of 0.75 ng were set up in triplicate for the 3130*xl* run using three total injections. This setup was duplicated on a separate plate. One plate was subjected to the recommended 3 minutes of denaturation at 95 °C followed by 3 minutes of snap cooling on an ice block before being run on the 3130*xl*. The other plate was run without denaturing or snap cooling the samples. Both setups contained formamide to denature the samples. Samples that were denatured and snap cooled prior to the run were compared to samples that were not by looking at allele calls, peak heights, and general peak morphology. Peak morphology was analyzed by estimating the peak to be a rectangle. The "width" of the peak was calculated by dividing the peak area by the peak height, with the sharper, smaller width peaks to be more optimal. Peak height and morphology data determined if the denaturing and

snap cooling protocol was necessary and if it was even beneficial or unfavorable for the DNA typing.

Analytical Threshold Study

Twenty amplification negatives were analyzed at an analytical threshold of 1 RFU for the blue, green, yellow, and red dyes. Peaks that were within 2 basepairs of the size standard were deleted out. All remaining peaks were separated by color, and the average, standard deviation, minimum peak height, and maximum peak height of the noise was calculated per color. The analytical threshold was calculated using three different methods. Method 1 followed SWGDAM guidelines and calculated analytical threshold as twice the range of the noise (Butler 2010). Method 2 was recommended by John Butler, looking at limit of detection (LOD) and limit of quantitation (LOQ) (Terrill and Butler 2006). LOD was calculated by taking the average and adding 3 times the standard deviation. LOQ was determined by taking the average and adding 10 times the standard deviation. The third method was suggested in a presentation by NIST, taking three times the maximum peak height of the noise (Coble 2011). The different analytical thresholds calculated were compared per method and an overall analytical threshold was chosen for all dye colors.

Stochastic Threshold Study

Data from samples amplified and run in studies previously described were used for this study, as well as 5 additional control blood samples amplified at targets of 0.25 ng, 0.125 ng, and 0.0625 ng. Resulting data was analyzed using three methods to determine the stochastic threshold. The first method, suggested by the Massachusetts State Police Crime Laboratory, used three times the analytical threshold (Sgueglia 2011). The second method, suggested by

SWGDAM, determined the stochastic threshold by taking the highest false homozygote peak height and adding 1 RFU (Butler 2010). The final method, again suggested by the Massachusetts State Police Crime Laboratory, determined the stochastic threshold by looking at the average peak height of a target input amount where stochastic effects begin to occur, which was a target of 0.125 ng in this study (Sgueglia 2011).

Sensitivity Study

In addition to analyzing control blood samples at all target inputs from studies previously described, three additional control blood samples were amplified at targets of 0.0625 ng, 0.125 ng, 0.25 ng, and 0.50 ng. The results were analyzed to determine at what target dropout occurred and at which target reliable and reproducible results were routinely obtained.

Mixture Study

Mixtures were made with a total target of 0.75 ng with the minor component comprising 5%, 10%, 25%, and 50% of the mixture, including male/female and male/male mixtures. The mixtures were analyzed to determine if all allele calls were present for the major and minor components of the mixture. The minimum percentage of minor component to produce a full minor component profile was determined, as well as the minimum percentage of minor component needed to detect the sample as a mixture.

Precision Study

Twenty-four allelic ladder samples were run in three different injections. The first ladder per injection was labeled as an allelic ladder in the GeneMapper® software with the remaining ladders treated as samples. Sizing of each allele of the 'sample' ladders was examined, and the

standard deviation for sizing of each allele calculated to determine the precision of sizing in samples run in the same injection, as well as between samples run in different injections.

Concordance Study

Four control blood extracts previously amplified with the Identifiler® amplification kit according to the validated Boston Police Department protocol were amplified with the Identifiler® Plus amplification kit, typed, and compared to determine if the kits generated the same allele calls. In addition, profiles of the samples from the degraded and touch DNA studies that were amplified with both kits were compared to determine if the same allele calls were made between the Identifiler® and Identifiler® Plus amplified samples.

Reproducibility Study

Five control blood samples were amplified at a total target of 0.75 ng for 28 cycles and were run on the 3130*xl* on day 0, day 1, day 3, day 7, and day 14 using a new plate dilution every time. Day 0 represented the same day the samples were amplified. Allele calls and peak heights were examined over time to determine if trends could be seen as the amplified samples aged.

