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A Quantifiler Trio-based HRM Mixture Screening Assay for the QuantStudio 6 Flex qPCR platform

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Abstract

At present, the forensic DNA workflow is not capable of providing information about the contributor status (single source vs. mixtures) of evidentiary samples prior to end-point analysis. This shortcoming can exacerbate the challenges inherent to mixtures and low-template DNA samples. Provided additional sample information earlier in the workflow, protocols could be implemented to mitigate these challenges. High-resolution melt (HRM) curve analysis is a technique used to detect genetic variation in DNA fragments and in the last decade has been evaluated for use in differentiation of samples by genotype and/or contributor status. To this end, a proof-of-concept HRM assay using the STR loci D5S818 and D18S51 and EvaGreen® intercalating dye was integrated into the Qiagen Investigator Quantiplex® qPCR kit. When tested on the ABI QuantStudio™ 6 Flex qPCR platform, resulting melt curve datasets and statistical analyses were capable of distinguishing single-source samples from mixtures at an 87.88% rate. Given this initial success, integration of the HRM assay into a more commonly used chemistry, the Quantifiler™ Trio kit, was pursued. Unfortunately, the presence of EvaGreen® dye caused substantial increase in small autosomal and Y-target quantification values, rendering this data unreliable. SYTO™ 17 and SYTO™ 64 fluorescent intercalating dyes with spectral emissions in the IPC target channel were tested in the assay to minimize spectral overlap with quantification target dye channels. However, dye channel sharing of the SYTO™ dyes with the IPC target dye, JUN™, resulted in a change in the expected cycle threshold values. Additionally, testing with SYTO™ 17 revealed inflation of the passive reference dye Mustang Purple™ and was not pursued further.

Integration of the HRM assay into the Quantifiler™ Trio was tested with two different reaction condition chemistries. After optimization of reaction condition, additional testing was conducted with adjustments in the assays data analysis settings. The standard Quantifiler™ Trio assays inter-run variation was compared to the percent differences of quantification values obtained from the standard Quantifiler™ Trio and integrated Quantifiler™ Trio HRM assays. Overall, percent differences between the assays were comparable to one another. Further, DNA profiles generated with the standard Quantifiler™ Trio and integrated Quantifiler™ Trio HRM assays were evaluated and revealed fully concordant profiles that were not statistically different from one another. Moreover, both assays reported similar male-to-female ratios and degradation indices. Lastly, an inhibition study conducted to discern whether dye channel sharing with the IPC target dye influenced its ability to detect inhibition was conducted and revealed no drastic difference in inhibition detection between the assays.

For the classification of single source vs. mixture samples in the optimized Quantifiler™ Trio HRM assay, the best performing prediction model was used for D5S818 (SVM linear) and D18S51 (SVM radial) loci. D5S818 reported a single source prediction accuracy of 86.76% and a mixture prediction accuracy of 25%. D18S51 reported a single source prediction accuracy of 79.41% and mixture prediction accuracy of 62.5%. The overall prediction accuracy of the assay using a combined metric was 73.8%.

Key words: Forensic science, qPCR, High resolution melt curves (HRM), Support vector machine modeling (SVM), Investigator Quantiplex® kit, Quantifiler™ Trio kit, DNA analysis

Introduction

Statement of the Problem

As a result of the improved sensitivity of short tandem repeat (STR) profiling, an increasing number of low-template DNA samples are being submitted to crime laboratories for testing. Standard inputs of DNA for STR amplification range between 0.5 and 1ng, whereas low-template (i.e., touch DNA) samples are defined as having available DNA template levels below 0.1ng (1–3). When assessing low-template DNA profiles, there are two primary challenges: stochastic effects are more pronounced and mixture profile interpretation is convoluted. Together, these associated challenges add an immense obstacle for forensic DNA examination expressly because the associated challenges are not typically identified until the end of the workflow process.

Stochastic Effects and Mixture DNA Profiles

Stochastic effects in low-template DNA samples are more pronounced due to the intermittent success of primer binding and amplification of DNA fragments during PCR, resulting in amplification peak variability. This is a major challenge as profiles significantly impacted by stochastic effects cannot undergo statistical analysis as it is difficult to distinguish true allele peaks from artifacts, which may lead to inconclusive results (4). Strategies designed to mitigate the frequency and impact of stochastic effects include modifications to the traditional forensic DNA workflow. For example, PCR amplification cycles may be increased from 28 to 34 (2), post-PCR purification techniques are conducted to increase signal, and adjustments to capillary electrophoresis (CE) settings are utilized in an effort to obtain more complete profiles (1,2). While these protocol modifications are established to aid in the resolution of low-template DNA samples, many samples still result in incomplete profiles that are not revealed until the endpoint of analysis leading to CODIS ineligible profiles that are often not accepted in court.

Additional challenges with low-template DNA profile interpretation arise when more than one contributor is present in a sample, known as a 'mixture.' Mixtures rarely contain equal parts of contributor(s) DNA, often producing a profile with a major and minor contributor(s). As a result of the unequal DNA amounts between contributors, alleles from a minor contributor may be below the analytical threshold and thus are indistinguishable from background noise, further complicating interpretation for analysts. To aid in mixture interpretation, several groups have issued mixture interpretation guidelines (5–7). However, even with established guidelines, mixture deconvolution is fallible to subjectivity. In one study, 17 DNA analysts were tasked with examining an evidence profile and were provided reference DNA profiles of the victim and three suspects. Although suspect three was the known contributor, only one examiner correctly determined suspect three could not be excluded as a contributor (8). Further, it is widely accepted that profiles containing 3 to 4 alleles at more than one locus are at least a two-person mixture, 5 to 6 alleles at least a three-person mixture, and 7 to 8 alleles at least a four-person mixture (4). Based on these assumptions, an empirical analysis of conceptual mixtures revealed that of out of 57,211,376 possible four-person mixtures, derived from 194 13-STR Caucasian profiles, 76.34% were mischaracterized as two- or three- person (9). For these reasons, mixtures add a layer of complexity that can make profile interpretation extremely challenging and potentially subjective.

