Development of a Direct Amplification Method for Exemplar and Pseudo-exemplar Samples Using Identifiler® Plus

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ABSTRACT

Crime laboratories are evaluating methods to help reduce DNA backlogs of biological evidence. One way of doing this is by directly amplifying reference samples. Reference samples are of higher quality and produce predictable results, so the typical DNA analysis workflow can be modified to save time in sample processing.

Pilot studies comparing the AmpFlSTR® Identifiler® Direct PCR Amplification Kit and AmpFlSTR® Identifiler® Plus PCR Amplification Kit were conducted to determine which amplification system best fits the needs of the New York City Office of Chief Medical Examiner (NYC OCME). The goal of these experiments was to establish a standard protocol that will account for variability of DNA amounts among reference samples.

Identifiler® Direct is a PCR amplification kit optimized for amplifying blood samples spotted onto FTA® cards that have not been extracted or quantified. Use of non-FTA® collection methods, according to Applied Biosystems (ABI), requires pretreatment with Prep-n-Go Buffer™. To potentially bypass this step, various sampling techniques and thermal cycling parameters were tested. Initial results showed that partial profiles were obtained for buccal samples and non-FTA® blood cards. Due to the additional time required to process samples using the Prep-and-Go Buffer™, other less costly amplification kits were considered.

Another method of direct PCR amplification uses Identifiler® Plus, which is optimized to overcome inhibition. The chemistry of the kit enables unpurified extracts to be directly

amplified. Buccal swabs and blood spotted onto Whatman® non-FTA® paper were incubated in 0.2% Tween® 20, 0.1 mg/mL Proteinase K and 2.4% Trehalose in TE^{-4} for 30 minutes at 56°C followed by 5 minutes at 99°C. An aliquot of neat extract was directly amplified using a half reaction of Identifiler® Plus. Experiments with various cutting sizes, extraction volumes, aliquots for amplification, and thermal cycling parameters were conducted using samples containing a wide range of DNA. First success rates ranged from 70-80% for exemplar samples. After re-injections and dilutions, 100% profiles could be reported and no re-amplifications were necessary.

Methods were also optimized for pseudo-exemplar samples such as bottles, cans, cups, straws, cigarette butts and chewing gum. One uniform protocol that encompassed all of these samples types was developed with the exception of different sized cuttings for each substrate. Preliminary results showed that there was a 78% first pass rate with over 98% of all samples yielding full profiles after re-injections and dilutions.

In brief, implementing a short extraction step followed by direct amplification with Identifiler® Plus proved to be a cost effective method to profile true and pseudo exemplar samples in a single day or less. For the overwhelming majority of samples, full profiles were generated from standard parameters. For a select number of samples, an additional injection on the 3130*xl* Genetic Analyzer with more or less sensitive parameters was required. In both cases, the initial STR results could be evaluated to accurately predict the additional step(s) needed to preserve both time and cost. Future studies will help to validate this method so that it can be implemented at the NYC OCME.

1. INTRODUCTION

Crime laboratories throughout the U.S. suffer from backlogs in DNA analysis. Increasing amounts of biological evidence submitted to labs require more time and cost for laboratories to analyze evidence. Methods are currently being developed to help reduce the time and cost required to analyze a DNA sample. Direct amplification of reference samples is one way to do this. Typical DNA analysis consists of extraction, purification, quantitation, amplification, separation and data analysis. Direct amplification eliminates the purification and quantitation steps, reducing the processing time significantly. Reference samples are generally of higher quality and give predictable results. Therefore, deviations from the typical DNA analysis workflow can be implemented to shorten processing times.

Two methods of direct amplification were tested to develop a method that best fits the needs of the New York City Office of Chief Medical Examiner (NYC OCME). The first method used Identifiler® Direct, a direct amplification chemistry that amplifies the 13 core CODIS loci, 2 additional pentanucleotide loci (D2S1338 and D19S433) and the sex-determining Amelogenin.[1] This kit has been optimized for buccal and blood samples applied to FTA® pretreated cards. Punches of the FTA® cards are directly added to amplification reactions without extraction. Applied Biosystems offers a Prep-n-Go™ Buffer that can be used in place of FTA® treated paper. Non-FTA® treated substrates (swab, etc.) can be incubated in the Prep-n-Go™ Buffer and the extract can be amplified. Incubation time for a swab is 20 minutes at room temperature.[2] Previously published research has tested Identifiler® Direct for non-FTA® substrates without use of Prep-n-Go™ Buffer, resulting in reportable profiles.[3] Identifiler® Direct was tested with substrates used by the NYC OCME (buccal swabs and blood spotted onto Whatman® non-FTA® treated cards). No Prep-n-Go™ Buffer was added to determine if this additional cost could be avoided.

The second direct amplification method involved Identifiler® Plus, which tests the same 16 loci as Identifiler® Direct. Identifiler® Plus has been optimized to overcome inhibition, which allows for samples that contain inhibitors, such as heme, to be directly amplified without need of purification.[4] Buccal and blood reference samples were extracted with an in-house buffer and directly amplified with Identifiler® Plus. The in-house buffer was modified from a pervious study testing direct amplification for sperm cells.[5] In addition, pseudo-exemplar samples were tested. Pseudo-exemplar samples are not professionally collected, but are observed to be handled by a particular individual, typically consisting of saliva samples. Pseudo-exemplar samples tested included bottles, cans, cups, straws, chewing gum and cigarette butts.

The amount of DNA obtained in a reference sample will vary from person to person. Without quantifying the DNA, it is impossible to know how much DNA is being added to a PCR reaction. By testing the two direct amplification methods, a direct amplification method using Identifiler® Plus was developed for exemplar and pseudo-exemplar reference samples that can withstand varying amounts of DNA found in various reference samples.. When the method was used, a DNA sample could be completely analyzed within one day. This means that when evidence comes into a laboratory, it can be extracted, amplified, separated and analyzed, resulting in a final profile by the end of the day. Implementing this method for reference samples can greatly reduce the time and cost of processing such samples.

2. MATERIALS AND METHODS

2.1 Sample collection

Buccal swabs were collected from individuals (2 swabs per collection) and dried in a hood 3h-overnight. They were stored at 4°C until extracted.