Reinjection Study

Five control blood samples were amplified at a total target of 0.75 ng for 28 cycles and ran on the 3130*xl* on day 0. Reinjections were run on day 0, day 1, day 2, and day 3. Allele calls and peak heights were evaluated for each reinjection to determine if trends could be seen with each new reinjection.

Touch DNA Study

The performance of Identifiler® Plus on difficult, very low level samples was explored using mock casework samples. A variety of surfaces which had the potential for touch DNA were swabbed, extracted, quantitated, and amplified with both Identifiler® and Identifiler® Plus to make a comparison of results between the two amplification kits. Extracts were concentrated down to 10 µL when a sample's concentration was otherwise unable to reach the DNA input target, and 5 µL of the extract was amplified with Identifiler® while the other 5 µL was amplified with Identifiler® Plus. The allele calls that were made were compared to determine the differences in sensitivity between the two amplification kits.

Degraded Sample Study

Extracts from known reference samples that previously displayed a high quantitation value but degraded profile quality with Identifiler® at a target of 1.5 ng were amplified with Identifiler® Plus kit at a target of 0.75 ng. Allele calls were compared between the results with the two kits to determine which amplification kit performed better on degraded samples.

Contamination Study

Allelic ladders were set up in a checkerboard pattern with amplification negatives. The positions of the ladders and amplification negatives changed with each injection so that an individual capillary alternated between ladder and amplification negative with each injection. Clean amplification negatives would demonstrate that no contamination was introduced through the pipettes, the kit, the capillaries, or the setup protocol, and that no bleed through occurred in the capillaries between samples. In addition, all negative controls used in the Identifiler® Plus

validation were examined to see if any peaks consistent with a DNA profile were present, suggesting contamination had occurred.

RESULTS

DNA Target Input/Cycle Number Study

Full profiles above the analytical threshold were generated at a target of 0.125 ng and higher when amplified for 28 cycles. However, a target of 0.125 ng did not have all peaks above the stochastic threshold. Peaks due to pull up were present when targeting 1.0 ng and 2.0 ng. When amplified for 29 cycles, full profiles were also obtained at a target 0.125 ng and higher above the validated thresholds for Identifiler® (100 RFU in blue, green, and yellow and 75 RFU in red). This data was not reanalyzed with the new Identifiler® Plus thresholds. Extra peaks due to pull up were present beginning at a target of 0.75 ng and the number of pull up peaks increased with increased target. A target of 1.0 ng at 28 cycles produced the highest peak height ratios (Table 1). However, since a target of 1.0 ng at 28 cycles produced some pull up peaks and the maximum peak height was over 7000 RFU, which was well above the desired maximum peak height (Table 2), the next highest peak height ratios were considered to determine the optimal target input. A target of 0.75 ng for 28 cycles and 0.5 ng for 29 cycles had the next highest peak height ratios. Amplification at 29 cycles generally had more artifacts and peak height ratios were not as high as 28-cycle amplifications. The 0.75 ng target amplified at 28 cycles had an optimal peak height range of 706-3673 RFU in all samples tested.

9947A Input Study

Full profiles were obtained at 28 cycles with a target of both 1.0 ng and 0.5 ng. However, the 1.0 ng target caused significant pull up. The target of 0.5 ng produced a better profile with

fewer artifacts than the recommended 1.0 ng. At 29 cycles, the 1.0 ng input caused excessive pull up and the 0.5 ng target generated a cleaner profile with less pull up peaks and other artifacts.

Injection Time Study

An increase in injection time resulted in an increase in peak heights for the normal 0.75 ng target (Table 3). This raised peak height averages up to 2845 RFU at an injection time of 20 seconds, well above desired peak height averages for a profile. Peak height ratios did not change for the 0.75 ng target at an increased injection time. For the low level DNA samples targeting 0.125 ng and 0.0625 ng, the peak height ratio averages decreased with an increased injection time (Table 4 and 5). The minimum peak height ratios reached as low as 33.3%, so stochastic effects need to be heavily considered at an increased injection time. The increased injection time did increase peak heights over the analytical and stochastic thresholds in some cases. The analytical and stochastic thresholds used, however, had not been reevaluated with data at increased injection times.