A laboratory's standard operating procedures or protocols may further exacerbate these obstacles in the interest of sample preservation. For instance, laboratory policy may prevent an analyst from consuming the entire sample DNA yield for initial analysis, even if the sample is known to be low-template. As described above, amplification of low-template samples often leads to inconclusive results. Similarly, policy may prohibit combining multiple touch swabs

collected from a single item of evidence as it could artificially generate mixture profiles. Consequently, it can instead result in multiple incomplete, low, or absent single-source profiles (10). Conversely, if a sample is identified as a mixture prior to DNA typing, a protocol change could dictate that more DNA be added to the initial amplification, and potentially result in minor contributor(s) peak heights that are more easily distinguished from background noise.

Path Forward and Existing Research

An assay capable of discerning single-source samples (vs. those containing mixtures), prior to the final step of profile interpretation, would be advantageous by providing analysts more information earlier on in the workflow process. Discerning evidence samples earlier in the forensic DNA workflow based on contributor status may be possible using high-resolution melt (HRM) curve analysis, which is typically achieved using a quantitative PCR instrument (11,12). Quantitative polymerase chain reaction (qPCR) methodology and instrumentation is common in forensic laboratories. Its use occurs early in the forensic workflow and is typically completed for the purposes of quantification of human DNA present in a sample.

At present, forensic laboratories do not routinely make use of the innate qPCR HRM capability and instead only use qPCR instrumentation for quantitative assessment of the concentration of genomic DNA present in an evidentiary sample. By design, however, the multiplex capabilities of qPCR instruments allow for more than one PCR target to be detected and quantified simultaneously (13). For example, qPCR can also provide a qualitative assessment of DNA degradation and detect the presence of some PCR inhibitors while simultaneously measuring the amount of human DNA present by adding targets that are detected with dyes that fluoresce in different channels (14–16). Likewise, HRM analysis could be pursued using a dye whose emission spectra falls within an open fluorescent channel. With HRM

analysis, DNA is detected by means of an intercalating dye that binds and fluoresces solely in double-stranded DNA (dsDNA). Fluorescence is diminished as the dsDNA amplicons denature during HRM and the temperature at which 50% of the fluorescence is no longer detected by the qPCR instrument is defined as its melting temperature (T_m). The T_m of DNA varies with its length and its specific nucleotide sequence, thus, HRM analysis can potentially be used to characterize differences in DNA amplicons based on their melt curve morphologies and subsequent statistical analysis (17).

As qPCR instruments with HRM analysis capabilities became available to forensic laboratories (early 2010s), researchers began developing HRM assays to test the ability of this approach for genotyping human identification targets (11,12,18). Initially, some groups began their work by targeting single-nucleotide polymorphism (SNP) and mini-STR loci (12,18) because their small amplicon sizes lends itself to more efficient amplification of low-template DNA samples. In 2012, Nguyen et al. used amplification and HRM analysis of mini-STR targets to evaluate the uniqueness of melt curves from various genotypes (12). Initially, HRM data was interpreted using Lightscanner®-96 HRM software (BioFire Diagnostics; Salt Lake City, UT), which groups morphologically similar melt curves using an "unbiased hierarchical clustering scheme." Eight CSF1PO and 10 TH01 genotypes were tested, and eight distinct melt curve morphologies were identified for each locus. A blind study revealed that samples with identical genotypes were correctly grouped together through the genotype-grouping algorithm used by the software. While these results seemed promising, the technology and classification algorithms used by the accompanying software were limited to grouping curve morphologies rather than calling individual sample genotypes or identifying mixtures.

More recently, Venables et al. 2014 assessed the ability of a commercial HRM kit, the MeltDoctor™ HRM Mastermix (ThermoFisher™; Waltham, Massachusetts), to accurately genotype SNP human identification targets based on homozygous or heterozygous classifications at individual target loci. In this study, one SNP site (rs733559) was targeted across 11 DNA samples and two SNP sites (rs310850 and rs3892905) were targeted in the positive control (9947A). Amplification and HRM analysis were conducted on a ViiA™ 7 Real-Time PCR system (ThermoFisher). Results of the HRM assay indicated 100% concordance in SNP genotype classification for all three SNPs across all samples and controls (18). However, the assay was limited in part by the low power of discriminations of individual SNP loci and its inability to target multiple SNPs in one run due to its restricted melt temperature range. This resulted in significant depletion of the DNA samples (7ng), which would be disadvantageous in forensics, especially in cases with touch or low-template DNA evidence.