Whole blood was provided from autopsies conducted at the NYC OCME and prepared on Whatman® collection cards that were non-FTA® treated. Cards were prepared by spotting 25µL whole blood in the middle of each circle and drying the cards overnight in a hood. These cards were stored at 4°C until extracted.

Pseudo-exemplar samples consisted of swabs from bottles, cans, cups, and straws. Swabs were moistened with distilled water and wiped along the openings and rims of the substrates. 1 swab was collected for each submission. In addition, chewing gum and cigarette butts were also collected after use. Samples were submitted after use by volunteers and all samples collected were stored at 4°C until extracted.

2.2. Initial Studies Testing Identifiler® Direct and Identifiler® Plus Direction Amplification Methods

For Identifiler® Direct studies, full amplification reactions were used (12.5µL reaction mix + 12.5µL primers) with manufacturer recommended thermal cycling parameters. This consisted of a 95°C initial hold for 11 min, followed by 27 or 28 cycles of a denature step for 20 sec at 94°C, an anneal step for 2 min at 59°C, and an extension step for 1 min at 72°C. This was succeeded by a final elongation at 60°C for 25 min and the samples were held at 4°C after amplification.

Identifiler® Plus studies used half reactions (5.0µL reaction mix + 2.5µL primers). Thermal cycling parameters consisted of a 95°C initial hold for 11min, followed by 28 or 29 cycles of a denature step for 20 sec at 94°C, an anneal step for 3 min at 59°C, and an additional extension step for 30 sec at 72°C. This was followed by a final elongation at 60°C for 60 min and the samples were held at 4°C after amplification.[6]

All samples were amplified on the GeneAmp® PCR system 9700 thermal cycler and CE was performed using the Applied Biosystems 3130*xl* Genetic Analyzer. Initial injection conditions were 1kV for 22 sec. If drop out occurred in a sample, it was re-injected at 5kV for 20 sec to increase peak heights. Data was analyzed with Genemapper® ID software v3.2.1. Since initial studies tested single source reference samples, a 20% filter was applied with a 75RFU threshold.

During data analysis, a profile was considered reportable if all alleles were called and true allele peaks were easily distinguished from artifact peaks (pull-up, stutter, etc.). This meant that no further lab work was necessary for the sample. If artifact peaks were tall enough to interfere with distinguishing true allele peaks, the sample was not reportable and required dilution before being re-run. When testing various variables, success rates were evaluated based on how many reportable profiles were obtained under initial injection conditions, how many had to be re-injected due to drop out and how many had to be diluted because of artifact peaks. It was decided that re-injections should be avoided because even if re-injected, all alleles in a profile may still not be called. If this occurs, the sample would require re-amplification. It is easier and takes less time to dilute a sample where all alleles have already been called.

2.2.1 Identifiler® Direct

Buccal Swabs

Buccal swabs were obtained from 2 individuals and samples were extracted in duplicate (4 samples total). For initial amplifications, 1/3 of a swab was cut, placed in Phosphate-buffered saline (PBS) or irradiated water and incubated at room temperature for 1 hour. The sample was then vortexted for 2 minutes and centrifuged at 1000rpm for 3 minutes to pellet the swab. 2µL of the extract was added to a PCR reaction and amplified for 27 cycles.

Further analyses consisted of using buccal swabs from 5 individuals, extracted in duplicate. 1/3 of a swab was cut and incubated in 100µL PBS. The same procedure was followed as previously described with the exception that the sample was amplified for 28 cycles.

Blood

Whole blood was collected from 5 individuals and extracted in duplicate. ¼ of a 6mm punch was directly added to an amplification reaction and amplified for 27 cycles.

2.2.2 Identifiler® Plus

Samples were extracted using an in-house extraction buffer consisting of 0.2% Tween® 20, 0.1mg/mL Proteinase K and 2.4% Threhalose in TE^{-4} buffer. Cuttings were incubated in extraction buffer for 30 min while shaking at 56°C. The samples were then transferred to a heat block at 99°C for 5 min to inactivate the Proteinase K. Once extracted, an aliquot of neat extract was added directly to a PCR half reaction. Various cutting sizes and PCR input volumes were tested to determine the best combination for each substrate.

Buccal Swabs

Buccal swabs from 5 individuals were used for initial studies and extracted in duplicate. Cuttings were incubated in 200µL extraction buffer. Various cutting sizes, PCR input volumes and amplification cycles were tested:

- 1/3 swab; 5µL; 29 cycles
- 1/3 swab; 2µL, 1µL and 5µL (of a 1/10 dilution); 28 cycles
- 1/6 swab; 3µL, 2µL and 1µL; 29 cycles

Blood

First, the appropriate cutting size to extraction buffer volume proportion was determined. This was performed to minimize inhibitory effects from heme contained in blood samples. One

blood card was used so that variation in DNA concentration between individuals would not affect the results. 200µL extraction buffer volume was used with a 1 punch (6mm), 0.5 punch, 0.25 punch, 2mm x 2mm cutting and 1mm x 1mm cutting. 2µL of extract was added to a PCR reaction and amplified for 29 cycles.

It was established that the 1mm x 1mm cutting was the only one to yield full profiles. The next step was to test various extraction buffer volumes and PCR input volumes. Blood cards from 5 individuals were tested in duplicate with the following combinations of extraction buffer volumes and PCR input volumes:

- 200µL; 5µL
- 200µL; 3µL
- 200µL; 2µL
- 300µL; 2µL

<u>Pseudo-exemplar Samples</u>

Bottles, Cans, Cups and Straws

For each substrate (bottles, cans, cups, and straws) 5 individuals provided samples after use. The 5 individuals varied, depending on the substrate. Swabs were cut by placing a razor blade on the bottom of the swab on one side and sliding the razor blade across the top to the bottom of the other side, cutting the swab in half. Tweezers were used to peel off the outer layer of half the swab, leaving most of the lightly packed inner swab on the stick. Each half was separately extracted to make duplicates.

The outer layer of the swab was incubated in 100µL extraction buffer. PCR input volumes of 3µL, 2µL and 1µL were tested.