Denature/Snap Cooling Study

All samples typed correctly regardless of whether the samples were denatured and snap cooled or not prior to run on the 3130*xl*. Normalized peak height averages (homozygote peak heights divided by 2) were slightly higher with denaturing and snap cooling, but no striking difference was seen (Table 6). Peak height ratio averages were virtually identical between the two conditions (Table 7). A smaller width (signifying a sharper peak) was observed in the samples that were not denatured and snap cooled, though the rectangle approximation may not have been very accurate.

Analytical Threshold Study

All negative controls used in the study were clean. The method suggested by NIST, where the analytical threshold is determined by 3 times the maximum height of the noise, gave the highest RFU per color, with blue at 18 RFU, green at 51 RFU, yellow at 36 RFU, and red at 33 RFU. Limits of quantitation ranged from 9.4 RFU in blue to 18.7 RFU in red. SWGDAM's method gave a maximum analytical threshold at 32 RFU in green.

Stochastic Threshold Study

Three times the analytical threshold suggested a stochastic threshold of 150 RFU. 28 false homozygotes were observed at a target of 0.0625 ng or less, with the maximum false homozygote at 153 RFU. This suggested a stochastic threshold of 154 RFU. The normalized peak height average (homozygote peak heights divided by 2) for samples targeting 0.125ng was 210 RFU.

Sensitivity Study

Full, reliable profiles were generated from a DNA input of 0.125 ng up to 1.0 ng when amplified for 28 cycles. However, at a target of 0.125 ng, not all peaks were above the stochastic threshold of 155 RFU. All peaks were above the stochastic threshold at a target of 0.25 ng and above.

Mixture Study

In a two person mixture targeting 0.75 ng, a mixture was able to be detected when minor component allele peaks crossed the analytical threshold at all ratios tested. This occurred as low as when the minor component comprised of only 5% of the total DNA present in the mixture.

Full profiles from the minor component were generated 10 out of the 12 times tested in mixtures where the minor component made up 25% of the mixture. A full profile of the minor component was rarely generated when the minor component only made up 10% of the mixture (3 out of 12 times tested). A 50/50 mixture always generated a profile with all peaks crossing the stochastic threshold, and all major and minor component peaks crossed the stochastic threshold when the minor component made up 25% of the mixture in 4 of the 12 samples tested.

Precision Study

All ladders contained all of the alleles that should have been present. However, in ladder dilutions that were called samples in the software, extra calls were made due to stutter peaks and other artifacts. The standard deviation was calculated for every allele in the allelic ladder for the 24 ladders run as part of the precision study. The average standard deviation was 0.061 bp. The minimum standard deviation was 0.028 bp for the 12.2 allele at D19 and the maximum standard deviation was 0.149 bp for the 45.2 allele at FGA.

Concordance Study

The Identifiler® and Identifiler® Plus amplification kits achieved full concordance in profiles of the four control blood samples. Concordance was also found between the two kits in the eight known degraded samples and three touch DNA samples.

Reproducibility Study

Allele calls were consistent between the initial injection and the new sample preparations on days 1, 3, 7, and 14. One of the samples exhibited unexpectedly low peak heights from the initial injection, suggesting an error in the concentration of the sample used by way of

quantitation or dilution. Overall, there was no trend in peak heights over time, with three samples having increased peak heights over time up to a 9.8% increase per new setup and two samples having decreased peak heights over time up to an 8.6% decrease per new setup (Figure 1).

Reinjection Study

Allele calls were consistent between the initial injection and reinjections on days 0, 1, 2, and 3. Over time, peak heights decreased with each reinjection, with a decrease of 3-12% in peak height average per reinjection (Figure 2). The sharpest decrease in peak heights occurred between the Day 2 and Day 3 reinjections. Peak heights of the samples set up on Day 3 were compared to the peak heights of the samples reinjected on Day 3. Peak height averages were higher for the samples that were newly set up than the samples that were reinjected (Figure 3).

Touch DNA Study

To compare the Identifiler® Plus and Identifiler® kits initially, touch DNA samples amplified with both kits were analyzed using the Identifiler® thresholds of 75 RFU in red and 100 RFU for blue, green, and yellow dyes. When analyzed at the same thresholds, more alleles were called when amplified with Identifiler® Plus than with Identifiler®, demonstrating Identifiler® Plus to be the more sensitive kit. The Identifiler® Plus data was then reevaluated at its lower analytical threshold of 50 RFU. The combination of the more sensitive kit plus the lower threshold of the Identifiler® Plus protocol led to many more alleles that were called in the sample amplified with Identifiler® Plus versus Identifiler®, especially in low level samples. However, many of the additional alleles called fell below the stochastic threshold of 155 RFU.