While work using mini-STRs and SNPs were important for proof-of-concept, a HRM screening method using traditional STR amplification may be more beneficial due to their higher power of discrimination, the wide scale availability of STR-typing databases, and the popularity of standardized STR primer sets. In a 2012 study by Nicklas et al., HRM analysis of 22 STR loci was conducted on the Rotor-Gene® Q (QIAGEN: Germantown, MD), after amplification (11). Initially, HRM data was interpreted using Rotor-Gene® ScreenClust HRM® Software (QIAGEN). However, the software frequently failed to make genotype calls for unknown samples, thus the group transitioned to a simpler qualitative inspection. Of the 22 STRs analyzed, three loci were found to produce distinct melt curves that could be used for accurate genotype grouping (19). Although only three of the 22 STR loci were found to produce decisive melt curve patterns, the authors concluded that, when used together as a triplex, the power of discrimination would be

sufficient to act as a screening tool for exclusionary information. In the same study, a two-person mixture sample at varying major:minor ratios were tested, and the researchers found that at a 1:1 ratio, the melt curves were distinct from the melt curves of the single-source samples used to create the mixture. However, as the ratios of each contributor's DNA varied more (i.e., 3:1), melt curves returned to a morphology that was more like the single source melt curve generated from the major contributor (11,19). Despite the studies promising preliminary evidence for the use of HRM to identify mixtures and genotype STR loci, there was subjectivity in the qualitative visual inspection of melt curves and limitations in the small number of genotypes sets/mixture samples tested.

While these early papers provide solid preliminary support for the use of a HRM assay in the forensic workflow, several additional considerations must be addressed in order for an assay to be viable in the forensic community. Key points of consideration include its ease of integration into the existing workflow without the addition of labor, time, or expense. This would be most easily achieved if a HRM assay was incorporated into the existing qPCR-based human quantification step. For this, it would be important to assure that the two assays are able to function independently of one another. The overall integration of an HRM assay into qPCR requires additional thermal parameters, consisting of a transition and melt stage, (20–22) and additional reagents including an intercalating dye and locus specific primers (11,12). These modifications are relatively simple to execute, but their effects are unknown and must be carefully evaluated before wide-scale adoption in the forensic community.

Proposed Solution and Preliminary Data

Previous research in the Dawson Green laboratory has shown that HRM analysis can be conducted successfully when integrated into an existing commercially available qPCR human

DNA quantification kit (22). D5S818 and D18S51 STR loci were selected as targets for the integrated HRM assay, based in part on the previously reported work by Nicklas et al. (11), and EvaGreen® (Biotium, Fremont, CA) was selected as the interrelating dye. Using the published primer sets from the PowerPlex® 16 System for each locus, D5S818 produces smaller amplicon lengths, ranging from 115 to 178 base pairs (bp) while D18S51 produces larger amplicons, between 262 and 342 bp (23). Due to differences in amplicon length, the two loci produce distinct melting ranges with no overlap between their melt curves. Further, while D5S818 is less discriminatory with fewer known alleles, its small size lends itself to an easier amplification in challenged samples. Alternatively, the D18S51 locus is larger but has more known alleles making its power of discrimination higher. A standalone singleplex HRM assay, with optimized melt stage settings, identified three and eight visually distinct and reproducible melt curves for D5S818 and D18S51, respectively (11,21,26). Given these promising results, integration of the assay into a commercially available human DNA quantification qPCR kit was pursued using the Investigator Quantiplex® kit (QIAGEN). Initially, the integrated Investigator Quantiplex® HRM assay was tested on the Rotor-Gene® Q qPCR platform (QIAGEN) (22,23,26–28). However, this assay was later transitioned to the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems: Foster City, CA), a more modern, emerging qPCR platform utilized within forensic community that also offers an HRM extension (20,22,27).

The Investigator Quantiplex® kit quantifies the total amount of amplifiable human DNA in a sample. Targets are detected for polymerization using Scorpion® primers that are covalently linked to their own probe, reporter, and quencher. The Quantiplex® kit has three targets and corresponding dyes: total DNA (FAM™), internal PCR control or IPC (VIC™), and the passive reference (ROX™) (14,29). In addition to the standard Investigator Quantiplex® reaction

components, Primer Mix IC FQ and Reaction Mix YQ, the integrated Quantiplex® HRM assay consisted of primers for the D5S818 and D18S51 STR loci and EvaGreen® intercalating dye. The modified, optimized Quantiplex® amplification protocols for the integrated Quantiplex® HRM assay included a hotstart activation of Taq polymerase at 95°C for 10 min followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. This was followed by a transition stage consisting of a final extension of 72°C for 2 min followed by 95°C for 20 sec, 55°C for 20 sec, and 56°C for 2 min. Finally, the proceeding melt stage was added with a 0.015°C/s ramp rate from 60°C to 95°C with 20 sec at the beginning and end temperature stages (22).

Prior to experimentation, quantification and melt data from the integrated Quantiplex® HRM assay was analyzed to ensure no interferences occurred with quantification accuracy and reproducibility of the assay. Further, the melt data was evaluated to determine whether the Quantiplex® assay targets produced melt products and if so whether they fell within the melt range of the added HRM STR targets. Standard curve R^2 values from the integrated Quantiplex® HRM assay on the Rotor-Gene® Q remained within the manufacturer's expected values (≥ 0.9926). Though the integrated HRM assays slope values were lower than the acceptable range (-2.5459 average versus the suggested range of -3.0 to -3.3) this was inconsequential as a value of -3.0 corresponds to ~100% PCR efficiency and values greater than this would demonstrate an increased efficiency (14,22,29). Further, the integrated Quantiplex® HRM assay exhibited lower inter-run variability than normally observed using the standard Quantiplex® assay protocols, averaging 17.74% variation across runs (versus 20.72% under normal conditions) (22). Finally, no melt products were produced by the Quantiplex® kit's targets precluding any overlap with the added D5S818/D18S51 STR targets (22,27). Additional testing of the assay on the QuantStudio™ 6 Flex displayed no significant difference in quantification values from samples tested with the

standard Quantiplex® and integrated Quantiplex® HRM assays (p=0.21). Also, distinct melt curves for both loci were produced at the same temperature range seen on the Rotor-Gene® Q and had no observed amplification product melt curves (22).