Chewing Gum

Chewing gum was collected from 5 individuals and stored at room temperature overnight to dry. Once dry, the middle of the gum was cut into thin pieces using a razor blade. The pieces were placed into a 1.5mL tube and pushed down until the pieces filled the tube to the 0.1mL mark. 100µL extract buffer was then added and the gum was extracted. 2µL and 1µL PCR input volumes were tested.

It was determined that storing the gum at room temperature presented problems when extracting the gum. New chewing gum samples were obtained from 5 more individuals, but stored at -20°C overnight. The same extraction procedure was used. 3µL, 2µL and 1µL PCR input volumes were tested.

Cigarette Butts

3 individuals provided 3 cigarette butts each for testing. 3mm x 3mm cuttings of the outer paper (making sure not to include the filter) were incubated in 100µL extraction buffer. PCR input volumes of 3µL and 2µL were tested.

2.3 Validation Studies with Identifiler Plus

PCR cycling parameters for validation studies followed manufacturer recommended settings with the exception that the final extension was extended to 60 min instead of 10 min. Initial CE injection conditions were 1kV for 22 sec. If drop out occurred, a sample was re-injected at 5kV for 25 sec. The same conditions used for analyzing samples for initial studies were also used for analyzing validation samples.

15 Samples Study

15 individuals provided buccal swabs to test that the method could be applied to varying concentrations of DNA from a significant number of buccal swabs. Samples were extracted in

duplicate. 1/6 swab was cut and incubated in 200µL extraction buffer. 2µL extract was added to a PCR reaction and amplified for 29 cycles.

10 Blood Cards Study

Blood cards prepared from blood from 10 individuals were tested to show that the method developed for blood cards could yield reportable profiles when applied to a greater number of samples. 10 samples were extracted in duplicate. 2mm x 2mm cuttings were incubated in 800µL extraction buffer. 5µL extract was amplified for 29 cycles.

Reproducibility Study

A reproducibility study was performed to demonstrate that the direct amplification method could result in consistent profiles analyzed over separate days. The reproducibility study consisted of two parts. For the first part, 5 individuals provided buccal swabs on three different days. The second part involved taking cuttings from the same swab on three different days. All samples were extracted in duplicate. 1/6 swab was cut and incubated in 200µL extraction buffer. 2µL extract was amplified for 29 cycles. Profiles were compared over the three days to show that consistent profiles were obtained. The second part of the study could not be performed due to time limitations.

Concordance Study

A concordance study was conducted to show that consistent profiles could be obtained from the same amplified samples on different instruments. Three 3130*xl*s of varying sensitivity were used: Galileo (very sensitive), Esther (moderately sensitive) and Rudy (least sensitive). Varying the sensitivities of the instruments also showed how the method's success rate would be affected by the sensitivity of the Genetic Analyzer used. Buccal swabs from 5 individuals were extracted in duplicate. 1/6 swab was cut and incubated in 200µL extraction buffer. 2µL extract

was added to a PCR reaction and amplified for 29 cycles. All samples were run on Galileo, Esther and Rudy and profiles were compared to ensure that consistent profiles were obtained.

In addition, a concordance study was performed to test the method between different platforms: the 3130*xl* and 3500*xl* Genetic Analyzers. The *3500xl* has a greater range of sensitivity that it can handle. Peaks that are oversaturated on the 3130*xl* around 8000-9000 RFUs are well distinguished on the 3500*xl*, which can handle saturation up to 60,000 RFUs. 3500*xl* data was analyzed using Applied Biosystems GeneMapper® *ID-X* software v1.2. A 75 RFU threshold was used with a 20% filter. The same 10 amplified samples that were used for the previous concordance study were run on the 3500*xl*. Profiles were compared to those obtained on the 3130*xl*s and success rates and the number of edits required were evaluated.

3. RESULTS

3.1 Identifiler® Direct

Buccals

The first samples were incubated in 200µL PBS or H_2O and amplified for 27 cycles. From the first injection at 1kV for 22 sec, only a couple of alleles were called for three of the samples, and none were called for one of the samples. Upon re-injection at 5kV for 20 sec, the number of called alleles greatly increased, however no samples yielded a full profile. There was drop out in every sample. It was determined that there was so apparent difference between using PBS or H_2O. Therefore, PBS was used for later analyses.

To increase the concentration of DNA in each sample, 100µL PBS was used to incubate the sample in. In addition, samples were amplified for 28 cycles to increase the amount of amplified product obtained. This theoretically quadrupled the amount of DNA obtained for run on the CE. Out of 10 samples run at 1kV for 22 sec, 4 yielded full profiles and 6 had partial

profiles. These profiles were significantly more complete, however due to the inability to gain full profiles for most of the samples under initial injection conditions further testing of Identifiler® Direct for buccal swabs was not continued.

Blood

For 10 samples run at 1kV for 22 sec, 4 profiles gave full profiles. However there were split peaks at most loci and approximately 50% of the time, the OL allele peak was taller than the true allele peak. 4 profiles had some alleles called, also with split peaks. 2 profiles had no alleles called. Due to the abundance of split peaks and lack of full profiles, testing of Identifiler® Direct for blood cards ceased. It was hypothesizes that the heme in from the punch may have affected the efficiency of the polymerase since the heme was not diluted before adding to the PCR reaction.

3.2 Identifiler® Plus

Buccals

The first samples tested for buccal swabs tested started with 1/3 swab cutting and a 5µL PCR input volume that was amplified for 29 cycles. Samples were overblown and there was an abundance of artifact peaks. Some samples were so oversaturated that additional peaks were pulled up into the internal size standard and no sizing could be assigned. Two changes were tested to decrease the concentration of DNA being injected. First, decreasing the cycle number for amplification was tested. Second, a smaller cutting size with the same 29 cycles amplification was tested. Results were compared to determine which approach worked best.

To start off, manufacturer recommended amplification cycle number of 28 cycles was used. 1/3 swab was extracted and amplified for 28 cycles with various PCR input volumes. 2µL

and 1µL PCR input volumes resulted in the most profiles that were reportable after the first run (Table 1), however 2uL did not require any re-injections.

