Document Title:	**Towards Commercialization: Preliminary Developmental Validation of a High Resolution Melt Curve Mixture Prediction Assay and SVM Tool**
Author(s):	**Tracey Dawson Green, Ph.D., Andrea Publow, MFA, CRA**
Document Number:	306166
Date Received:	**March 2023**
Award Number:	**2019-DU-BX-0003**

COVER PAGE

National Institute of Justice
FY19 Research & Evaluation for the Testing & Interpretation of Physical Evidence in Publicly Funded Forensic Laboratories

Award 2019-DU-BX-0003

Towards Commercialization: Preliminary developmental validation of a high resolution melt curve mixture prediction assay and SVM tool

Tracey Dawson Green (*formerly* Cruz), PhD (PI)
Professor & Chair, Department of Forensic Science
Virginia Commonwealth University
(804) 828-0642
tcdawson@vcu.edu

Andrea Publow, MFA, CRA
Director of Sponsored Programs – Gov't/Non-Profit Support
Virginia Commonwealth University
(804) 828-6772
ospgold@vcu.edu

Final Technical Summary
Submission Date: 3/6/2023

DUNS:
EIN:

Virginia Commonwealth University
Box 980568
800 East Leigh Street, Suite 3200
Richmond, VA 23219

VCU PT No.
VCU Index No.
Award Amount: $399,699

Project/Grant Period: 1/1/2020 – 12/31/2022

_____   _____
Signature of Submitting Official   Date

1

PROJECT SUMMARY

Introduction

The current method of generating a forensic DNA profile utilizes the PCR amplification of short tandem repeats (STRs) to allow for capillary electrophoresis (CE)-based detection of alleles at specific loci (1). The use of the PCR technique coupled with capillary electrophoresis has enabled analysis of samples containing degraded or trace amounts of DNA, such as typing of DNA extracted from saliva on cigarette butts (2). With recent increases in sensitivity of these analytical techniques, there has been a subsequent increase in submission of 'touch DNA' samples that come from the transferal of skin cells that occur during contact with a surface (3). These touch DNA samples often contain low levels of template DNA (less than 100pg available for STR amplification) which becomes problematic during PCR as some target regions may be preferentially amplified over others. This can result in a number of undesirable effects that complicate profile interpretation such as allele drop-in, allele drop-out, and peak imbalances (4). Further, because these touch DNA samples originate from surfaces that may have been touched by numerous individuals, these samples are prone to mixtures – i.e. DNA present from more than one source contributor. When a mixture is present, along with low amounts of DNA, resulting data often includes one or more of the contributors' allele peaks falling below the analytical threshold, further confounding the profile interpretation process and often leading to "inconclusive" reporting.

In the current forensic laboratory workflow, both allele genotype assignment and mixture detection occur at the end-point of analysis, after DNA separation and detection (CE analysis), during the data review process when allele fragments are sized, allele values are assigned, and data quality evaluated. At that point, intra- and inter- locus imbalances and the presence of three or more peaks at multiple loci indicate to the examiner that multiple contributors are present in the DNA sample (5). Because this information is not available until the last step (end- point analysis), it is not possible to make earlier analytical adjustments to the protocols or workflow that may increase the likelihood of generating a profile with a distinguishable minor contributor. While reamplification of a low, mixed DNA sample may be possible, it is time consuming and risky – often providing little-to-no new information. Additionally, with low template or touch samples, the samples are more often consumed during initial testing, leaving little remaining DNA for a second analysis. Further, no meaningful comparisons or conclusions can be made with respect to identification of the contributor of a DNA evidence sample until after the CE run (hours to overnight), import of the raw data into a genotyping software package, a careful data review by at least one trained examiner, STR profile interpretation and export, and formal reporting of the case conclusions – a process that can easily take weeks, from start (initial sample evaluation) to finish (case report issued).

A mixture prediction assay that could characterize a sample as either a single source or mixture sample, and potentially provide early exclusionary information (for single-source samples, based on predicted genotype), earlier in the forensic DNA workflow would be useful to both the forensic DNA and investigative communities - particularly when sample consumption is a concern or when multiple surface swabs are available from a single evidence item (8). For example, the majority of the samples analyzed by the Bureau of Alcohol, Tobacco, Firearms, and Explosives (ATFE) are touch DNA samples collected from items such as guns, explosive debris, and ammunition (6). These often include multiple swabs from various areas of the evidence, for example, swabs from the trigger, safety lever, hand grips, slide, and/or hammer area are often collected as separate samples from a gun submitted for testing. Unfortunately, 50% of this type of evidence analyzed at the ATFE results in inconclusive results, low level data, and/or complicated mixtures with indistinguishable minor contributors (7). ATFE protocol currently dictates that low level DNA samples be concentrated down to 10µL, and that only half (5 µL) may be used for initial amplification. Unfortunately, if a mixture is detected at the final CE/data analysis step and minor

2

contributors are indistinguishable, there is no way to reamplify with more template, as only half of the DNA sample remains. However, if a mixture prediction tool was available early on in the workflow to confirm that the sample was a mixture (and not single source), then a request could be made for a deviation from the standard consumption policies to allow for more than half of the sample to be used in the initial amplification. By allowing for more of the sample to be used in the initial testing, there should be an increase in amplified product, thus increasing the likelihood that resulting minor allele peaks are above the analytical threshold and can be easily identified. Alternatively, if multiple sample swabs taken from the same evidence item can be determined early-on to be from a single contributor, examiners could be empowered to more confidently combine DNA extracts from those items to increase the amount of template DNA available for STR amplification during the initial DNA testing, while avoiding the creation of accidental mixtures. Both scenarios include the use of screening information to redirect the DNA workflow in an effort to improve the first round pass (success) rates associated with the testing of low level DNA and/or mixture samples, and would subsequently also reduce retest rates. This, in turn, could save valuable examiner time as well as reduce consumable expenses.

A 2015 NIJ award (2015-MU-MU-K026) paved the way for significant progress towards the goal of developing a forensic DNA mixture detection assay (8). The goal of the previously funded project was to design an assay for mixture detection that could be multiplexed with the quantitation step of the forensic DNA workflow. The assay developed utilizes a post-qPCR melt-curve analysis to detect the presence of double-stranded amplicon products from two targeted STRs (D5S818 and D18S51). The two year grant allowed for significant optimization and testing of the STR amplification/melt assay on two qPCR platforms, preliminary reproducibility testing, evaluation of melt curves for genotype prediction using numerous statistical models, and finally, integration of the assay into an existing commercially-available qPCR human DNA quantitation kit (Quantiplex® kit) (9,10). Initial testing on a limited set of single source and 2-person 1:1 mixed samples using the Qiagen Rotor-Gene Q platform revealed that this quantitation-HRM integrated assay was able to accurately distinguish between single-source and mixture samples 94% or 100% of the time, depending on the analytical approach (8-10). While this work has successfully produced a viable qPCR-based melt curve assay for prescreening identification of mixtures, there remained several considerations that needed to be addressed prior to crime lab testing and implementation.

Goals & Objective

In order to de-risk and make more broadly applicable, the previously developed HRM qPCR-based assay for mixture detection would need an evaluation/retooling on a more common, modern qPCR platform more commonly utilized in forensic DNA laboratories. Similarly, integration of the HRM qPCR-based assay into a more commonly utilized commercial quantification kit would be beneficial to expand the applicability of the new assay and prediction tool. Additionally, key developmental validation studies must be completed, including, a comprehensive assessment of accuracy and reproducibility, an assessment of assay performance using mixtures that contain more than 2 persons and across a spectrum of mixture ratios, and testing of the assay using DNA from non-probative compromised forensic evidence samples. Further, depending on the results observed, larger reference data sets (standards) may be needed to improve upon the statistical predictions using the developed approach and, ultimately, a web-based tool would be required in order to facilitate broader access to the prediction tool. In order to more fully develop and assess the value of this emerging laboratory method, the goals below were set forth in the original proposal.

1) Test and evaluate the developed integrated mixture screening assay on the QuantStudio™ qPCR platform, which is more consistent with instrumentation used in forensic practice.