In order to determine how well the integrated Quantiplex® HRM assay was able to distinguish between single source and mixture DNA samples, D18S51 and D5S818 genotype classification analysis using the resulting raw melt curve data was conducted with R statistical software (R Foundation for Statistical Computing; Vienna, Austria). Initially, melt curve data of 8-10 known samples, representing each of seven different genotypes for D5S818, and each of seven different genotypes for D18S51, were used to train the software ('training set'). For predictive analysis, three machine learning methods were compared for predictive accuracy: support vector machine (SVM) linear, SVM radial, and linear discriminant analysis (LDA). A mixture sample group consisting of ten 1:1 two-person mixtures was subsequently added to the training data set to allow for a classification category of "mixture" in addition to the genotype classifications for single source samples (22,30).

Analysis of Rotor-Gene® Q melt data from 56 single source and 10 1:1 two-person mixtures ("validation set") revealed that 100% of mixtures and 87.5% of single source samples tested were accurately predicted as such using the SVM radial for D5S818 and SVM linear algorithm for D18S51. While 100% of single source samples were correctly classified as such, they were genotyped correctly only 60.3% and 46.15% of the time (for D5S818 and D18S51, respectively) (21,22,27). Thereafter, the integrated Quantiplex® HRM assay was tested on the QuantStudio™ 6 Flex qPCR platform. As a higher resolution instrument, it collects more data points per run than the Rotor-Gene® Q and thus was expected to achieve improved prediction accuracies. To achieve the highest number of datapoints obtained per sample, HRM data

collection settings on the QuantStudio™ 6 Flex were adjusted to 'continuous' which collected data every 0.015°C/s between 60°C and 95°C (22). Testing revealed 90% of mixtures and 87.5% of single source samples were accurately predicted as such using the SVM radial algorithm for both D5S818 and D18S51(22,27). However, as seen with the previous Rotor-Gene® Q data, with the mixture group in place, samples identified as single source were often not correctly genotyped (at only 16% and 22.64% for D5S818 and D18S51, respectively).

The results obtained from the integrated Quantiplex® HRM assay readily demonstrated that HRM analysis, with the two selected STR targets, can accurately distinguish between mixtures and single source samples at the qPCR stage. Thus, the primary goal of this project was met with the initial proof-of-concept data. However, the Investigator Quantiplex® kit alone is not an ideal commercial quantification kit for this assay, as it has not been implemented by many laboratories in the forensic community. HRM integration into a more widely used commercial quantification kit, such as the Quantifiler™ Trio (Applied Biosystems™), would be more valuable to the practitioner community, particularly if validated for use on an updated qPCR platform, such as the QuantStudio™ 6 Flex.

Quantifiler™ Trio is a qPCR assay capable of both quantitative and qualitative analysis of amplifiable human DNA in a sample. The assay incorporates three quantification targets: a small autosomal target (80 bp), a large autosomal target (214 bp), and Y chromosome target (75 bp). The small autosomal target quantifies the total amount of amplifiable human DNA in a sample, while the large autosomal target is used, in combination with the small autosomal target, to discern the degradation index (DI). A DI value greater than one indicates degradation is present and a DI greater than 10 indicates that the sample is more highly degraded and/or compromised and that STR amplification may be negatively impacted (31). The Y target represents the amount

of human male DNA that is present in a sample and is favorable for use with sexual assault samples as a way to indicate male genomic DNA contribution in the total DNA contribution. Amplification of all Quantifiler™ Trio targets are measured using a TaqMan™ probe coupled with a reporter and quencher dye engaged in fluorescence resonance energy transform (FRET). At the extension step, the TaqMan™ probe is cleaved, the reporter is released from the quencher, and fluorescence proportional to the amount of amplified product is emitted. Quantifiler™ Trio employs five fluorophores: VIC™ for small autosomal targets, ABY™ for large autosomal targets, FAM™ for Y targets, JUN™ for the IPC, and Mustang Purple™ for the passive reference (15,31).

Preliminary work into an integrated Quantifiler™ Trio HRM assay on the QuantStudio™ 6 Flex revealed two challenges. First, spectral emission overlap of the HRM intercalating dye, EvaGreen®, with FAM™ and VIC™ fluorophores used in the commercial kit was observed (27). Unfortunately, this emission overlap between the dyes resulted in inflated quantification values of both the Y and small autosomal targets. Considering that downstream profile generation is contingent upon the accuracy of DNA quantification, a new intercalating dye would be needed to replace EvaGreen® in the integrated HRM assay in order to move forward. However, as the Quantifiler™ Trio utilizes all five available dye channels and the QuantStudio™ 6 Flex, the candidate HRM intercalating dye would be required to share a channel. Secondly, initial studies also revealed that a distinct melt curve was produced from the Quantifiler™ Trio targets; these HRM products overlapped in the same temperature range of the added STR targets, potentially interfering with subsequent downstream HRM-based classification analyses (27). If the HRM product overlap resulted in a less accurate classification of samples as single source or mixture samples, primers for D5S818 and/or D18S51 STR loci would need to be redesigned in order to isolate their melt ranges away from those of Quantifiler™ Trio products.

Methods

Buccal samples were collected on sterile cotton swabs previously from donors in accordance with the approved Institutional Review Board Human Subjects Research Protocol (VCU-HM20002931, renewed in December 2021), as a part of the IRB forensic biological sample registry. STR profiles of samples used in this study (previously generated) were obtained from the laboratory's IRB registry database. 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 the studies that follow (1,26).