The NYC OCME has validated Identifiler® Plus for 29 cycles, so the next step was to modify the method for 29 cycles. This was done by reducing the cutting size in half, taking a 1/6 of a swab instead of 1/3. Results are found in Table 1. 3µL PCR input volume resulted in profiles that were overblown and were difficult to distinguish true allele peaks from artifact peaks (pull-up, split peak, etc.). 1µL resulted in the most profiles that were reportable, 70%, however a re-injection was required. 2µL resulted in 60% reportable profiles and no re-injections were required.

Since the NYC OCME uses 29 cycles for Identifiler® Plus, it was concluded that using 1/6 of swab with 29 cycles would serve as the best method. Since 2µL PCR input volume gave the best results, a final method was developed for buccal swabs:

- 1/6 of swab cutting
- Incubate in 200µL extraction buffer
- 2µL PCR input volume
- Amplify for 29 cycles

<u>Blood</u>

1mm x 1mm cutting was determined to give the best results (Figure 1). It was the only cutting size where all alleles were called. It was hypothesized that a 1mm x 1mm cutting diluted the concentration of heme so that the reaction was not significantly inhibited. A trend to note is that as cutting size increased, the percentage of alleles decreases to a certain point. However once you pass that point, the percentage increases, though never to yield a full profile (Figure 1). It is unknown why having a greater cutting size would yield better results because the amount of

heme would be greater. Possibilities might include that there is a greater amount of DNA as well or that something occurs during the centrifugation of the sample before aliquoting for amplification. There was a larger amount of "junk" found at the bottom of the tube when the punch size increased. Despite not knowing the reason for this trend, it did not affect results because none of these cutting sizes yielded full profiles.

Testing several combinations of extraction buffer volumes and PCR input volumes with 1mm x 1mm cuttings resulted in 200µL/5µL and 300µL/2µL having the most reportable profiles (Table 2). 200µL extraction buffer with 5uL PCR input had an 80% success rate with no re-injections needed. 300µL extraction buffer with 2µL PCR input had a 60% success rate and 20% of samples required re-injection. It was suggested by analysts at the NYC OCME that 1mm x 1mm cuttings would be hard to manage due to their small size. Therefore, a bigger cutting size of 2mm x 2mm incubated in 800µL extraction buffer was considered to give the same ratio. The final procedure developed for blood cards was:

- 2mm x 2mm cutting
- Incubate in 800µL extraction buffer
- 5µL PCR input volume
- Amplify for 29 cycles

Pseudo-exemplar Samples

Buccal and blood samples are expected to yield a large amount of DNA since they are professionally collected. For pseudo-exemplar samples, DNA concentrations will vary depending on how easily a person sheds cells and how much they used a particular item, such as a cup. The more contact they have with the item, the more DNA they will leave on it. It is expected that pseudo-exemplar samples will have a significantly lower amount of DNA so

sampling methods were modified. By choosing to sample only the outer part of the swab, collection is focusing on taking the part of the swab that is highly concentrated with DNA. Avoiding including the less dense fluff part of the swab results in a smaller cutting that can incubated in a smaller extraction buffer volume. This helped to concentrate the DNA.

Bottles

1µL and 2µL did not have 100% success rates even after re-injection (Table 3). Even though 2µL gave the best first pass rate of 90%, the re-injected sample still contained drop out. This means that the sample would have to be re-amplified. 3µL had a slightly lower first pass rate of 80%, however after re-injection all full profiles could be obtained. No re-amplifications were necessary. Two re-injections were required for 3µL because for one of the samples one allele dropped out at a heterozygous locus that was previously called when amplified with 2µL and 1µL. It would be expected if the sample had all alleles called for 1µL and 2µL, that it would be the same for 3µL. An explanation could be that there was a primer binding site mutation.

Cans

1µL had a 60% first pass rate and an overall 80% pass rate when samples containing drop out were re-injected (Table 4). 2µL had the highest first pass rate of 90% and required one re-injection. After re-injection all profiles were obtained without need for amplification. 3µL had a first pass rate of 80% and required two dilutions due to overblown peaks. If dilutions were performed, a 100% final pass rate would be expected without any re-amplifications. Due to 2µL requiring a re-injection and the 3µL needing none, it was decided that 3µL would be the best option for cans.

Cups

1µL had a 70% first pass rate with 30% re-injections required (Table 5). After re-injections, only 90% of the profiles were obtained. 2µL had the highest first pass rate of 90% and required 1 dilution. 3µL had a 70% first pass rate with three dilutions needed. If dilutions were performed, it would be expected that 100% profiles could be obtained for both 2µL and 3µL without any re-amplifications. Since 2µL had the highest first pass rate, it gave the best results.

Straws

The first amplifications performed on straws showed contamination in the negative control. When re-run, the contamination still appeared. The extracts and extraction negative were re-amplified and no contamination was observed. The contamination must have occurred in the amplification tube of the original amplification, however the original extracts and extraction negative were not contaminated.

1µL required the most re-injections with a 70% first pass rate (Table 6). When re-injections were performed, 3µL was the only one to yield a final 100% success rate without need for re-amplification. For one of the samples using 2µL, there was an imbalance of sister alleles at one locus that caused one peak to drop below threshold. This lowered the first pass rate to 80%. From the results obtained, 3µL yielded the best results.

Chewing Gum

The first samples that were stored at room temperature presented problems during extraction. Though dried overnight, the inside of the gum was still sticky when cut into. It was difficult to get the gum to the bottom of the extraction tube without it sticking to the sides. Both the 1µL and 2µL had a 50% first pass rate (Table 7). It was suggested storing the chewing gum samples in a freezer would help harden the gum. New gum samples were collected and storing

them at -20°C greatly helped during extraction. The gum no longer stuck to the tube and it was much easier to cut and sample. 1µL had decent results with an 80% first pass rate, however profiles containing drop out could not be recovered upon re-injections. 2µL had the highest first pass rate of 80% and required two dilutions. 3µL had a 60% first pass rate and required four dilutions. If dilutions were performed, it would be expected that 2µL and 3µL would have 100% success rates without need for re-amplification. 2µL had the best results with needing the least amount of additional lab work.