Note: *Since the submission of this proposal, ThermoFisher has announced a plan to convert HID qPCR products and support to an updated platform, the QuantStudio. This platform is operationally and mechanically identical to the 7500, but with significant improvements, including high resolution melt curve capability. Given the plans to discontinue the 7500 model and our previous data, suggesting that the dissociation function on the 7500 may not be powerful enough for the developed mixture assay, we have converted all proposed 7500 studies to the QuantStudio platform.*

 a. Integration of STR melt curve assay into the Quantifiler™ Trio qPCR human DNA quantitation kit; evaluate & optimize testing conditions for the QuantStudio™ platform
 b. Conversion of existing integrated Quantiplex®-HRM assay to the QuantStudio™ platform
 c. Evaluation of mixture vs. single source prediction accuracies using identified best-performing statistical models
 d. Development of a formal protocol for both assays on the QuantStudio™ platform

2) Complete select preliminary developmental validation studies to supplement the previously obtained proof-of-concept data. This will include:
 a. Reproducibility, accuracy and reliability testing
 b. Sensitivity testing to determine the linear range of detection
 c. Testing of assay using >2+ person mixtures
 d. Testing of mixture across a spectrum of DNA ratios
 e. Testing of compromised mock forensic evidence samples

3) Development of an easy-to-use free, online tool for mixture prediction analysis, including:
 a. Generation of larger single-source reference datasets for examination of a 10-fold cross validation support vector machine (SVM)-learning approach (versus single cross validations)
 b. Evaluation of additional machine learning approaches, such as Artificial Neural Networks, as needed
 c. Development of user interface for access to reference datasets, prediction analysis, and exportable reporting

4) Testing of the developed integrated mixture screening assay with analysis tool in partner public forensic laboratory to assure direct applicability to lab practice. This will include:
 a. Training of ATFE staff on-site using formal developed protocols
 b. Evaluation of mixture vs. single-source sample prediction capabilities using actual forensic casework samples

Project Design & Methods

Initial Sample Selection and DNA Analysis

 Buccal swab samples for this work were part of the VCU forensic biological sample registry and were previously collected using sterile cotton swabs from donors in accordance with the approved VCU Institutional Review Board, Human Subjects Research Protocol (VCU-HM20002931). Sample DNA was purified using a Qiagen QIAcube liquid extraction robot using the standard manufacturer's Buccal Swab Spin QIAcube Protocol using QIAamp® DNA Blood Mini kit reagents (Qiagen). Samples DNA extracts were quantified using half-reactions of the Investigator Quantiplex® kit on the Rotor-Gene® Q following manufacturer's recommended protocols. Sample STR reference profiles were developed by amplifying 1ng

of DNA extract from each sample with the AmpFLSTR® Identifiler® PCR amplification kit (Thermo Fisher Scientific) on a GeneAmp 9600 thermal cycler (PerkinElmer, Waltham, MA). The 15µl reaction consisted of 5.7µl of PCR Reaction mix, 2µl of Primer set, 2.1µl Tris-EDTA (TE), 0.2µl of AmpliTaq™ Gold Polymerase (5U/µl) (Thermo Fisher Scientific), and 5µl of template DNA. Thermal cycling parameters were: activation at 95°C for 11min followed by 28 cycles of 94°C denaturation for 60s, 59°C annealing for 60s, and 72°C elongation for 60s, finished with a 60°C final extension for 90min. Amplified STR products were separated and detected on an Applied Biosystems™ 3130 Genetic Analyzer (Thermo Fisher Scientific) using a 36-cm capillary array and a 10s injection with an analytical threshold of 75 relative fluorescent units (RFUs). For capillary electrophoresis analysis, 1.5µl of amplified DNA or 1µl of allelic ladder was mixed with 0.1µl of GeneScan™ 500-LIZ™ size standard (Thermo Fisher Scientific) and 12µl of Hi-Di™ formamide (Thermo Fisher Scientific). STR profiles were analyzed using GeneMapper™ ID software v4.1 (Thermo Fisher Scientific). Samples that expressed genotypes of interest at the D5S818 [(10,11), (11,11), (11,12), (11,13), (12,12), (12,13) and (13,13)] and D18S51 [(12,13), (12,14), (12,15), (12,16), (13,14), (13,16), and (14,15)] loci were used in all experimental studies.

Initial evaluation of prediction models for STR genotype determination

In order determine what prediction modeling algorithms were best suited for STR genotype prediction, samples whose genotypes fell within both of the aforementioned genotype groups were tested using D5S818 and D18S51 singleplex amplification and melting on three qPCR platforms. Amplifications included a 38µl reaction mix composed of 1X PCR Gold Buffer without $MgCl_2$ (ThermoFisher), 3mM $MgCl_2$, 250µM dNTPs, 1µM forward and reverse primer (each), 1µM AmpliTaq Gold DNA polymerase (ThermoFisher), 1x EvaGreen® intercalating dye, and 0.25mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). Template DNA (2 µl) was added to each reaction for a total reaction volume of 40µl. Primer sequences used for D5S818 amplification were (F) 5'-GGGTGATTTTCCTCTTTGGT-3' and (R) 5'-AACATTTGTATCTTTATCTGTATCCTTATTTAT-3'; primer sequences used for D18S51 amplification were (F) 5'-CAAACCCGACTACCAGCAAC-3' and (R) 5'-GAGCCATGTTCATGCCACTG-3 (11-13). The thermal cycling parameters used consisted of an initial 10min 95°C denaturation followed by 45 cycles of: 95°C for 5s, 56 °C for 20s, and 65°C for 30s with fluorescence detected during the extension cycle. Following the amplification cycles, samples underwent a transition cycle consisting of 72°C for 2min, 95°C for 20s, 55°C for 20s and 56°C for 2min, after which the amplicons were melted. For the melt analysis on the Applied Biosystems™ 7500 platform (ABI 7500), amplicons were melted from 60-95°C using the "continuous" option (for 0.5% incremental increases in the temperature) as these conditions were determined to provide the highest resolution melt curves for this platform. Fluorescent signal was detected throughout the melt cycle in filter 1 when using the ABI 7500 platform. The raw dissociation data was exported using the ABI 7500 System Detection Software (SDS) v2.0.6 (ThermoFisher). For analysis on the Rotor-Gene® Q, reaction conditions, amplification cycle, and transition cycle parameters used were identical to those described above. However, following the transition cycle, the amplicons were melted from 60-95°C at a 0.1° incremental increase (2s hold), with fluorescent detection in the HRM channel. For STR melt analysis on the QuantStudio™ 6 Flex qPCR platform, reaction conditions, amplification cycle, and transition cycle parameters were identical to those described above.

Negative derivative data from every temperature point along the entire melt curve for each tested sample was modeled using a quadratic spline in R statistical software (©The R Foundation, Vienna, Austria) (14), which captured the full features of each melt curve (**Figure 1**). The spline allow for the formation of a mean melt curve for each genotype tested for each STR locus; the splines generated were then used to obtain coefficients could subsequently be used in the classification process. Three machine learning tools were tested within R statistical software to determine which model provided the highest genotyping classification rates for each qPCR platform used. These models were: LDA, SVM with linear basis functions (SVM Linear), and SVM with radial basis functions (SVM Radial). In order to determine

5

genotype accuracies, sample data were separated evenly into known "training" and unknown "validation" data sets. The data from the training sets were used to train the software for classification using all three modeling tools; all other samples were treated as experimental unknowns and were included in the validation data sets. The training set consisted of seven to eight samples for each common genotype selected for testing for both D5S818 and D18S51 loci. The validation set included additional unique samples who had known genotypes that fell within the group of genotypes selected for testing. Confusion matrices generated for each modeling algorithm tested were used to determine the STR genotype prediction accuracy of each model for both STR loci tested. Confusion matrices included known genotype of the samples on the vertical axis and the predicted genotype on the horizontal axis (**Table 1**). Genotyping accuracy of the validation data was calculated by taking the sum of samples that accurately classified (Table 1, seen along the diagonal) divided by total number of samples tested in the validation set. The best models for each qPCR platform were selected for use with all subsequent studies. Given the relatively low success with the lower resolution ABI 7500, further work on this platform was discontinued.