Initial Evaluation of the Integrated Quantifiler™ Trio HRM Assay

To confirm the observations that were previously reported (27), the integrated Quantifiler™ Trio HRM assay with newly optimized melt cycling parameters (22) was tested with two sets of standard samples (a five sample dilution series ranging from 50 to 0.005ng/µl). One set included the EvaGreen® dye (as described) and one with no additional dye. Additionally, another two sets of five single-source DNA samples were tested using the previously described method (22,27); one set included the D5S818 and D18S51 STR primers and the other did not include the STR primers. As reported previously, the integrated amplification chemistry included 4µl Quantifiler™ HP Primer Mix, 5µl Quantifiler™ THP PCR Reaction Mix, 0.11µl each of 100µM forward and reverse primers (1.0µM final concentration of each primer), 0.55µl of 20X EvaGreen®dye (1X final concentration), and 1µl DNA input. The STR 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 (27). Amplification cycling parameters were based on the Quantifiler™ Trio's manufacturer's recommendation of 40 cycles of: 95°C for 9 sec and 60°C for 30 sec with a 2.5°C/s ramp rate. The amplification cycles were followed by a transition cycle of 72°C for 2 min, 95°C for 20 sec, 55°C for 20 sec, and 56°C for 2 min (with a 1.6 °C/s ramp rate), which was followed by a melt cycle. The melt cycle included a continuous ramp rate of 0.015°C/s from 60°C to 95°C (with a 20-second hold at the beginning and end temperature stages). For data analysis on the QuantStudio™ 6 Flex software, the threshold, baseline start and baseline end for all targets were based on the manufacturer recommended settings (31). To assess inflation of quantification values, quantification values obtained from the small autosomal, large autosomal, and Y targets of the standard samples were evaluated. The percent increase in quantification values observed from standard samples tested with EvaGreen® versus those without EvaGreen® were calculated. To assess potential HRM overlap of the Quantifiler™ Trio targets with the STR targets, the resulting melt curves from the five single-source DNA samples analyzed with and without D5S818 and D18S51 STR primers were qualitatively compared.

Reduced amplification and consequently low melt curves for the D5S818 target were observed with integration of the HRM assay into the Quantifiler™ Trio. Thus, in order to determine whether the low-level amplification and melt observed at the D5S818 locus was due to a single limiting reaction component, the integrated Quantifiler™ Trio HRM assay was tested as described above with five single source DNA samples but with additional dNTPs or additional D5S818 STR primers added to the reaction cocktail. The final concentration of the added components tested consisted of either 100µM dNTPs, 1.5µM forward and reverse D5S818 STR primers, or both dNTPs and D5S818 primers. A final concentration of 1.5µM D5S818 primers

was chosen for all subsequent studies based on a qualitative inspection of the D5S818 primary melt peak height and peak resolution as well, avoiding added volume to the reaction.

New Intercalating Dye Calibration & Evaluation

Once inflated quantification values were confirmed, alternate commercial intercalating dyes with spectral emissions in the orange channel (x4-m4 filter) and minimal spectral emission overlap with the IPC target dye, JUN™ (λEx/Em: 580/623nm) were explored. Two candidate dyes, SYTO™ 64 (λEx/Em: 621/634nm) and SYTO™ 17 (λEx/Em: 599/619nm), were selected for further evaluation within the integrated Quantifiler™ Trio HRM assay. Prior to testing of the new dyes in the integrated Quantifiler™ Trio HRM assay, calibration of the dyes on the QuantStudio™ 6 Flex system was conducted by first determining the optimal dye concentration, followed by a custom dye calibration plate, and a HRM calibration plate, as described below.

Optimal Dye Concentration

Optimal candidate dye concentration was assessed as per the Custom Dye Calibration of Applied Biosystems™ Real-Time PCR Instruments protocol (32). A quadruplicate series of dye dilutions (SYTO™ 64 range of 2.5mM to 0.625mM and SYTO™ 17 range of 5mM to 0.04µM) was evaluated. Dyes were diluted to the appropriate concentration with sterile 18 MΩ H_2O. A 19µl aliquot of each dilution was combined with 1µl of amplified dsDNA for total reaction volume of 20µl. Aliquots of each dilution were dispensed into a 96-well plate and processed using the manufacturers recommended protocol on the QuantStudio™ 6 Flex (32).

Custom Dye Calibration Plate

Calibration of the candidate dyes on the QuantStudio™ 6 Flex was conducted following the manufacturers recommended protocol (30). In two independent runs, with SYTO™ 64 and

SYTO™ 17, a full 96-well plate was loaded with 20µl aliquots consisting of 19µl of each dye at the optimal dye concentration and 1µl of amplified dsDNA.

Custom HRM Dye Calibration

Intercalating dyes for use in high-resolution melt require an additional HRM dye calibration plate. The HRM calibration plate consisted of a 40µl reaction volume of 1X Taq Gold Buffer, 3mM $MgCl_2$, 25µM dNTPs, 1µM of each forward and reverse primer, 2 units AmpliTaq Gold DNA polymerase, 0.25mg/ml BSA, 12.5 µM SYTO™ 64 or 100 µM SYTO™ 17 dye, and 1X MeltDoctor™ (ThermoFisher) (final concentrations). Sterile 18 MΩ H_2O was used to bring the total reaction volume to 2ml. Aliquots of 20µl each were dispensed into all wells of a 96-well reaction plate, which was then processed on the QuantStudio™ 6 as a 'Non-MeltDoctor™ calibration', as described in the manufacturer's protocol (20).