Cigarette Butts

After testing of the other substrates, it was found that 1µL did not appear promising. Therefore for cigarette butts, only 2µL and 3µL were considered. 9 samples were tested, and 2µL and 3µL had the same first pass rate of 56% (Table 8). Only the 3µL could recover a profile that had drop out, however neither 2µL nor 3µL resulted in 100% full profiles. 3uL required one sample that needed to be re-amplified. Increasing the cutting size to 4mm x 4mm or 5mm x 5 mm could increase the number of alleles called in a profile. This was not studied due to time restriction, however further studies will test a bigger cutting size. It is expected that with a larger cutting size, 100% full profile recovery is possible.

Pseudo-exemplar Samples Summary

2µL and 3µL had the best results. 2µ had the highest first pass rate with 86% of profiles being reportable without additional lab work (Table 9). 3µL had a lower first pass rate of 78%, due to significantly more dilutions needed, however 3µL had the highest final pass rate of 98%. With 3µL, only one sample did not yield a full profile after re-injection. This sample would have to be re-amplified. For 2µL, two samples did not yield full profiles and required re-amplification. If each substrate was to be considered (2µL for some and 3µL for others), then

the first pass rate could be 88% with a final pass rate of 98% (Figure 2). It was determined that one common method should be developed to encompass all pseudo-exemplar substrates tested. Therefore, since 3µL had the highest final pass rate it was used. The final procedure developed for pseudo-exemplars was:

- Cutting
    - Bottles, cans, cups and straws: ½ outer layer peeled swab
    - Chewing gum: storage at -20°C, cuttings to fill to 0.1mL mark of 1.5 tube
    - Cigarette Butts: 3mm x 3mm cutting (larger sizes to be tested in future)
- Incubate in 100µL extraction buffer
- 3µL PCR input volume
- Amplify for 29 cycles

3.3 Identifiler® Plus Direction Amplification Validation Studies

15 Samples Study

Analyzing 30 buccal samples, 70% resulted in reportable profiles the first run. 27% had to be diluted and re-run and 3% required re-injection at 5kV for 25sec. Dilutions and re-injections were not performed due to time constraints. Further studies will evaluate the overall success rate and determine if any re-amplifications are required.

10 Blood Cards Study

20 samples were analyzed. There was an 80% first pass rate, 10% needed dilutions and 10% needed to be re-injected. Due to limited time, additional work could not be performed.

Reproducibility Study

For the first part of the reproducibility study, samples were collected on three different days to determine if consistent profiles could be obtained. For Day 1, one sample required re-

injection (Table 10). For Day 2, one sample needed re-injection and two required dilutions and for Day 3, two samples required dilutions. Out of the 30 samples analyzed over the 3 days, 7% needed re-injection and 13% needed dilutions. This gave an overall first pass success rate of 80%. For samples that gave reportable profiles the first run, profiles were consistent over the three separate days. The second part of the reproducibility study could not be performed, but future work will complete this study.

Concordance Study

The first part of the concordance study tested samples on three different instruments of varying sensitivities to ensure that consistent profiles could be obtained. On the most sensitive instrument, Galileo, 50% samples needed dilutions and 10% needed re-injection (Table 11). One of the samples could not be analyzed due to mis-migration. This sample would need to be re-injected. On the moderately sensitive instrument, Esther, 10% needed dilutions and 10% needed re-injections. Finally on the least sensitive instrument, Rudy, only 10% needed re-injection. This study showed that the success rates obtained from the direct amplification method varies depending on the sensitivity of the instrument it is applied to. The method may have to be modified to fit the limitations of a specific instrument used.

The new 3500*xl* Genetic Analyzer on the market has the ability to handle samples that would be oversaturated on the 3130*xl*. Overblown samples on the 3130*xl* will not be overblown on the 3500*xl*. The second part of the concordance study tested samples on the 3500*xl* that were previously run on the 3130*xl* to see if it changed the success rates. When the 30 samples from the 15 Buccal Samples Study was run on the 3500*xl*, only one sample needed dilution. This resulted in a 96.7% first pass rate. Overall, only one edit was required (due to pull-up) out of all 30 samples. This samples was the one requiring dilution. Therefore, if the direct amplification

method were applied to the 3500*xl*, success rates are expected to increase from 70-80% to nearly 100%.

4. DISCUSSION

4.1 Identifiler® Direct

Previous research conducted by Brito et al. showed that Identifiler® Direct could be used for non-FTA® treated samples and yield desired results. It included buccal swabs that have not been treated with Prep-n-Go™ Buffer. The NYC OCME does not use FTA®-treated substrates for collection of reference samples and similarly aimed to test Identifiler® Direct on buccal swabs and blood cards that were not-FTA® treated. The results obtained, however, demonstrate that there must be a lysis step before amplification. This would require the use of Prep-n-Go® Buffer, which presents an additional cost, or using an in-house buffer to extract the DNA. It was determined that if this additional step was required, it would be more cost-efficient to use an in-house buffer with Identifiler® Plus direct amplification since it would be more cost effective (half reactions are validated in the laboratory for Identifiler® Plus).

4.2 Identifiler® Plus

Samples extracted with the in-house buffer and amplified with Identifiler Plus for 29 cycles yielded promising results. Once all of the optimal conditions were found for each substrate, only one sample required re-amplification. For buccals, blood, bottles, cans, cups, straws and chewing gum, 100% full profiles could be obtained after re-injections and dilutions. Only for cigarette butts was there a sample that had drop out which could not be recovered with re-injection. Once final protocols were developed for each substrate the first pass rates were as follows:

- Buccals: 70-80%
- Blood: 80%
- Pseudo-exemplar samples: 78%

Initial studies showed that the direct amplification method involving an extraction with the in-house buffer and amplification with Identifiler® Plus could give reliable results. Analysis could be complete in one day. Approximately 75% of profiles can be reported at the end of the day and about 25% will need additional lab work, however only <1% of the samples required re-amplification. Having to re-amplify <1% of the samples and re-inject or re-run 25% of the samples would take less time than purifying and quantifying all reference samples. Considering all samples that were used for analysis, this method had a >99% success rate for avoiding the need of re-amplification.