Integration and functional testing of the Quantiplex®-HRM assay

To evaluate the success of the melt curve assay within the qPCR-based quantification step of the forensic DNA workflow, the D5S818 and D18S51 primers and EvaGreen® dye (Biotium) were integrated into the Investigator™ Quantiplex kit and tested on the Rotor-Gene® Q and QuantStudio™ 6 Flex qPCR platforms using HRM analysis. Integrated Quantiplex®-HRM reactions included a 16.16µl master mix comprised of 7.36µl of the Quantiplex® primer mix, 7.36µl of the Quantiplex® reaction mix, 0.16µl of 100µM forward and reverse primer for each STR locus (as described above), and 0.8µl 20x EvaGreen® intercalating dye. To each reaction, 1µl template DNA was added for a total reaction volume of 17.16µl. In order to assure proper amplification of the Quantiplex® targets, the thermal cycling program was slightly altered from that described above. Thermal cycling parameters used for the integrated Quantiplex®-HRM assay included a 10 min 95°C denaturation followed by 40 cycles of 95°C for 5s and 60°C for 30s. Following amplification, samples underwent a transition cycle consisting of 72°C for 2min, 95°C for 20s, 55°C for 20s and 56°C for 2min, after which the amplicons were melted. Amplicon melt parameters for the Rotor-Gene® Q were identical to those previously detailed. The QuantStudio™ 6 Flex melt program included a ramp from 60°C to 95°C using the continuous setting with a ramp rate of 0.015°C/s, which allowed for maximum resolution.

In order to determine if the Quantiplex® amplicons themselves produce melt products when the transition and melt cycles were added to the amplification parameters an additional set of Quantiplex® standards were amplified on the Rotor-Gene® Q using the manufacturer's recommended reaction conditions (without STR primers or EvaGreen® dye) and amplification parameters, but with the added transition and melt program described above. Resulting melt curves were qualitatively compared to those obtained when using the integrated Quantiplex®-HRM assay, as described above. To compare melt curves, 10 samples with D5S818 and D18S51 genotypes within the selected study set were amplified using the integrated Quantiplex®-HRM assay, as described above. The mean and standard deviation of the D5S818 and D18S51 primary melt peak temperatures were calculated and compared those obtained in the initial evaluation studies describe above using a two-tailed students t-test (α=0.05). In order to determine if alterations in Quantiplex® reaction chemistry would affect resulting human DNA quantification estimates expected, two sets of Quantiplex® standard samples were analyzed across two separate Rotor-Gene® Q runs using both the traditional Quantiplex® chemistry (with half reactions) and the integrated Quantiplex®-HRM assay (described above). On each run, one set of standards were used to generate the standard curve while the other set of standards were evaluated as unknowns. Resulting standard curve quality metrics and inter-run variation were compared. The inter-run variation was determined by calculating the average differences in quantification values between runs and the percent differences observed in quantification values obtained for each standard sample from each run, as described above. The percent difference was

6

calculated by taking the absolute value of the difference in quantification values obtained across runs, dividing by the average, and then multiplying by 100.

Single source vs. mixture prediction using the integrated Quantiplex®-HRM assay

Available DNA samples were split into training and validation sample sets and subsequently tested using the newly optimized integrated Quantiplex®-HRM assay and two qPCR platforms (Rotor-Gene® Q the QuantStudio™ 6 Flex). The training set was comprised of 101 single source DNA samples with D5S818 and D18S51 genotypes of interest (see above). Additionally, 10 1:1 two-person mixtures (made of contributors with genotypes of interest) were included in the training set. The validation set was comprised of 56 single-source samples, each having genotypes of interest at both loci, as well as 10 different 1:1 two-person mixtures. Sample data was imported into the R statistical software as described above and tested using only the best performing genotype prediction model, as determined above. Confusion matrices were generated and subsequently used to determine the accuracy of the predictions. Single source typing prediction accuracies were determined for each locus by calculating the total number of samples classified as a single source genotype (regardless of whether the correct genotype was obtained) divided by the total number of single source samples tested in the validation set. Similarly, mixture accuracy was determined by dividing the number of mixture samples correctly classified by the total number of mixtures tested in the validation set. For combined accuracy of the integrated Quantiplex®-HRM assay, predictions for both STR loci tested were considered. If either STR locus was classified as a mixture for a given sample, then the final classification for that sample was indicated as a mixture. Finally, the number of samples that classified accurately (as either a single source or mixture) was divided by the total number of samples tested in order to determine the overall accuracy of the integrated Quantiplex®-HRM assay for both qPCR platforms.

Quantifiler™ Trio-HRM reaction optimization

In order to determine the optimal reaction conditions for the integrated Quantifiler™ Trio-HRM assay, increased reaction volumes were tested; each set of reaction conditions tested kept the manufacturer's recommended sample Quantifiler™ Trio component concentrations. Two reaction conditions were tested, each with a different total volume: 16µl and 11µl. The 16µl volume reaction included 5.8µl of Quantifiler™ Trio Primer mix, 7.2µl of Quantifiler™ Trio THP Reaction mix, 0.10µl of 100µM D5 and D18 primers, 0.63µl of 5µM SYTO™ 64, and 2.0µl of sample DNA or standard sample DNA input (as recommended by the manufacturer). Alternately, the 11µl reaction included 4.0µl of Quantifiler™ Trio Primer mix, 5.0µl of Quantifiler™ Trio THP Reaction mix, 0.11µl of 100µM D5 and D18 primers, 0.55µl of 5µM SYTO™ 64, and 1.0µl of sample DNA or standard sample DNA input. All reactions followed the amplification and melt cycling parameters as described above. To evaluate each reaction volume, five single source DNA samples along with a set of the Quantifiler™ Trio standard DNA samples were tested. Additionally, the same samples were also tested using the standard Quantifiler™ Trio assay following the manufacturer's recommend protocol (but with half-volume reactions). Resulting data was assessed for quantification accuracy by calculating the percent difference between these values and comparing to the normal inter-run variation observed using the standard Quantifiler™ Trio assay. The percent difference (inter-run variation) was calculated by taking the absolute value of the difference in quantification values obtained across runs, divided by the average, and then multiplied by 100. Additionally, the resulting D5 and D18 loci melt curve morphologies for samples analyzed using each reaction volume were carefully qualitatively assessed. The reaction condition which produced quantification values (based on the small autosomal target) most similar to those observed using the standard Quantifiler™ Trio assay was selected for use in all subsequent studies (16µl reaction).

Testing of the optimized Integrated Quantifiler™ Trio-HRM assay reaction

The final reaction conditions selected for the integrated Quantifiler™ Trio-HRM assay consisted of: 5.8µl Quantifiler™ HP Primer Mix, 7.2µl Quantifiler™ THP PCR Reaction Mix, 0.63µl of 128µM SYTO™ 64 (5µM final concentration), 0.1µl of 100µM D18S51 forward and reverse primers (0.62µM

7

final concentration), and 0.1µl of 240.45µM D5S818 forward and reverse primers (1.5µM final concentration) in a total volume of 16.03µl per well. This includes a DNA input of 2µl for all samples tested. Data analysis settings included baseline start and end values of 3 and 17, respectively, for all targets and a threshold of 0.4, 0.08, and 0.1 for IPC, large autosomal and small autosomal/Y targets, respectively. These final reaction conditions and settings were used for all subsequent testing and prediction analyses using the integrated Quantifiler™ Trio-HRM assay.

Inter & Intra run variation

A set of 10 single-source DNA samples were analyzed over two different runs on two different days to determine the inter-run variation observed using the integrated Quantifiler™ Trio-HRM assay and optimized data analysis settings. Additionally, five of the samples were analyzed in duplicate on the same run. The inter-run variation of the integrated Quantifiler™ Trio-HRM assay was determined by calculating the average percent differences observed in quantification values obtained for each sample from each run, as described above. The intra-run variation was determined by calculating the average percent differences observed in quantification values obtained from each sample run in duplicate on the same plate (on the same day), as detailed above.

Quantification precision

Quantification accuracy of the integrated Quantifiler™ Trio-HRM assay was evaluated by testing 10 single source samples using the finalized reaction conditions and data analysis settings; quantification values from all three quantification targets were compared to those obtained when the same samples were tested using the standard Quantifiler™ Trio assay per manufacturer's recommended protocol (with half volume reactions). The percent difference between these values was calculated, as described above, and values were compared to the normal inter-run variation of the standard Quantifiler™ Trio assay.