In order to confirm the proper integration of these dyes into the assay products and to select the best dye to use going forward, two sets of five single-source DNA samples were analyzed using the integrated Quantifiler™ Trio HRM assay protocol described above. In the first set of samples, SYTO™ 64 at a final concentration of 12.5µM was included instead of EvaGreen®; the second set of samples included SYTO™ 17 at a final concentration of 100µM instead of the EvaGreen®. The resulting multicomponent plots for each dye were reviewed and any spectral emission overlap (with other targets in the Quantifiler™ Trio assay) was noted. SYTO™ 64 was selected for use in all subsequent studies due to its minimal spectral emission overlap with other targets as compared to SYTO™ 17.

Testing of the SYTO™ dyes in the integrated Quantifiler™ Trio HRM assay also revealed IPC cycle-thresholds (Ct) values that were out of expected passing range of 27 to 31. IPC amplification curves also failed to uniformly cross the threshold as expected and as routinely

observed from samples amplified using the standard Quantifiler™ Trio assay protocol. Additionally, standard curve values revealed out of range slopes and Y-intercepts as compared to those recommended by the manufacturer and observed in the laboratory. Thus, further optimization in dye concentration, reaction conditions, and data analysis settings was pursued.

Optimization of Reaction Chemistry & Analysis Settings

To identify the best dye concentration to be used in the integrated Quantifiler™ Trio protocol, a dye dilution series of SYTO™ 64 was tested using the protocol described above except for the substitution of SYTO™ 64 at final concentrations of 2μM, 2.5μM, 5μM, and 10μM (20,25). One single source female DNA sample was tested at each of the four dye concentrations. Primary peak heights from the resulting melt curves were qualitatively evaluated. This information, along with the cost implications associated with each dye concentration, was used to determine the dye final dye concentration to be used for all subsequent studies.

To test the manufacturer's recommended DNA/standard input volume of 2μl, while keeping the Quantifiler™ Trio components at the recommended concentration, a different reaction condition was tested in the integrated Quantifiler™ Trio HRM assay (**Table 1**). This alteration in chemistry resulted in a reaction volume (16μl) different than the integrated Quantifiler™ Trio reaction chemistry described above (11μl) (**Table 1**). Thus, for comparison purposes both reaction conditions are referred to throughout as either the 11μl reaction or the 16μl reaction. Two sets of five single source DNA samples were tested with both reaction conditions following the amplification and melt cycling parameters as described above except data analysis settings were modified from the manufacturer's recommendation. For these experiments, the IPC threshold was set to 0.95 and large autosomal, small autosomal, and Y target thresholds were set to 0.05. Additionally, the IPC baseline start and end values were

adjusted to 5 and 20 and the values for the large autosomal, small autosomal, and Y targets were adjusted to 3 and 18, respectively. A set of the five samples was analyzed with the standard Quantifiler™ Trio assay following manufacturer's recommended protocol, using half-volume reactions, for comparison purposes. The two reaction conditions produced primary melt curve peaks with similar peak heights, peak height locations, and resolution (data not shown) thus further evaluation between the two conditions was conducted. For this, the accuracy of the quantification values produced from the large autosomal, small autosomal, and Y targets in the assay were compared. Quantification accuracy was determined by calculating the average variation of target quantification values obtained from the integrated Quantifiler™ Trio HRM assay and from those obtained from the standard Quantifiler™ Trio assay. The percent difference between these values were calculated by taking the absolute value of the difference in quantification values, divided by the average, and then multiplied by 100. These values were then compared to the normal inter-run variation of the standard Quantifiler™ Trio assay. The inter-run variation of the standard Quantifiler™ Trio assay was determined by calculating the average percent differences observed in quantification values obtained from the same five samples tested on two different days. 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, see **Table 1**).

While quantification values from the small autosomal target using each new integrated Quantifiler™ Trio HRM reaction condition were within the normal range of variability, quantification values for the large autosomal and Y-targets were considerably higher than those observed with the standard assay. To assess whether further modifications in data analysis

settings could mitigate these issues, two additional sample DNA sets each consisting of 10 samples (5 female/5 male) were tested. The first set of samples was tested using the standard Quantifiler™ Trio manufacturer's protocol (half volume reactions), including recommended data analysis settings (31). The second set of samples was tested using the 16µl reaction condition using the melt cycling parameters as described above and modified data analysis settings. Modifications in data analysis settings of the IPC threshold values were incrementally increased from 0.1 to 0.4 and quantification target thresholds incrementally decreased from 0.2 to 0.08 for the large autosomal target and 0.1 for the small autosomal and Y targets. The baseline end value for all targets were also incrementally increased from 15 to 17. Quantification accuracies from the samples tested using the standard Quantifiler™ Trio assay and those tested using the integrated Quantifiler™ Trio HRM with the new data analysis settings were compared using the inter-run variation as described above. The data analysis settings that resulted in the most accurate quantification values were selected for use in all subsequent studies.

In order to determine if the new data analysis settings would mitigate observed out-of-range IPC Ct values and standard curve quality values, five standard samples (ranging from 50 to 0.005ng/µl) were tested using the integrated Quantifiler™ Trio HRM 16µl reaction condition (**Table 1**). Resulting IPC Ct values and IPC amplification plots were compared between data analyzed using the manufacturer's settings and those analyzed using the optimized, modified settings determined above. Lastly, two sets of 10 single source DNA samples were tested with the integrated Quantifiler™ Trio HRM 16µl reaction condition (as described above) using the optimized, modified data analysis settings. The resulting standard curve slope values were compared to passing values recommended by the manufacturer and Y-intercept values were compared to the previously validated range in the lab. To determine the inter-run variation,

quantification values obtained from each set of samples analyzed were compared using the percent difference, which was calculated as described above. The modified data analysis settings selected for subsequent use with the integrated Quantifiler™ Trio HRM assay were based on those that produced the most accurate quantification values, in-range, and uniform IPC Ct values, and relatively in-range standard curve quality metrics.