Degraded Sample Study

None of the degraded samples generated a full profile at a target of 0.75 ng with Identifiler® Plus, but at least one new allele was called per sample compared to the amplification with Identifiler® three years prior. However, some Identifiler® samples did have additional alleles called that were not present in the Identifiler® Plus samples. In addition, many alleles that had been called with Identifiler® now fell below the stochastic threshold with the Identifiler® Plus amplification.

Contamination Study

All negative controls throughout the validation studies were clean. One negative control had apparent amplified DNA present in the injection, but when that amplicon was rediluted and reinjected, no peaks were visualized or detected. Therefore, the amplified DNA present was attributed to well-specific contamination.

DISCUSSION AND CONCLUSION

The Identifiler® Plus amplification chemistry was successfully validated for use in the Boston Police Department Crime Laboratory. The kit produced reliable, reproducible, and robust results demonstrated by the various validation studies. Optimal conditions for the analytical procedure included amplifying the DNA extracts for 28 cycles with a DNA target input of 0.75 ng. It was determined that 5 µL of 9947A would be added to each amplification set as a positive control. Based on the desired peak height averages for the normal target, 10 seconds would be the injection time for the normal Identifiler® Plus protocol, as recommended by the manufacturer. An increase in injection time could be beneficial in generating a more complete DNA profile for low level DNA samples, but a separate protocol would need to be

validated prior to implementation to examine stochastic effects and determine appropriate thresholds. The denaturation and snap cooling study showed that formamide is sufficient to denature the samples so that the DNA is single-stranded when run on the genetic analyzer. However, since the Identifiler® Plus User's Manual recommended denaturing and snap cooling the samples before the run, this procedure would be performed on samples amplified with Identifiler® Plus. Based on the analytical threshold study results, an analytical threshold of 50 RFU was chosen for use at the Boston Police Department Crime Laboratory with Identifiler® Plus. This analytical threshold would be used for all dye colors. Examination of the stochastic threshold study data using the average peak heights at a target where stochastic effects occur method suggested a threshold of 210 RFU when using a target of 0.125 ng. However, a target of 0.125 ng always produced full profiles above the analytical threshold and peak height ratios averaged 81.1%. Therefore, this stochastic threshold of 210 RFU was considered to be too high since stochastic effects were not too widely seen for samples at 0.125 ng. Therefore, a stochastic threshold of 155 RFU was chosen based on the highest false homozygote method.

Once the optimal protocol for the kit was established, the performance and limitations of the Identifiler® Plus amplification kit were evaluated. The sensitivity study demonstrated that although the normal target was 0.75ng, the range of target DNA producing full profiles was 0.125 ng to 1.0 ng. Mixture data showed that minor components in mixtures, especially in low levels, may be used for exclusions since the allele peaks cross the analytical threshold. However, when minor component allele peaks fail to cross the stochastic threshold, which often occurs when present in low percentages, they cannot be used in statistics and for inclusions. Mixtures were able to be detected when only 5% of the minor component was present, demonstrating the sensitivity of the Identifiler® Plus kit. The precision study showed that allelic ladders should be

designated as allelic ladders in GMID software when run to avoid extra allele calls due to stutter or other artifacts. A standard deviation calculated to be 0.15 bp or less demonstrated the precision in sizing necessary so that alleles in samples being compared to the allelic ladder would be binned correctly and not be designated as off-ladder. All alleles in the ladder met this condition. Therefore, the precision study demonstrated that precise typing could be performed. The concordance study showed that the results of the amplification were accurate. The Identifiler® Plus amplification kit typed samples in concordance with the Identifiler® amplification kit. The reproducibility study was able to show the reproducibility of the Identifiler® Plus amplification kit for both allele calling and peak heights over a period of 2 weeks. This supported the Boston Police Department Crime Laboratory protocol that amplified samples can be run on the 3130*xl* up to two weeks after they were originally amplified. The sharp drop seen in peak heights between day 2 and day 3 of reinjections supported the Boston Police Department Crime Lab protocol that samples should be freshly diluted on day 3 instead of being reinjected if samples needed further testing. Peak heights were also higher on day 3 when a new set up was used versus a reinjection. The results of the contamination study showed that no contamination was present in the pipettes, the capillaries, the Identifiler® Plus amplification kit, or the setup protocol.