As the small autosomal target is used as the quantification measure for downstream STR-amplification, STR amplification and analysis was pursued to assess the performance of the quantification values obtained from the integrated Quantifiler™ Trio-HRM assay. For this, two sets of five DNA samples were amplified using the PowerPlex® Fusion 5C (Promega; Madison, WI) multiplex STR amplification kit on the ProFlex PCR System (Thermo Fisher) following manufacturer's recommended protocol but using half-volume reactions. Each reaction included 2.5µl of PowerPlex® 5X Master Mix, 2.5µl of PowerPlex® 5X Primer Mix, 5µl amplification-grade water, and 2.5µl of 0.1ng/µl DNA (0.25ng total) per reaction. The first set of five samples were diluted using small autosomal quantification values reported from the standard Quantifiler™ Trio assay and the second set of samples with values reported by the new integrated Quantifiler™ Trio-HRM assay, using the optimized conditions described above. Amplification parameters were based on the manufacturer's recommendation of a 96°C hot start for 1 min and 30 cycles of: 94°C for 10 sec, 59°C for 1 min, 72°C for 30 sec with a final extension at 60°C for 45 min. The genetic fragments were separated by size using the ABI 3500 Genetic Analyzer. Each sample analyzed included 9.7µl Hi-Di formamide, 0.3µl WEN ILS 500, and 1µl of amplicon product or allelic ladder per reaction with a 1.2 kV, 5 second injection. The generated STR profiles from each sample set were compared (with a stochastic threshold of 300RFU and analytical threshold of 100RFU) using the total percent of expected STR alleles recovered, heterozygote peak balance (flags for peak height ratio <70%), and mean allele peak heights calculated in MS Excel (Microsoft; Redmond, WA). Mean peak heights for each sample were calculated by taking the sum of the peak heights and dividing by the sum of STR alleles observed. Heterozygote peak balance was calculated by taking the smaller allele peak height and dividing by the larger allele peak height, multiplied by 100.

Degradation Index and Male: Female Ratio

In order to determine if the integration of the HRM components and subsequent alterations in data analysis settings associated with the integrated Quantifiler™ Trio-HRM assay altered the capability of this assay to accurately assess degradation indices (DI) and the male-to-female ratios (M:F), 10 single source

8

DNA samples were analyzed (5 male, 5 female) and these metrics were calculated. Resulting values were compared to those obtained from the same samples when tested using the standard Quantifiler™ Trio assay per manufacturer's recommendations, with half volume reactions. DI's for each sample were calculated by dividing the small autosomal quantification values by the large autosomal quantification values. The DI values for all 10 samples were then averaged for comparison purposes. Male-to-female ratios were calculated for each of the five known male samples analyzed. For each male sample, the M:F was calculated by subtracting the absolute value of the small autosomal target average quantification values from the male DNA (Y target) values and then dividing this value by the male DNA value.

IPC Inhibition Assessment

In order to determine whether dye channel sharing of the IPC target dye (JUN™) with the added intercalating dye (SYTO™ 64) affected the IPC's ability to detect the presence of inhibitors an inhibition study was conducted. The selected inhibitors, hematin (Sigma-Aldrich®; St. Louis, MO) and humic acid (Alfa Aesar®; Haverhill, MA), were dissolved in 0.1N NaOH and water, respectively. Two sets of control 2800M DNA samples (0.1ng/μl) were prepared and each was spiked with a different known inhibitor. The first set included a range of hematin concentrations (200μM, 500μM, 750μM, 1000μM, and 1250μM final in sample concentrations) while the second set included a range of humic acid concentrations (200ng/μl, 300ng/μl, 400ng/μl, 600ng/μl, and 800ng/μl final in sample concentrations). Each dilution set was then analyzed in duplicate. One set of each inhibitor dilution series was tested using the newly optimized integrated Quantifiler™ Trio-HRM assay and the other was tested using the standard Quantifiler™ Trio assay, as described above. Control 2800M DNA at 0.1ng/μl, with no inhibitor spike, was also analyzed in duplicate and served as the control. IPC Ct values from each sample tested were compared to the average IPC Ct values obtained from the corresponding DNA standards; those that were "undetermined" or more than two Ct units from this value were flagged for severe PCR inhibition (15).

Single source vs mixture prediction accuracy testing

Available DNA samples were split into training and validation sample sets and subsequently tested using the newly optimized integrated Quantifiler™ Trio-HRM assay and the QuantStudio™ 6 Flex qPCR system. The training set was comprised of 74 single source DNA samples with D5S818 genotypes of interest (see above) and 70 single source DNA samples with D18S51 genotypes of interest (see above); together, this included 114 unique single source samples when overlap was accounted for. Additionally, 16 1:1 two-person mixtures were included in the training set. The validation set was comprised of 56 single-source samples, each having genotypes of interest at both loci, as well as 16 different 1:1 two-person mixtures. For each sample, the negative derivative melt data was exported from the QuantStudio™ 6 Flex software, organized by STR locus, converted to a CSV file, and then imported into R-statistical software for the analysis by three different prediction modeling tools, including linear discriminate analysis (LDA), support vector machine (SVM) linear, and SVM radial as described above for the integrated Quantiplex®-HRM assay.

Reproducibility Testing

In order to determine if samples were consistently providing the sample predictions using the integrated Quantifiler™ Trio-HRM assay, three sets of 10 single source and 17 two-person 1:1 mixtures were analyzed as described above. Two sets of samples were run on the same qPCR plate while the third set was run on a different plate on a different day.

To determine if variation between runs could be attributed to Quantifiler™ Trio lot number, the entire training set (described above) was retested using a new lot of Quantifiler™ Trio and the integrated Quantifiler™ Trio-HRM assay. This data was used to generate new splines for analysis. All validation samples were retested using the new lot of Quantifiler™ Trio and the integrated Quantifiler™ Trio-HRM assay and all three machine learning models were assessed. Prediction accuracies were evaluated for each lot using the best performing models for each STR locus.

9

Generation of an expanded reference dataset & user interface development

Sample allocation for the creation of 10 different training and validation sets was completed. The 10-fold cross validation HRM code has been run successfully on the D5 locus for the reference dataset. Due to improvements in the measurement accuracy of the derivatives of the melt curve, revisions were necessary for the computer code. The higher accuracy measurements produced samples that are on a different sampling schedule across the temperature spectrum. To standardize the samples to a common sampling schedule, a quadratic spline model was fit to the integrated Quantiplex®-HRM assay observed data using basis functions on the desired sampling schedule of every 0.15°C beginning at 77.5°C to 95°C, and the standardized data was obtained by interpolating the melt curve values on this schedule. Each melt curve was then scaled so that the maximum value is 1 and the minimum value is 0 in order to be able to ensure to eliminate any issues with magnitude of the curves influencing the classifier. Each melt curve was then fit using a quadratic spline with 112 knots to obtain the features of the melt curve while reducing the size of the problem from 1119 values per curve to 112 values. The spline coefficients for each curve serve as the set of variables for input into the classification algorithms and was used to explore feature patterns across genotypes. To generate synthetic data the spline coefficients the spline models were utilized in the following manner. For each of the genotypes the mean and variance covariance matrix of the spline coefficients was obtained. Using this mean and variance random draw from a multivariate normal distribution is obtained which will be the spline coefficients for the synthetic observation. This set of spline coefficients was applied to the basis functions to create a new synthetic melt curve for the given genotype.

To determine if samples with genotypes not observed in the training set could be accurately predicted using this assay, 20 single source and 20 new 1:1 mixtures were evaluated using the integrated Quantifiler™ Trio-HRM assay, as described above.

While most of the progress to-date has focused on the data modeling, several web interfaces have been designed to function with the integrated assay. Each interface is being tested to identify the best approach for ease-of-interaction and melt data upload.

Results and Discussion

Initial evaluation of prediction models for STR genotype determination

In order determine what prediction modeling algorithms were best suited for STR genotype prediction, samples were tested using D5S818 and D18S51 singleplex amplification and melting on three qPCR platforms (ABI 7500, Rotor-Gene® Q, and the QuantStudio™ 6 Flex). Resulting melt data from all three qPCR platforms were analyzed using three machine learning tools: LDA, SVM Linear, and SVM Radial. For melt data generated on the ABI 7500, SVM Linear and SVM Radial performed best for the D5S818 data and D18S51 data, respectively. This trend was also observed for those samples tested on the QuantStudio™ 6 Flex. However, for samples tested on the Rotor-Gene® Q, SVM Radial performed best for predicting single source genotypes using the D5S818 melt data, whereas SVM Linear outperformed the other methods for single source genotyping using the D18S51 data (data not shown).