Testing of the Final, Optimized Reaction for the Integrated Quantifiler™ Trio HRM assay

After all optimization studies were complete, the reaction conditions and data analysis settings were finalized. The final reaction conditions selected 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 (1µM final concentration), and 0.1µl of 240.45µM D5S818 forward and reverse primers (1.5µM final concentration) in a total volume of 16.03 µl per well (with a DNA input of 2µl). Data analysis settings included baseline start and end values of 3 to 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 our newly optimized integrated Quantifiler™ Trio HRM assay.

In order to determine if the integration of the STR primers and subsequent alterations in data analysis settings associated with the integrated Quantifiler™ Trio HRM assay altered the resulting male-to-female ratios (M:F) and/or degradation indices (DI), 10 single source 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 (using manufacturer's conditions with half volume reactions). DIs for each sample were calculated by dividing the average quantification values obtained from the

small autosomal target values) by the average quantification values obtained from the large autosomal target values. Male-to-female ratios were calculated for each of the five known male samples analyzed. For each 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.

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. Control 2800M DNA (0.1ng/µl) was spiked with a range of hematin concentrations (25µM, 62.5µM, 83.75µM, 125µM, or 156.25µM final concentrations) or humic acid concentrations (25ng/µl, 37.5ng/µl, 50ng/µl, 75ng/µl, or 100ng/µl final concentrations) and then each dilution set was analyzed in duplicate. One set was tested using the newly optimized integrated Quantifiler™ Trio HRM assay and the other was tested using the standard Quantifiler™ Trio assay using manufacturer's recommended protocol (but with half-volume reactions). Control 2800M DNA at 0.1ng/µl, with no inhibitor spike, was also analyzed in duplicate and served as the control. IPC Ct values were compared to the average IPC Ct value obtained from the DNA standards and those that were more than two Cts from this value were flagged (15).

As the small autosomal target is used as the quantification measure for downstream STR-amplification, to confirm the accuracy of the values obtained from the optimized integrated Quantifiler™ Trio HRM assay, STR amplification and analysis was pursued. 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 (ThermoFisher) 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 alleles recovered, heterozygote peak balance (peak height ratio <70%), and mean allele peak heights calculated in MS Excel (Microsoft; Redmond, WA). Mean peak heights for all five samples were calculated by taking the sum of the peak heights and dividing by the sum of alleles observed. Heterozygote peak balance was calculated by taking the lower peak height and dividing by the higher peak height, multiplied by 100.

Single Source vs. Mixture Prediction Accuracy Testing

Training and validation sample sets were tested using the newly optimized integrated Quantifiler Trio™ HRM assay on the QuantStudio™ 6 Flex System, as described above. The training set was comprised of 56 single-source DNA samples with D5S818 genotypes of interest (see above) and 62 single-source DNA samples with genotypes of interest (see above); together,

this included 99 unique samples when overlap was accounted for. Additionally, 16 1:1 two-person mixtures each were included in the training set. The validation set was comprised of 68 single-source samples, each having genotypes of interest for both loci, as well as 16 different 1:1 two-person mixtures. For each sample, the negative derivative melt data was exported as a MS Excel file, organized by STR locus, and converted into a CSV file. This data was then imported into R-statistical software and three different prediction modeling tools, including linear discriminate analysis (LDA), support vector machine (SVM) linear, and SVM radial analyses. Confusion matrices generated by these algorithms were used to determine the prediction accuracy of each model for both loci. Initially, to authenticate the accuracy of the models, the training set for each STR locus tested was tested against itself. Following this, the validation data was tested against the trained model. Genotyping accuracy of the validation data was calculated by taking the sum of samples that accurately classified divided by total number of samples. Single source typing prediction accuracies were determined from the resulting confusion matrix for each locus by calculating the total number of samples classified as a single source genotype (regardless if the correct genotype of was obtained) divided by the total number of single source samples tested. Similarly, mixture accuracy was determined by dividing the number of mixture samples correctly classified by the total number of mixtures tested. For combined accuracy of the integrated Quantifiler Trio™ 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. From this data, the best statistical prediction model was selected for each STR locus and the overall accuracy of the integrated Quantifiler Trio™ HRM assay using that model was determined.

Results and Discussion

Initial Evaluation of the Integrated Quantifiler™ Trio HRM Assay

When the integrated Quantifiler Trio™ HRM assay was initially tested, the multicomponent plot comparison of five standard DNA samples revealed appreciable inflation in the baseline fluorescence of the small autosomal target dye, VIC™ (mean increase of 248%), and of the Y target dye, FAM™ (mean increase of 2,080%) compared to testing using the standard Quantifiler Trio™ chemistry (**Figure 1, Table 2**). This phenomenon was not surprising given the significant spectral emission overlap of EvaGreen® in the green channel (λEm: 530-533nm) with VIC™ in the green channel (λEm: 547-569nm) and FAM™ in the blue channel (λEm: 505-535nm). Though fluorescence levels were normalized when standard DNA samples and buccal swab DNA samples were processed with the same integrated HRM assay components, inflation of the Y target dye (FAM™) occurred in both male and female samples resulting in false positives. Initial testing also revealed overlap of the Quantifiler™ Trio melt products with the D18S51 melt products produced with the integrated Quantifiler™ Trio HRM assay (**Figure 2**). Although some overlap was noted, the impact of this on prediction accuracies downstream was unknown. Thus, optimization and further testing was pursued; if the assay was unable to properly predict sample source information, then adjustments in the STR primer sequences would be made. Incidentally, melt curve overlap between amplification and STR products were not observed in the integrated Investigator Quantiplex® HRM assay that was previously developed in this laboratory, as its amplification products end with single-strand DNA which result in no melt products (1,22,27). Finally, a slight reduction in the peak height of D5S818 was noted from samples tested with the integrated Quantifiler Trio™ HRM assay versus those observed with the previously reported integrated Investigator Quantiplex® HRM assay (**Figure 2**) (1,27). This is likely attributable to the higher number of amplification targets in the Trio™ chemistry as