If a larger cutting size is tested for cigarette butts, the overall success rate could potentially be increased to 100% without any need for re-amplification.

4.3 3500*xl* Platform

Results on the 3500*xl* proved to be even more promising. Due to the ability to better distinguish peaks in highly concentrated DNA samples, previously oversaturated samples on the 3130*xl* were no longer oversaturated on the 3500*xl* and would not have to be diluted. The first pass rate on the 3500*xl* with initial runs was 96.7%. The 3500*xl* also better detects low quantities of DNA so the chance of drop out is less likely. If the direct amplification method were applied to a 3500*xl* platform, reference samples would easily be processed in the period of a day. The 3500*xl* can process 24 wells in a run compared to the 16 wells a 3130*xl* can process. With the increase first pass success rate and more samples being analyzed in a shorter period of time, the time and cost it takes to analyze reference samples will be greatly reduced.

4.4 Final Elongation Time

Previous scientific papers have noted that Identifiler® Plus tends to result in split peaks at Amelogenin when used for direct amplification. Meyers et al. suggests extending the final elongation time from 10 minutes to 60 minutes. To further test options to reduce this effect, the thermal cycling parameters were considered for Identifiler®, Identifiler® Plus and Identifiler® Direct. Both Identifiler® and Identifiler® Direct have a separate anneal step for 2 minutes at 59°C and extension step for 1 minute at 72°C. Identifiler® Plus has shortened this by combining the anneal and extension steps into 1 step at 59°C for 3 minutes (shortens the time it takes to ramp between temperatures). Therefore, for the initial studies an additional 30 second extension step at 72°C was added to test if the separation of steps makes a difference in the split peak effect. It was determined that there was no apparent difference, so for the validation studies the manufacturer thermal cycling parameters for Identifiler® Plus were used with the exception of the extension of the final hold to 60 minutes. Extending the time to 60 minutes did have a great difference in the split peak compared to samples previously run with the recommended 10 minutes hold used in other ongoing projects at the NYC OCME.

4.5 Pseudo-exemplar sampling

Currently the NYC OCME consumes the entire swab for pseudo-exemplar samples. If the extraction is not successful or becomes contaminated, there is no additional effort that can be made to obtain results. The method developed of using half of the swab allows for a sample to be duplicated. If something happens with one extraction or no results are obtained, the second half can be extracted to be sure that all possible work on the sample could be done. In addition, having duplicates can help to confirm that the results obtained from pseudo-exemplar samples are reliable.

4.6 Future Studies

The validation studies that were started and could not be finished will have to be completed. In addition, the following studies will have to be performed to complete the validation:

Sensitivity Study

Since the method includes an extraction step, it was determined that the sensitivity study must include cells rather than a pristine positive DNA control. A buccal swab was smeared on a membrane slide and dried in a hood. Using the PALM® microscope, varying number of cells were catapulted into tubes (5, 10, 20, 50, 100, etc. cells in triplicate). Extraction buffer was added following the buccal swab procedure. Tests need to be performed to test how little cells are needed to yield a full profile. For small numbers of cells, they are to also be microconed and concentrated to see if profiles can be recovered. This will be done to help determine if a small quantity of cells can still give a full profile.

Stability Study

There are two parts to the stability study that will be performed. The first involves 10 buccal swabs that were stored in a 50°C heater to accelerate the aging of the DNA samples. They are to be extracted months after they were collected. The second part of the study involves re-amplifying extracts that have been previously analyzed. This will be done to test the stability of the extract and the buffer the DNA samples are stored in after extraction. Degradation of samples and allele recovery will be evaluated over time.

Pseudo-exemplar Samples Study

Similar with the Buccal and Blood Samples Studies, more pseudo-exemplar samples will be tested with the new validation conditions to show that the method withstands a variety of samples.

Compromised Samples Study

DNA concentrations from pseudo-exemplar samples will greatly vary. This is because the amount of DNA recovered depends on how easily a person sheds cells and how much they used an item. If an item was only drunk from once or smoked once, there would not be as much DNA as if they had drank the entire container or smoked the entire cigarette. Samples used for the initial studies in this project were provided after they were fully used. The Compromised Samples Study will test substrates that have only been drunk from once or twice or smoked from once or twice. This will test if the method can be applied to lightly used samples and if a modified method needs to be developed for these samples.

Mixture Study

Though the samples tested were reference samples, pseudo-exemplar samples may contain mixtures. The analyses conducted for this project treated samples as single source to initially evaluate if the method could be a viable option. While exemplar samples are professionally collected and expected to be single source, pseudo-exemplar samples are not guaranteed to be single source. The substrate may have been touched by another person before being used by the individual. Also, the substrates collected as pseudo-exemplars can typically be found disposed, such as in a trash or on the ground. Coming into contact with other surfaces may add other DNA sources that are found on the contacting surface. Therefore, a mixture study will need to be conducted to evaluate the significance and frequency of finding mixtures on pseudo-exemplar samples.

Contamination Study

Once all studies have been performed, the negative controls will have to be assessed to show that this method does not result in contamination. Using a direct amplification method reduces the number of tube transfers used for a sample as well as the amount of time a sample tube is opened. The sample is added directly to an extract tube and from there directly into the PCR reaction. For typical DNA analysis workflow, the sample is added to the extract tube, and then washed through a purification system in another tube. An aliquot is required to quantify the DNA and after quantification, manipulation of the sample may be required through dilutions and microcons. After all of these steps are performed, then the sample can be added to a PCR reaction.

Implementing a direct amplification method reduces the possibility for contamination to occur. This study will evaluate its effectiveness in reducing contamination.

5. CONCLUSION

After testing different amplification kits and different variables, a direct amplification method was developed for exemplar and pseudo-exemplar reference samples using Identifiler® Plus. It was determined that Identifiler® Direct did not fit the needs of the NYC OCME and that Identifiler® Plus yielded promising results. Since Identifiler® Plus has been validated for half reactions, the cost of processing a reference sample with this new method greatly reduces the time and cost for analysis. The in-house buffer used was created in house and the incubation time does not greatly differ from the incubation required for the use of Prep-n-Go™ Buffer.