Integration and functional testing of the Quantiplex®-HRM assay

To evaluate the success of the melt curve assay within the qPCR-based quantification step of the forensic DNA workflow, the D5S818 and D18S51 primers and EvaGreen® dye were integrated into the Investigator™ Quantiplex® kit and evaluated on the. Initially, standard samples were tested on the Rotor-Gene® Q platform and melt curves qualitatively evaluated to ensure that alterations to the reaction and amplification conditions did not affect the subsequent melt curves produced. Melt curves obtained were compared to those produced from the initial evaluation studies (described above). Samples amplified using

10

the integrated Quantiplex®-HRM assay produced curves which were indistinguishable from those developed previously using singleplex amplification and melt of the D5S818 and D18S51 amplicons (**Figure 2**). Further, there were no significant differences in primary peak melt temperature when the samples amplified using the integrated assay were compared to those obtained using the singleplex amplification and melt of D5S818 and D18S51 (**Figure 3**, p=0.8496 and 0.1895 for D5S818 and D18S51, respectively). Similar observations were noted when melt curves were examined using the same testing conditions on the QuantStudio™ 6 Flex qPCR platform. Together, these data demonstrate that the Quantiplex® chemistry does not alter the melt curves produced or contribute any additional melt products to the integrated assay.

In addition to studying the effects of the altered Quantiplex® reaction on the melt curves themselves, it was important to determine if the altered reaction conditions would impede the accuracy of the quantification reaction. When tested on the Rotor-Gene® Q platform, R^2 values and Y intercepts were unaffected by these alterations and consistently fell within the manufacturer's expected values (data not shown). However, the slope was slightly higher (-2.55) than the expected range (-3.0 to -3.6) (**Figure 4**). While this was unexpected, it was not expected to be problematic as the approximate 2 cycle change to the standard sample Ct values is observed consistently across all standards in the curve and thus, isn't expected to alter resulting quantification values. In order to determine if this was true, quantification values from samples tested using the integrated Quantiplex®-HRM assay were compared to those obtained when the same samples were tested using the standard Quantiplex® chemistry and reaction (**Table 2**). The values obtained using the integrated Quantiplex®-HRM assay were not significantly different from those obtained using the standard Quantiplex® chemistry and reaction (p= 0.7685, data not shown). Further, these values more similar to the expected values than were those obtained from duplicates run across multiple plates using the standard Quantiplex® chemistry and reaction (inter-run variation). Similar observations were noted when melt curves were examined using the same testing conditions on the QuantStudio™ 6 Flex qPCR platform. Taken together, these data indicated that human DNA quantification accuracies are not impacted by the changes to the reaction and thermalcycling parameters.

Single source vs. mixture prediction using the integrated Quantiplex®-HRM assay

The new integrated Quantiplex®-HRM assay was tested to determine its ability to accurately distinguish single source from mixture samples (containing DNA from two contributors). The best prediction models for each STR locus tested had been previously determined (detailed above). Thus, all further calculations of prediction accuracy used the confusion matrices from these algorithms. As the HRM assay incorporates two loci as a way to increase the power of discrimination in its predictions, a combined accuracy metric was used for overall prediction accuracies. With this approach, if a sample were to be inaccurately classified as single source at one locus and a mixture at the second locus, assuming the sample is single source may lead to combining of sample extracts and creation of artificial mixtures; thus, a this interpretation is a more conservative approach. With both loci considered, 87.5% of single source samples and 100% of mixtures were correctly classified as such when using the integrated Quantiplex®-HRM assay on the Rotor-Gene® Q, producing an overall accuracy of 89.39% (**Table 3**) in which 59 of the 66 samples tested were correctly classified. When the assay was tested in combination with the QuantStudio™ 6 Flex qPCR platform, 87.5% of the single source samples and 90% of the mixtures were correctly classified as such when using the same assay, producing an overall accuracy of 87.88% (**Table 3**) in which 58 of the 66 samples tested were correctly classified.

Testing of the final, optimized Integrated Quantifiler™ Trio-HRM assay reaction

<u>Inter and Intra-Run Variation</u>

Samples analyzed over two different runs were evaluated to determine the inter-run variation observed with the final, optimized integrated Quantifiler™ Trio-HRM reaction and data analysis settings. The observed values across all quantification targets from the integrated Quantifiler™ Trio-HRM assay

11

were less variable than those obtained when the same samples were tested across runs using the standard Quantifiler™ Trio chemistry (**Table 4**). A similar trend was noted for the intra-run variation, with the integrated Quantifiler™ Trio-HRM assay exhibiting less variability in quantification values across samples analyzed in duplicate on the same run (data not shown).

Quantification precision

The quantification values obtained from samples analyzed using the integrated Quantifiler™ Trio-HRM assay were compared to those obtained when the standard Quantifiler™ Trio assay was utilized. The large autosomal target and Y target quantification values were, on average, slightly higher than the normal variation in quantification values across runs when the standard Quantifiler™ Trio chemistry is used (**Table 5**). These differences are expected to have minimal practical impact when used in forensic casework. Alternately, the small autosomal quantification values produced by the integrated assay were, on average, within the normal range of variation seen when the same samples were analyzed using the standard Quantifiler™ Trio chemistry over two runs (8.17% vs. 9.11%, respectively, **Table 5**). To further evaluate the DNA profiles generated after PCR amplification, samples were diluted according to quantification values obtained from the standard Quantifiler™ Trio reaction as well as those obtained from the newly optimized integrated Quantifiler™ Trio-HRM assay. Template DNA from each dilution was amplified using the Promega® PowerPlex® Fusion 5C kit and STR profile quality was compared. All samples, regardless of quantification value used, produced 100% of the expected STR alleles above the analytical threshold and STR profiles were 100% concordant across sets (data not shown).

Degradation Index and Male: Female Ratio

Degradation assessments revealed that mean DI values were consistent and less than one, as expected, when DNA samples were tested with the Quantifiler™ Trio reaction and the newly optimized integrated Quantifiler™ Trio-HRM assay (**Table 6**). This data suggests that the modifications made to the kit chemistry and data settings did not alter the ability of this assay to detect non-degraded DNA. In our studies, both the standard Quantifiler™ Trio reaction and the integrated Quantifiler™ Trio HRM assay produced M:F ratios greater than 10:1 (17:1 and 26:1, respectively) when single source male samples were tested (**Table 6**).

IPC Inhibition Assessment

For humic acid, IPC flags were triggered for all concentrations of humic acid tested using the standard Quantifiler™ Trio assay and the integrated Quantifiler™ Trio-HRM assay (**Table 7**). For hematin, IPC flags were observed with all concentrations of hematin tested using the standard Quantifiler™ Trio assay. However, only the four highest concentrations of hematin (500µM, 750µM, 1000µM, and 1250µM) prompted IPC flag for severe PCR inhibition when using the integrated Quantifiler™ Trio-HRM assay (**Table 7**). Although, the IPC flag would not have been triggered, the IPC Ct values produced by each integrated Quantifiler™ Trio-HRM assay 200uM replicate consistently neared the IPC Ct cutoff mark. Overall, the alterations to the Quantifiler™ Trio assay did not appreciably alter the ability of the chemistry to detect inhibition from common forensic inhibitors, though this seems to vary somewhat with specific inhibitors.

Single Source vs. Mixture Prediction Accuracy Testing

The new integrated Quantifiler™ Trio-HRM assay was tested to determine its ability to accurately genotype single source samples and to distinguish single source from mixture samples (containing DNA from two contributors). Three different prediction algorithms were initially tested using the exported HRM data however, for the D5S818 locus data, sample genotypes were more accurately predicted using the SVM radial algorithm while the SVM linear method was most accurate for D18S51 (data not shown). Thus, all further calculations of prediction accuracy used the confusion matrices from these algorithms. Using only the D5S818 data, 94.64% of single source validation samples and 25% of mixtures were accurately predicted as such. Alternately, using the D18S51 HRM data alone, 92.86% of single-source samples and

12

31.25% of mixtures were accurately predicted as such (**Table 8**). However, as the HRM assay incorporates two loci as a way to increase the power of discrimination in its predictions, a combined accuracy metric was used for overall prediction accuracies. With both loci considered, 89.29% of single source samples and 43.75% of mixtures were correctly classified as such producing an overall accuracy of 79.2% (**Table 8**) in which 57 of the 72 samples tested were correctly classified.

Reproducibility Testing

In order to determine if samples were consistently providing the sample predictions using the integrated Quantifiler™ Trio-HRM assay, three sets of samples were run on the same qPCR plate while the third set was run on a different plate on a different day. Samples tested in duplicate on the same run provided similar prediction accuracies (71% overall); however, samples tested in duplicate over two separate runs were less likely to result in the same predictions (**Table 9**). In order to determine if variation between runs could be attributed to Quantifiler™ Trio lot number, the entire training set (described above) was retested using a new lot of Quantifiler™ Trio and the integrated Quantifiler™ Trio-HRM assay. When samples were compared to a training set that had been tested using the same kit, prediction accuracies increased to 83.3% and 79.2% for two different kit lots (**Table 10**). This data suggests that training standards may need to be rerun for each new kit lot obtained, along with development of new splines and determination of best prediction model to use. In an attempt to avoid this, we are currently working to combine training set data from all lots tested to generate consensus splines that could be used for classification of all unknowns, regardless of kit lot.