Comparisons between the Identifiler® and Identifiler® Plus amplification kits were also explored. Touch DNA samples demonstrated the higher sensitivity of the Identifiler® Plus amplification kit with more allele calls being made than with the Identifiler® amplification kit when analyzed at the same thresholds. Combining the higher sensitivity of the kit with the lower threshold for calling led to a large increase in alleles called with Identifiler® Plus. However, most of these alleles were below the stochastic threshold of 155 RFU, so these alleles would only

be able to be used for exclusions. One drawback seen to running the samples with Identifiler® Plus was that many alleles that were called in Identifiler® and able to be used in statistics now fell below the stochastic threshold in the Identifiler® Plus samples and would not be used in statistics and would only be able to be used for exclusions. The degraded samples also had many alleles that were called using Identifiler® now falling below stochastic threshold with the Identifiler® Plus amplification, so these alleles could now only be used in exclusions rather than inclusions and statistics. However, taking into account that the Identifiler® Plus amplification used half the target of the Identifiler® amplification and that the samples may have continued to degrade throughout the last 3 years, Identifiler® Plus appeared to be better at amplifying degraded samples than Identifiler®, since the two kits generated similar results. Using both kits at the same target input, one would predict Identifiler® Plus to have many more new alleles present compared to the Identifiler® kit. Overall, the Identifiler® Plus amplification kit provided more information and should be considered for use over the Identifiler® amplification kit. The Identifiler® Plus kit also demonstrated that a lower target input could be used with the same or better sensitivity compared to the Identifiler® kit. All of these improvements combined with a faster amplification time makes Identifiler® Plus a more desirable kit than Identifiler® for forensic DNA laboratories.

Ongoing studies should be performed to ensure that the validated protocol is working well for the needs of the Boston Police Department Crime Laboratory. Stutter artifacts were often called as part of the profiles instead of being filtered out by the stutter filter. Therefore, a stutter study should be done to see if any of the stutter filters should be raised based on their instrumentation. Future studies should also focus on whether an internal validation of the

Identifiler® Plus protocol for an increased cycle number to 29 cycles would be beneficial for low copy DNA samples often seen in casework.

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Table 1. DNA Target Input/Cycle Number Study: Peak Height Ratios from Item 5 using Identifiler® Plus

	28 cycles, 1.0 ng	28 cycles, 0.75 ng	28 cycles, 0.5 ng	28 cycles, 0.25 ng	29 cycles, 0.5 ng	29 cycles, 0.25 ng	29 cycles, 0.125 ng
Average Peak Height Ratio	91.7%	86.5%	85.1%	81.8%	87.3%	79.9%	66.6%
Minimum Peak Height Ratio	75.6%	72.6%	64.7%	57.7%	74.5%	55.1%	19.6%
Maximum Peak Height Ratio	98.9%	98.3%	96.7%	99.5%	98.9%	96.5%	96.6%

Table 2. DNA Target Input/Cycle Number Study: Peak Heights from Item 4 using Identifiler® Plus

	28 cycles, 1.0 ng	28 cycles, 0.75 ng	28 cycles, 0.5 ng	29 cycles, 0.5 ng
Average Peak Height	2790 RFU	1658 RFU	915 RFU	2266 RFU
Minimum Peak Height	1840 RFU	1099 RFU	472 RFU	1331 RFU
Maximum Peak Height	7017 RFU	3673 RFU	2151 RFU	4981 RFU

Table 3. Injection Time Study: 0.75 ng Target Peak Height Ratios and Peak Heights at 10, 15, and 20 Second Injection Times

0.75 ng	10 seconds	15 seconds	20 seconds
Average Peak Height Ratio	89.38%	89.68%	89.64%
Minimum Peak Height Ratio	80.78%	80.75%	80.88%
Maximum Peak Height Ratio	98.69%	98.73%	99.32%
Normalized Average Peak Height (RFU)	1436.0	1843.1	2845.4
Minimum Peak Height (RFU)	929.0	1173.0	1833.0
Maximum Peak Height (RFU)	2185.0	2837.0	4394.0
Average Peak Height (RFU)	1482.3	1902.5	2937.2