To implement Identifiler® Direct at the NYC OCME would require further testing with their current collection substrates (non-FTA® treated), testing to determine if half reactions

could also be used, an additional cost for Prep-n-Go™ Buffer and training for analysts to use a newly introduced kit. Implementing Identifiler® Plus with an in-house buffer extraction and half reactions for PCR is most cost effective. In addition, since Identifiler® Plus is validated at the laboratory and analysts are currently being trained with the kit, no significant additional training would be required to prepare analysts for this new direct amplification method. Therefore, it was determined that Identifiler® Plus would best fit the needs of the NYC OCME.

Testing with Identifiler® Plus gave promising results, especially when applied to the 3500*xl*. This new method can help crime laboratories with their need to reduce DNA backlogs. A sample can be processed from extraction to obtaining a reportable profile in one day. With future studies, this method can prove to be an extremely effective way to process reference samples.

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TABLES AND FIGURES

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/ Re-runs Required (%)	Require Edits (%)	Out of Profiles That Require Edits			
					Maximum Number of Edits	Minimum Number of Edits	Average Number of Edits	St. Dev. Of Number of Edits
1/3 Swab, 28 Cycles								
2uL	80	0	20	30	23	2	11	10.8
1uL	80	10	10	50	8	1	2.4	3.1
5uL of 1/10 Dilution	50	50	0	20	2	1	1.5	0.7
1/6 Swab, 29 Cycles								
3uL	20	0	80	100	29	3	17.3	8.7
2uL	60	0	40	90	17	1	9.7	7.1
1uL	70	10	20	60	8	1	4.5	2.7

Table 1. First success pass rates, required re-injection/re-run rates and required edits information on 10 buccal samples for Identifiler® Plus direct amplification method.

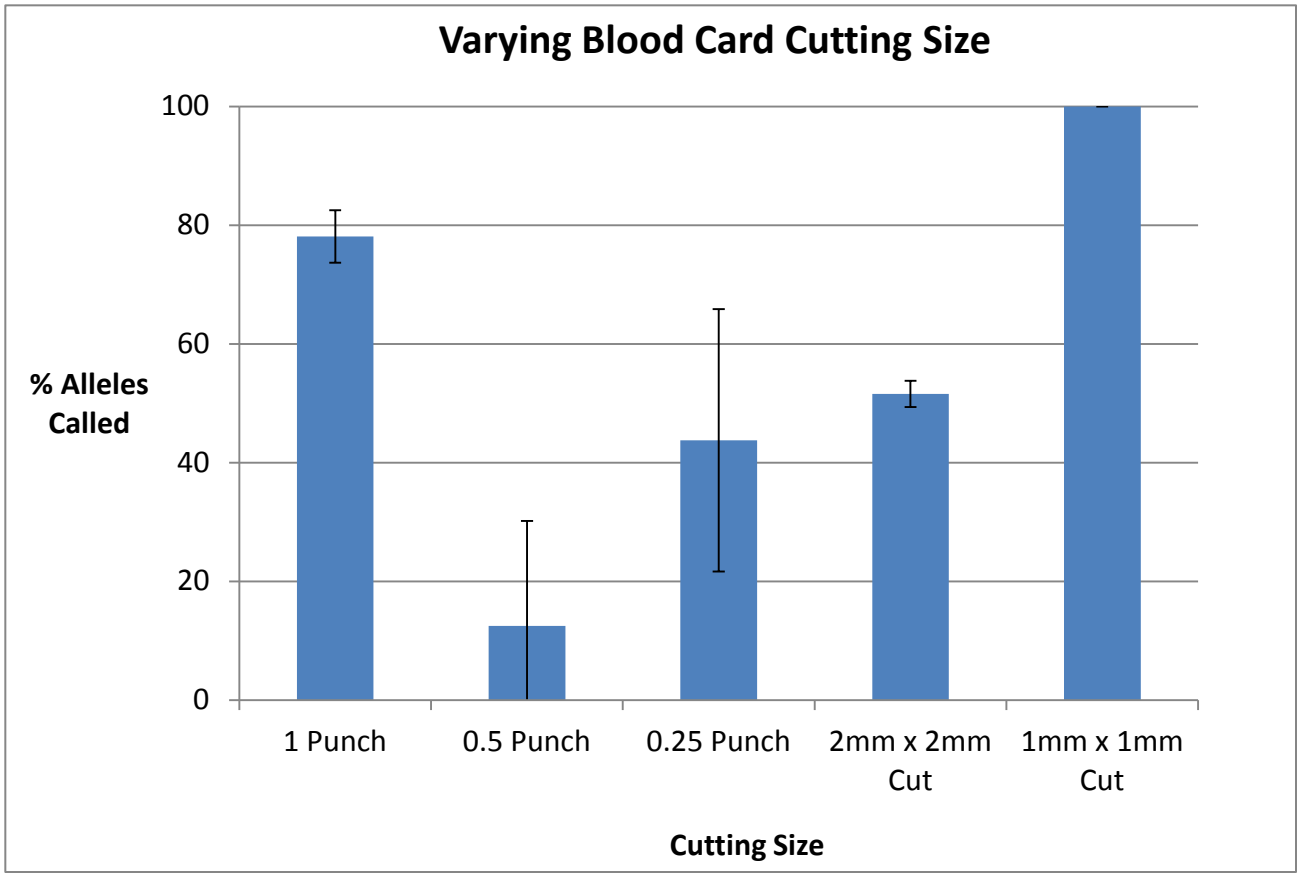

Figure 1. Percentage of alleles called per cutting size of blood card. The same blood card was used for all cutting sizes.