Generation of an expanded reference dataset & user interface development

A synthetic training set was created with help of real training data from the integrated Quantiplex®-HRM assay and polynomial regression splines and interpolation in order to determine the behavior of melt curves from samples with genotypes not represented in the training set. The synthetic data generated was incorporated into the existing training set (based on real data). When this hybrid training dataset was used to predict the genotypes of the same samples (as validation), prediction accuracy was 100%. Additionally, when a fully synthetic training set was created and used to test the same synthetic sample set, the prediction value was near 100%. Further, when the fully synthetic train dataset was used to predict the original validation sample set (using real data), the model was able to accurately predict if a sample was a single source or mixture sample in 93.7% of samples tested. However, when the fully synthetic training dataset was used to predict the nature of real samples who had genotypes the model had not seen before, prediction accuracies dropped to <20%. Therefore, different mathematical equations are being explored in an effort to create more realistic synthetic data. However, given the discontinuation of the Quantiplex® kit all synthetic data generation using this kit will cease and continue with the Quantifiler™ Trio kit.

Prior to the development of an expanded training dataset for the integrated Quantifiler™ Trio-HRM assay, a study was completed to evaluate the capability of the existing model (without expansion) to accurately predict a sample DNA as single source or mixture. Using only the D5S818 data, 80% of single source validation samples and 30% of mixtures were accurately predicted as such. Alternately, using the D18S51 HRM data alone, 100% of single-source samples and 20% of mixtures were accurately predicted as such (**Table 11**). With both loci considered, 80% of single source samples and 45% of mixtures were correctly classified as such producing an overall accuracy of 62.5% (**Table 11**). This data suggests that expansion of the training dataset may not be needed to achieve high prediction accuracies using this model. Thus, additional samples with non-observed genotypes will be added to the validation set and tested to see if this trend holds.

To-date, only modest work has been completed on the web interface for this prediction tool, however, several basic interface designs have been developed. Basic interface designs will be tested to determine a best path forward based on user ease-of-use and ability to upload melt curve data. The R Shiny application can be used to do this which would be advantageous as the modeling aspect is also in R.

However, if this platform does not seem to be robust to large scale use (after testing), a PHP database approach may be needed to upload and store data and an automation script may be needed to link to R for the analysis.

Applicability to Criminal Justice

The completion of the project moves this mixture vs. single source qPCR tool a step closer to being released to our partner labs for testing. Ultimately, we aim to incorporate the expanded training set into a single web-based tool to facilitate broader access of the melt curve database and to provide an easy-to-use, free, on-line tool for quick assessment of qPCR melt curve data. Subsequently, when this integrated qPCR-HRM tool is implemented in the forensic field it will provide forensic examiners with a powerful way to assess all types of evidence items containing biological material as either a single source or mixture sample without the need for additional steps in the workflow and minimal additional costs. The knowledge of the mixture status of a forensic sample is beneficial as it allows for procedural modifications that could further elucidate the major and minor contributor/s within a sample, to be made. Additionally, the developed tool may also provide genotype prediction information for identified single source samples. With early genotyping information from two STR loci, this assay could provide early exclusionary data – possibly preventing the need for additional labor-intensive investigations, saving investigative time. This approach results in only a negligible increase in reaction setup time and reaction costs, while providing key sample information for the bench scientist and investigative information for the investigating officers much more quickly. This assay will contribute to saving time and resources, which are limiting factors in forensic labs, that are consumed in the lengthy touch DNA and mixture detection, analysis and reanalysis process. Additionally, since this tool gives information into the probative, and potentially exclusionary, nature of a sample, it may also help triage which samples to move forward for STR analysis thus minimizing additional downstream work.

Excitingly, this work could also be useful for applications well beyond forensic science. In a broader sense, the method used to generate the synthetic melt curves could be used in any application that uses HRM, such as mutation scanning, population studies, pathogen detection, and species identification. The algorithms implemented in the proposed project will be a useful guide for the development and optimization of similar genetic tools for these biomedical applications. Beyond the biological sciences this information gleaned herein could even be used for any situation where the measurable outcome is functional data such as tracking an object through an image, animal tracking via sensors, human sleep patterns, and human activity patterns.

Products

Scholarly Products:
1. Formal protocol: Integrated Quantiplex®-HRM assay using Qiagen Rotor-Gene® Q qPCR platform (2019).
2. Formal protocol: Integrated Quantiplex®-HRM assay using ThermoFisher QuantStudio™ 6 Flex qPCR platform (2021).
3. Formal protocol: Integrated Quantifiler Trio™-HRM assay using ThermoFisher QuantStudio™ 6 Flex qPCR platform (2022).
4. A. L. Williams, "Expanded Testing of an Integrated qPCR Mixture Screening Assay Using

High Resolution Melt Curve Analysis and Support Vector Machine Modeling," Virginia Commonwealth University [Thesis], Richmond, Virginia, 2020.

5. D. A. Torres, "A Quantifiler™ Trio-based HRM Mixture Screening Assay for the QuantStudio™ 6 Flex qPCR platform," Virginia Commonwealth University [Thesis], Richmond, Virginia, 2022.

6. C. Smith, "Redesign, Evaluation, and Validation of a Commercially Viable High-Resolution Melt Based Mixture Screening Tool [Dissertation Proposal]," Virginia Commonwealth University, Richmond, Virginia, 2022.

7. Cloudy DC, Smith CJ, Boone EL, Kuehnert K, Cox JO, Williams SJS, Dawson Green T. Statistical Methods for Discrimination of STR Genotypes Using High Resolution Melt Curve Data. Int J Legal Med [*in preparation*].

8. Smith CJ, Williams AL, Wines HE, Cox JO, Williams SJS, Boone EL, Dawson Green T. Integration of a high-resolution melt curve assay into a commercial quantification kit for preliminary mixture detection. Int J Legal Med [*in preparation*].

9. Torres D, Smith CJ, Williams AL, Cox JO, Williams SJS, Boone EL, Dawson Green T. A Quantifiler™ Trio-based HRM screening assay for the accurate prediction of single source versus mixed biological samples. Int J Legal Med [*in preparation*].

Other Dissemination Activites:

1. "Towards Commercialization: Preliminary developmental validation of a high resolution melt curve mixture prediction assay and SVM tool". February 2023. Dawson Green T (presenter). 2023 NIJ Symposium (Oral Presentation).

2. "Sample Analysis using Prediction Modeling for Early Mixture Detection and Expansion of a Reference Training Set". November 2022. Smith C (presenter), Torres D, Williams AL, Seashols-Williams SJ, Dawson Green T. 33rd International Symposium on Human Identification (ISHI) Washington, D.C. (Poster presentation)

3. "Helping Solve "Whodunnit": Determining the Number of Contributors in a Sample Earlier in the Forensic Workflow". Smith C (presenter), Williams AL, Wines HE, Cloudy DC, Boone EL, Seashols-Williams SJ, Dawson Green T. April 2022. 25th Annual Graduate Research Symposium, VCU, Richmond, VA (Poster presentation)

4. "Helping Solve "Whodunnit": Determining the Number of Contributors in a Sample Earlier in the Forensic Workflow". Smith C (presenter), Williams AL, Wines HE, Cloudy DC, Boone EL, Seashols-Williams SJ, Dawson Green T. April 2022. ILSSO Spring Research Showcase, VCU, Richmond, VA (Poster presentation)

5. "Integration of an HRM mixture-prescreening assay into the Quantifiler Trio™ quantification chemistry using the QuantStudio™ 6 Flex Real-Time PCR System". February 2022. Torres D (presenter), Smith C, Williams AL, Boone EL, Seashols-Williams SJ, Dawson Green T. 74th Annual AAFS Scientific Conference, Seattle, WA (Oral presentation)

6. "Proof-of-Concept Testing of the Quantiplex® -HRM Integrated Assay for Early Mixture Detection using Support Vector Machine Modeling for "Whole Curve" Analysis". February 2022. Smith C (presenter), Williams AL, Wines HE, Cloudy DC, Boone EL, Seashols-Williams SJ, Dawson Green T. 74th Annual AAFS Scientific Conference, Seattle, WA (Oral presentation)