Table 4. Injection Time Study: 0.125 ng Target Peak Height Ratios and Peak Heights at 10, 15, and 20 Second Injection Times

0.125 ng	10 seconds	15 seconds	20 seconds
Average Peak Height Ratio	80.26%	77.71%	77.93%
Minimum Peak Height Ratio	38.91%	34.68%	33.33%
Maximum Peak Height Ratio	99.12%	98.32%	99.71%
Normalized Average Peak Height (RFU)	199.4	335.5	536.7
Minimum Peak Height (RFU)	114.0	103.0	160.0
Maximum Peak Height (RFU)	390.0	638.0	1004.0
Average Peak Height (RFU)	212.7	346.3	554.0

Table 5. Injection Time Study: 0.0625 ng Target Peak Height Ratios and Peak Heights at 10, 15, and 20 Second Injection Times

0.0625 ng	10 seconds	15 seconds	20 seconds
Average Peak Height Ratio	N/A	69.91%	67.85%
Minimum Peak Height Ratio	N/A	41.70%	42.27%
Maximum Peak Height Ratio	N/A	97.08%	94.92%
Normalized Average Peak Height (RFU)	58.5	94.7	122.3
Minimum Peak Height (RFU)	100.0	89.0	113.0
Maximum Peak Height (RFU)	134.0	259.0	317.0
Average Peak Height (RFU)	117.0	139.5	171.2

Table 6. Denature/Snap Cooling Study: Peak Heights With and Without Heating and Snap Cooling Prior to 3130*xl* Run

	No Heating/Snap Cooling	With Heating/Snap Cooling
Normalized Peak Height Average (RFU)	1454	1491
Minimum Peak Height (RFU)	458	536
Maximum Peak Height (RFU)	5296	5123
Average Peak Height (RFU)	1590	1631

Table 7. Denature/Snap Cooling Study: Peak Height Ratios With and Without Heating and Snap Cooling Prior to 3130*xl* Run

Blood Samples	No Heating/Snap Cooling	With Heating/Snap Cooling
Peak Height Ratio Average	87.44%	87.42%
Peak Height Ratio Minimum	70.83%	70.55%
Peak Height Ratio Maximum	99.89%	99.89%

Table 8. Denature/Snap Cooling Study: Peak Morphologies With and Without Heating and Snap Cooling Prior to 3130*xl* Run

	No Heating/Snap Cooling	With Heating/Snap Cooling
Average Peak "Width"	9.69	9.76
Minimum Peak "Width"	8.23	8.31
Maximum Peak "Width"	11.82	11.80

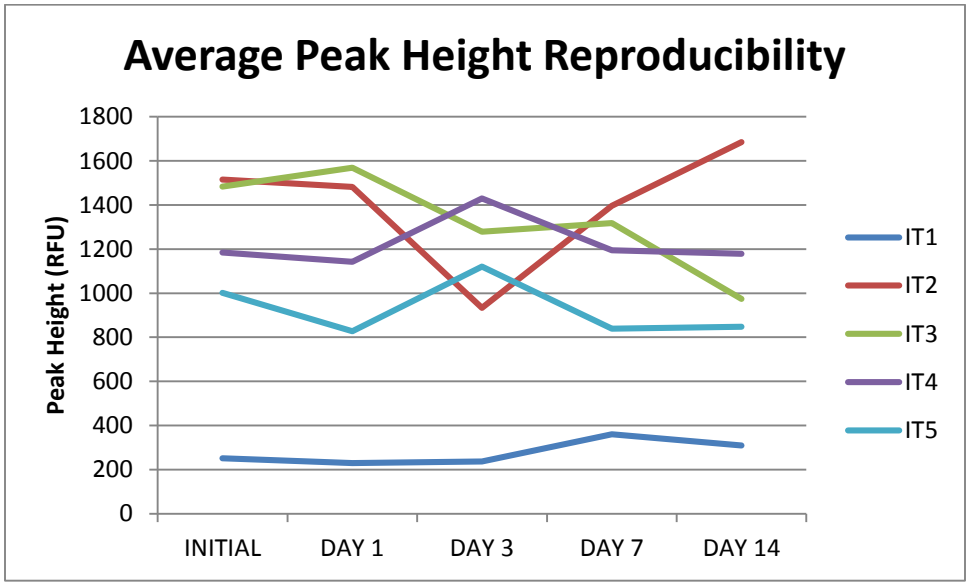

Figure 1. Reproducibility Study: No Trend in Peak Height Averages per Sample per New Setup