					Out of Profiles That Require Edits			
	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/ Re-runs Required (%)	Require Edits (%)	Maximum Number of Edits	Minimum Number of Edits	Average Number of Edits	St. Dev. Of Number of Edits
200uL, 2uL	30	50	20	20	14	3	8.5	7.8
200uL, 3uL	50	30	20	20	52	6	29	32.5
200uL, 5uL	80	0	20	20	57	23	40	24
300uL, 2uL	60	20	20	20	8	1	4.5	4.9

Table 2. First success pass rates, required re-injection/re-run rates and required edits information on 10 blood samples for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	80	20	0	100
2uL	90	10	0	90
1uL	80	20	0	90

Table 3. Success pass rates on 10 samples from bottles for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	80	0	20	100
2uL	90	10	0	100
1uL	60	40	0	80

Table 4. Success pass rates on 10 samples from cans for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	70	0	30	100
2uL	90	0	10	100
1uL	70	30	0	90

Table 5. Success pass rates on 10 samples from cups for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	90	10	0	100
2uL	80	20	0	80
1uL	70	30	0	80

Table 6. Success pass rates on 10 samples from straws for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/ Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
Room Temp				
2uL	50	30	20	N/A
1uL	50	50	0	N/A
-20°C				
3uL	60	0	40	100
2uL	80	0	20	100
1uL	80	20	0	80

Table 7. Success pass rates on chewing gum samples for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	78	22%	0	89%
2uL	78	22%	0	78%

Table 8. Success pass rates on 9 samples from straws for Identifiler® Plus direct amplification method.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)	Expected Reportable Profiles Obtained After Re-injections
3uL	78	9	12	98
2uL	86	11	5	97
1uL	72	28	0	84
Best Combo	88	9	5	98

Table 9. Summary of success rates for pseudo-exemplar samples for Identifiler® Plus direct amplification method.

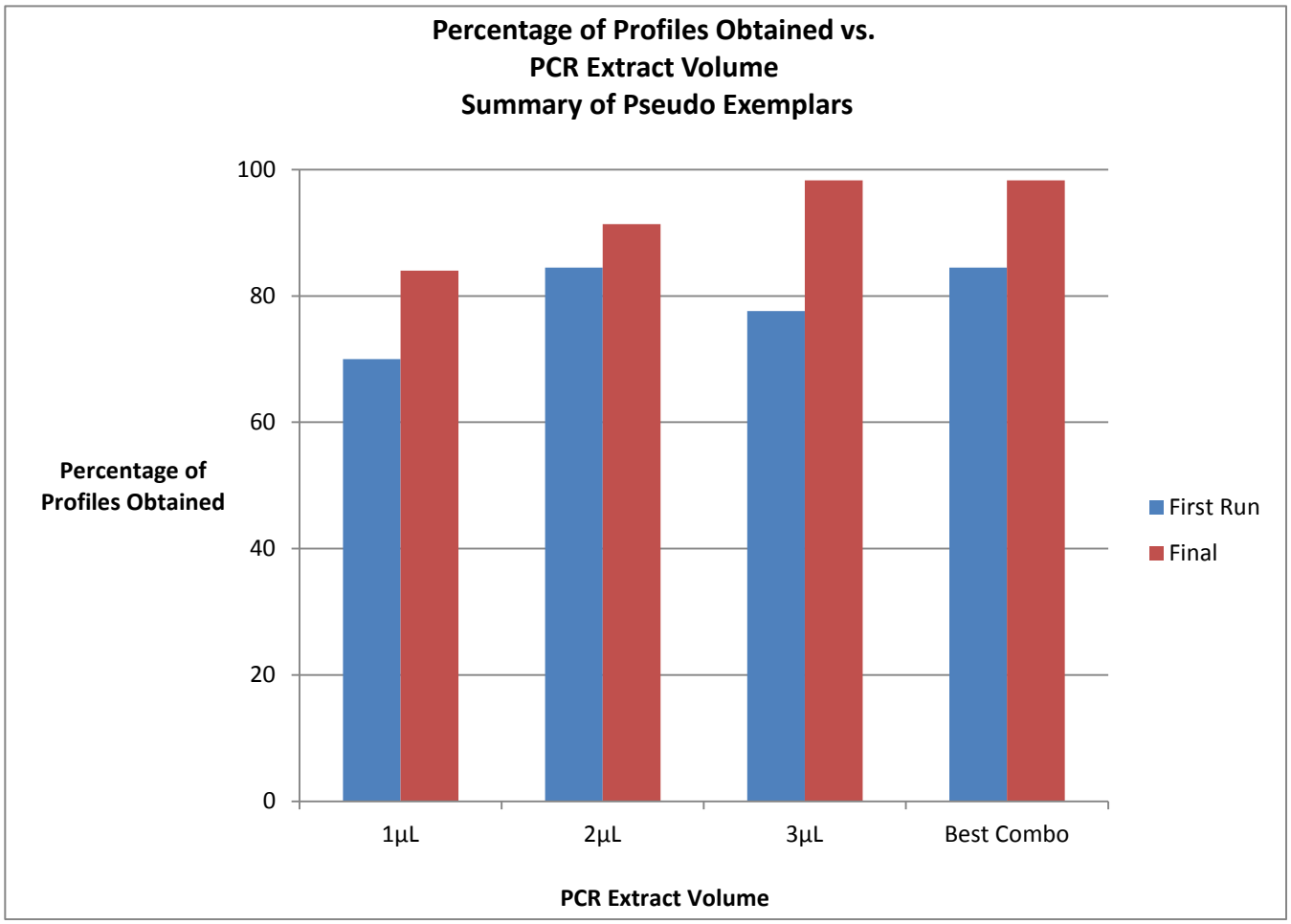

Figure 2. Summary of pseudo-exemplar studies. The blue indicates the percentage of profiles that were reportable after initial runs. The red indicates the percentage of profiles expected to be obtained after re-injections and dilutions are made.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)
Day 1	90	10	0
Day 2	70	10	20
Day 3	80	0	20

Table 10. Summary of Reproducibility Study, testing swabs collected on three different days.

	Reportable Profiles Obtained First Run (%)	Re-injections Required (%)	Dilutions/Re-runs Required (%)
Galileo	40	10	50
Esther	80	10	10
Rudy	90	10	0

Table 11. Summary of Concordance Study, testing different sensitivities. Galileo is most sensitive, Esther moderately sensitive and Rudy the least sensitive. For Galileo, one of the samples had mis-migration in the internal size standard and needs to be re-run.