7. "Helping Solve "Whodunnit": Determining the Number of Contributors in a Sample Earlier in the Forensic Workflow". Smith C (presenter), Williams AL, Wines HE, Cloudy DC,

Boone EL, Seashols-Williams SJ, Dawson Green T. VCU 2021 3 Minute Thesis (3MT) Final Round, VCU, Richmond, VA (Oral Presentation)

8. "High Resolution Melt Curve Analysis and Support Vector Machine Modeling for Identification of Two-Person Mixtures". Smith C (presenter), Williams AL, Wines HE, Cloudy DC, Boone EL, Seashols-Williams SJ, Dawson Green T. September 2021. MAAFS 2021 Annual Conference, Pocono Manor, PA (Oral presentation)
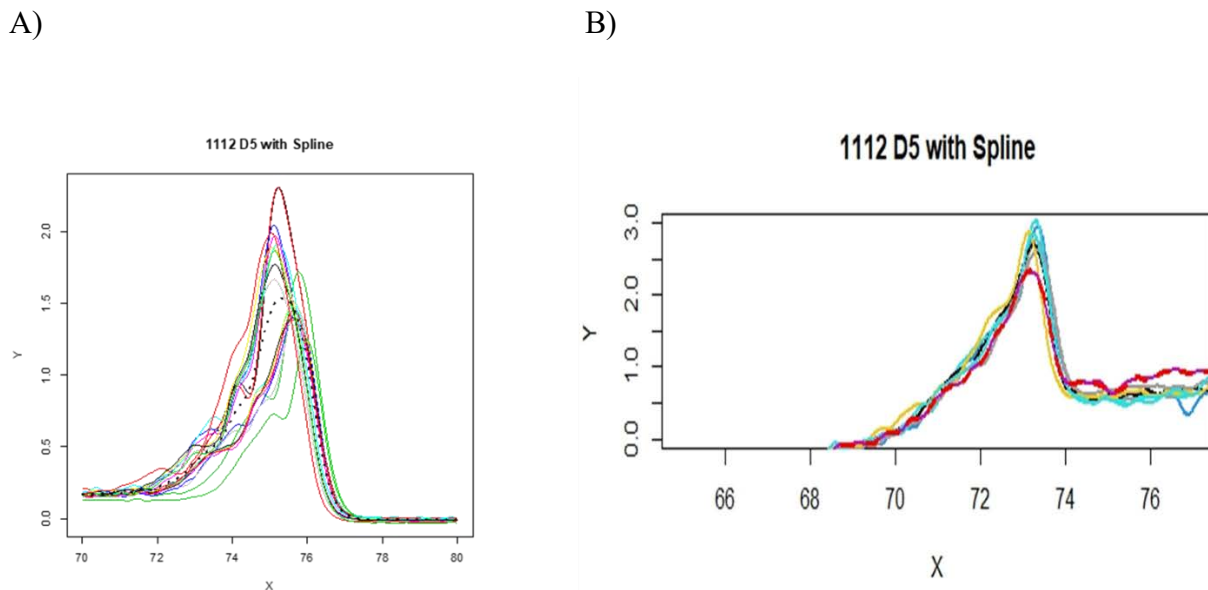
Tables & Figures:

A)

B)

Figure 1: Melt curves for D5S818 samples with 11,12 genotype using the Rotor-Gene$^{®}$ Q (A) and the QuantStudio™ 6 Flex (B). The dotted black line represents a fitted spline used for classification methods.

		Predicted Genotypes						
		(10,11)	(11,11)	(11,12)	(11,13)	(12,12)	(12,13)	(13,13)
Known Genotypes n= 185	(10,11)	2	1	1	5	0	2	0
	(11,11)	1	34	1	0	1	2	0
	(11,12)	1	0	35	3	10	0	0
	(11,13)	0	1	3	26	1	1	0
	(12,12)	0	2	4	1	7	1	0
	(12,13)	0	9	7	4	3	5	2
	(13,13)	0	5	0	2	1	1	0
	Accuracy Rate: 58.92%							

Table 1: Example of confusion matrix generated from R statistical software package.
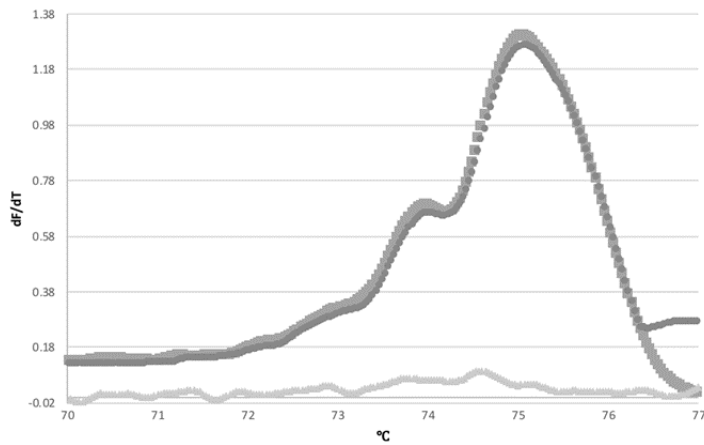
16

Figure 2. D5S818 melt curve for a single sample using two different amplification/melt parameters on the Rotor-Gene Q®. dF/dT represents change in fluorescence level (positive or negative) with respect to per unit change (increase) in temperature. The singleplex amplification and melt of D5S818 produced a melt curve that is similar in fluorescence and overall curve morphology to that obtained using the integrated Quantiplex®-HRM assay.
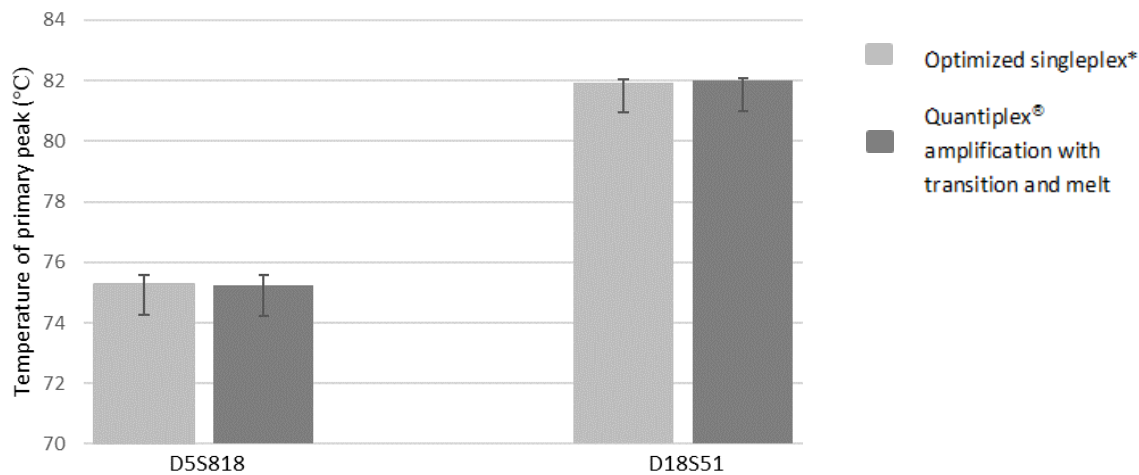

Figure 3. Primary peak melt temperature of two STR amplicons using two different amplification/melt parameters on the Rotor-Gene Q® (n=10). There are no significant temperature differences observed between values obtained using the integrated Quantiplex®-HRM assay and the simpler singleplex amplifications of the D5S818 and D18S51 loci (p=0.8496 and p=0.1895, respectively).
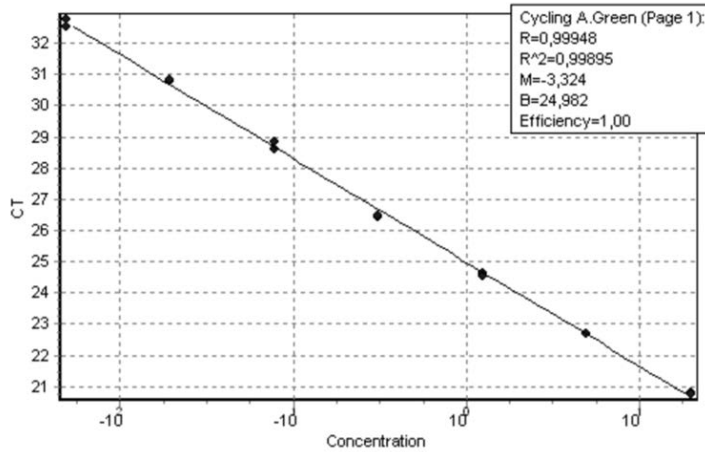
Figure 4. Representative integrated Quantiplex®-HRM assay standard curve and associated QC measures using the Rotor-Gene® Q. Slopes obtained using the altered chemistry and reaction conditions were slightly higher than the expected range (-3.0 to -3.6). However, the Ct difference was consistent across all standards in the curve, thus quantification is not expected to be impacted.