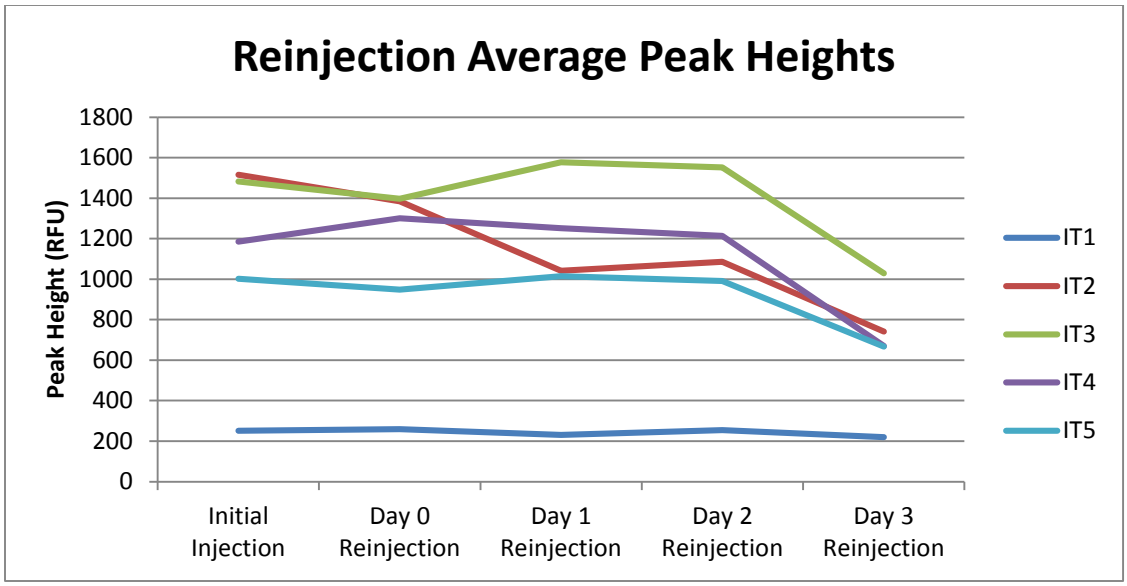

Figure 2. Reinjection Study: Peak Height Averages Decrease per Sample per Reinjection

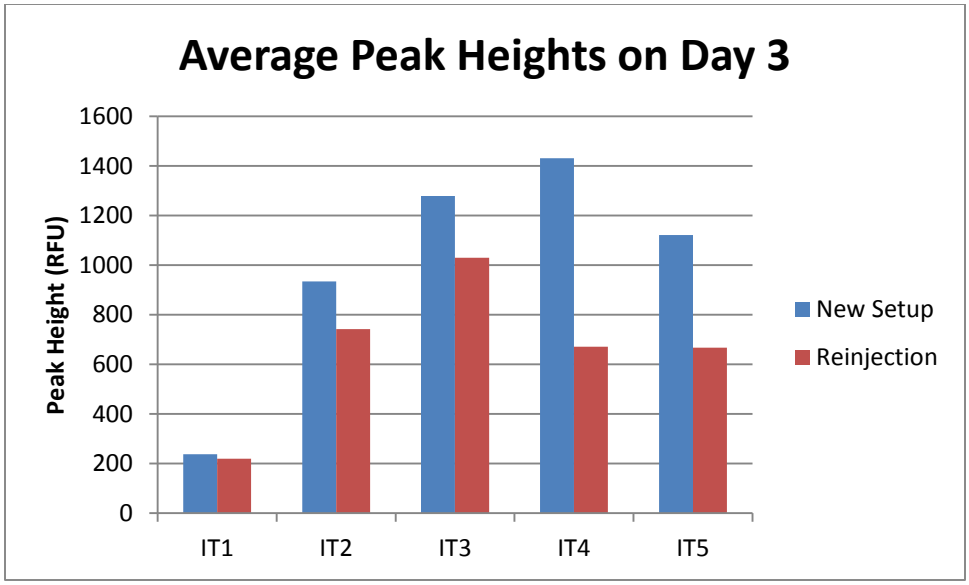

Figure 3. Reinjection Study: Average Peak Heights Are Higher on Day 3 Using a New Setup versus a Reinjection