	Quantiplex® reaction and amplification*	Quantiplex® reaction with STR primers & amplification with transition & melt◊
Average Difference between runs	0.5371 ng/μl	0.7808 ng/μl
% Variation between runs	20.72%	17.74%

*based on multiple runs in Dawson-Cruz laboratory
◊ as compared to standard Quantiplex® and amplification run
n=16
p= 0.2148

Table 2. Quantiplex® hDNA quantification vs. integrated Quantiplex®-HRM hDNA quantification

18

	Rotor-Gene® Q	QuantStudio™ 6
Single-Source N=56	87.5%	87.5%
Mixtures N=10	100%	90%
Overall Accuracy	**89.39%**	**87.88%**

Table 3. Single source and mixture sample prediction accuracy using the integrated Quantiplex®-HRM assay.

% Inter-run Variation between runs	Large Autosomal (n=10)	Small Autosomal (n=10)	Y-Target (n=5)
Standard Quantifiler™ Trio Assay	9.8%	9.1%	8.0%
Integrated Quantifiler™ Trio-HRM Assay	5.7%	4.9%	7.0%

Table 4: Inter-run variation of the integrated Quantifiler ™ Trio-HRM assay vs the standard Quantifiler™ Trio chemistry.

Variation Comparison	Large Autosomal % Difference (n=10)	Small Autosomal % Difference (n=10)	Y-Target % Difference (n=5)
Standard Quantifiler™ Trio Assay Inter-run	9.76	9.11	7.99
Integrated Quantifiler™ Trio-HRM Assay vs Quantifiler™ Trio	9.84	8.17	9.68

Table 5: Quantification using the integrated Quantifiler ™ Trio-HRM assay versus the standard Quantifler ™ Trio chemistry

	Degradation Index n=10	Male: Female n=5
Standard Quantifiler™ Trio assay	0.9	17:1
Integrated Quantifiler™ Trio-HRM assay	0.96	26:1

Table 6: Average degradation index (DI) and male to female ratios of samples evaluated using the integrated Quantifiler ™ Trio-HRM assay and the standard Quantifiler ™ Trio chemistry.

Sample (Inhibitor Concentration)	IPC Flag	
	Standard Assay n=3	Integrated Assay n=3
Control 2800M 0.1ng/µl (0)	N	N
Humic Acid (200 ng/µl)	Y	Y
Humic Acid (300 ng/µl)	Y	Y
Humic Acid (400 ng/µl)	Y	Y
Humic Acid (600 ng/µl)	Y	Y
Humic Acid (800 ng/µl)	Y	Y
Hematin (200 µM)	Y	N
Hematin (500 µM)	Y	Y
Hematin (750 µM)	Y	Y
Hematin (1000 µM)	Y	Y
Hematin (1250 µM)	Y	Y

Table 7: Effects of two common inhibitors, humic acid and hematin, on the standard Quantifiler™ Trio and integrated Quantifiler™ Trio HRM assays. "Y" or "N" indicates whether the IPC flag would have been triggered indicating severe PCR inhibition.

	D5S818	D18S51	Combined Accuracy
Single Source (n=56)	94.64%	92.86%	89.29%
Mixtures (n=16)	25%	31.25%	43.75%
		Overall Accuracy:	79.20%

Table 8: Single source and mixture sample prediction accuracy using the integrated Quantifiler™ Trio HRM assay.

Classification	Single-Source vs Mixture **Inter-run** Reproducibility	% Reproducibility
SS	5 of 7	71%
Mix	5 of 10	50%
Combined	10 of 17	59%

Classification	Single-Source vs Mixture **Intra-run** Reproducibility	% Reproducibility
SS	5 of 7	71%
Mix	7 of 10	70%
Combined	12 of 17	71%

Table 9: Variation of the integrated Quantifiler™ Trio HRM assay with (inter-run) and between (intra-run) runs.

Kit Lot	D5 Best Model	D18 Best Model	Combined Single Source Accuracy	Combined Mixture Accuracy	Overall Accuracy
1	LDA	SVM Radial	89.3%	62.5%	83.3%
2	SVM Radial	SVM Linear	89.2%	43.8%	79.2%

Table 10: Prediction accuracies of the integrated Quantifiler™ Trio HRM assay using two different kit lots and best performing machine learning models.

	D5S818	D18S51	Combined Accuracy
Single Source (n=20)	80%	100%	80%
Mixtures (n=20)	30%	20%	45%
		Overall Accuracy:	62.5%

Table 11: Prediction accuracies of samples with non-observed genotypes using the integrated Quantifiler™ Trio HRM assay using

References

1) Kimpton CP, Gill P, Walton A, Urquhart A, Millican ES, Adams M. Automated DNA profiling employing multiplex amplification of short tandem repeat loci. PCR Methods Appl. 1993 Aug;3(1):13-22.
2) Hochmeister MN, Budowle B, Jung J, Borer UV, Comey CT, Dirnhofer R. PCR-based typing of DNA extracted from cigarette butts. Int J Legal Med. 1991;104(4):229-33.
3) Aditya S, Sharma AK, Bhattacharyya CN, Chaudhuri K. Generating STR profile from "Touch DNA". J Forensic Leg Med 2011;18:295-298.
4) Gill P. Application of low copy number DNA profiling. Croat Med J. 2001 Jun;42(3):229-32.
5) Butler JM. Forensic DNA Typing. Elsevier Academic Press: New York, 2005.
6) Bureau of Alcohol, Tobacco, Firearms, and Explosives. "National Response Team". US Department of Justice. Accessed September 10, 2012.
7) Balding DJ, Buckleton J. Interpreting low template DNA profiles. Forensic Sci Int Genet 2009;4:1-10.
8) Dawson Cruz T. qPCR Genotype Determination and Mixture Detection Using High Resolution Melting Curve Analysis of STR Loci [Internet]. 2019 [cited 2021 Sep 30]. Available from: https://www.ojp.gov/library/publications/qpcr-genotype-determination-and-mixture-detection-using-high-resolution
9) H. Wines. qPCR Genotype Determination and Mixture Detection Using High Resolution Melt Curve Analysis on the Applied Biosystems 7500 and Qiagen Rotor-Gene ® Q qPCR systems. MS thesis. Virginia Commonwealth University, 2018.
10) Cloudy, D. Discrimination of D5S818 and D18S51 STR genotypes using LDA & PCA evaluation of high resolution melt curve data from the Qiagen Rotor-Gene® Q qPCR Instrument. MS thesis. Virginia Commonwealth University, 2017.
11) J. A. Nicklas, T. Noreault-Conti, and E. Buel, "Development of a Fast, Simple Profiling Method for Sample Screening Using High Resolution Melting (HRM) of STRs," *J. Forensic Sci.*, vol. 57, pp. 478–488, 2012, doi: 10.1111/j.1556-4029.2011.01981.x.
12) Q. Nguyen, J. Mckinney, D. J. Johnson, K. A. Roberts, and W. R. Hardy, "STR Melting Curve Analysis as a Genetic Screening Tool for Crime Scene Samples*," J. Forensic Sci., vol. 57, no. 4, pp. 887–899, Jul. 2012, doi: 10.1111/j.1556-4029.2012.02106.x.
13) D. Cloudy, "Discrimination of D5S818 and D18S51 STR genotypes using LDA & PCA evaluation of high resolution melt curve data from the Qiagen Rotor-Gene® qPCR instrument," Virginia Commonwealth University, Richmond, Virginia, 2017.
14) James G, Witten D, Hastie T, Tibshirani R. An Introduction to Statistical Learning: with Applications in R. First. Casella G, Fienberg S, Olkin I, editors. New York: Springer; 2013. 1–367 p.
15) A. Holt, S. C. Wootton, J. J. Mulero, P. M. Brzoska, E. Langit, and R. L. Green, "Developmental validation of the Quantifiler® HP and Trio Kits for human DNA quantification in forensic samples," *Forensic Sci. Int. Genet.*, vol. 21, pp. 145–157, 2016, doi: 10.1016/j.fsigen.2015.12.007.