compared to the Quantiplex® chemistry and therefore increased competition for PCR components. Thus, to increase D5S818 amplicon production and improve the peak height of the resulting melt peak, additional primers and dNTPs were tested with the integrated Quantifiler Trio™ HRM assay. Both the addition of a higher concentration of D5S818 primers, as well as the addition of supplemental dNTPs, clearly improved the peak height of resulting primary D5S818 melt peak (**Figure 3**). The addition of both also further improved the peak height of the primary D5S818 peak, however, this was not a substantial increase when compared to the melt curves resulting from the increase in D5S818 primer concentration alone. Consequently, 1.5µM D5S818 primer concentration was selected for use in the final integrated Quantifiler Trio™ HRM assay, without any additional dNTPS, to avoid the additional volume and costs associated with the addition of a new component (dNTPs).

New Intercalating Dye Calibration & Evaluation

Given the significant impact of the EvaGreen® dye on the quantification targets within the commercial Quantifiler™ Trio kit, two alternate intercalating dyes, SYTO™ 64 and SYTO™ 17, were selected for evaluation with the integrated Quantifiler™ Trio HRM assay. Considering that the Quantifiler™ Trio utilizes all dye channels on the QuantStudio™ 6 Flex, dye channel sharing with the selected interrelating dyes was expected and unavoidable. SYTO™ 64 and SYTO™ 17 were selected, as their primary spectral overlap would be limited to the IPC target dye, JUN™, avoiding any overlap with the quantification target dyes. Dye concentration experimental data revealed an acceptable fluorescence signal for SYTO™ 64 at the lowest concentration recommended (1,670,000rfu was observed at 62.5µM, data not shown). As such, 62.5µM SYTO™ 64 was chosen and the subsequent dye calibration passed. However, even at the highest concentration recommended, the fluorescence signal for SYTO™ 17 was below the

recommended acceptable fluorescence signal range (≤ 500,000rfu was observed at 5mM, data not shown). Still, lower fluorescence signaling may be advantageous with the Quantifiler™ Trio HRM assay, as it may minimize the impact on the IPC target dye JUN™, which shares a spectral channel. As such, 62.5µM SYTO™ 17 (with a signal at approximately 180,000rfu) was selected as the final concentration for calibration; the subsequent calibration passed.

Next, a custom HRM calibration would be needed for both prior to testing in the integrated Quantifiler™ Trio HRM assay. Calibration of both dyes in the HRM calibration plate was initially conducted with a final concentration of 100µM. However, using this concentration, only SYTO™ 17 passed (SYTO™ 64 did not). In fact, precipitate of the dye was observed in the HRM calibration reaction mix for SYTO™ 64 at this concentration. Upon closer inspection of the data, SYTO™ 64 was noted to have reached acceptable fluorescence signaling at 62.5µM. Thus, a lower final concentration of SYTO™ 64 was tested (12.5µM). Using this concentration, the custom HRM dye calibration passed for this dye. These final concentrations (100µM for SYTO™ 17 and 12.5µM for SYTO™ 64) were used for confirmation testing within the integrated Quantifiler™ Trio HRM assay.

Although some slight differences in HRM melt curve morphology and STR product temperature ranges were observed when using the new intercalating dyes in the Quantifiler™ Trio HRM assay, these differences were not appreciable. Not surprisingly, amplification plots of the IPC target dye (JUN™) revealed inflation of its normal fluorescence when using either of the new candidate dyes (**Figure 4**) as compared to those observed with the standard Quantifiler™ Trio HRM (**Figure 1A**). Unexpectedly, SYTO™ 64 also resulted in an elevation of the large autosomal target dye, ABY™ (**Figure 4A**). In order to determine if this apparent spectral overlap would be significant enough to alter quantification values, and if this effect could be mitigated,

further optimization studies were pursued. Alternately, samples whose reactions included SYTO™ 17 displayed a substantial elevation in the passive reference dye, Mustang Purple™, which is used to normalize the reporter signals for all quantification values (**Figure 4B**). Given that this could lead to interference of its ability to regulate the reporter fluorescence signal, and subsequently affect the quantification accuracy of all targets, no further testing with SYTO™ 17 was pursued. Lastly, these studies revealed several additional factors that would need to be addressed prior to prediction testing. First, IPC Ct values of the five standards samples analyzed in the integrated Quantifiler™ Trio HRM assay were outside the 27 to 31 Ct range expected. Further, the quality metrics of the standard curves resulting from the integrated Quantifiler™ Trio HRM assay tested with each new dye displayed slopes that were consistently lower than the manufacturer's recommendation and Y-intercept values that were consistently higher than the validated range (31). Because of these issues, further optimization in dye concentration, reaction condition, and data analysis settings was required.

Optimization of Reaction Condition Chemistry & Analysis Settings

To determine the ideal concentration of SYTO™ 64 dye to be used in the integrated Quantifiler™ Trio HRM assay, a dye dilution series for SYTO™ 64 was tested. Although the 2.5 µM, 5µM, and 10µM final concentrations of SYTO™ 64 dye all displayed comparable peak heights for the primary HRM melt curve peaks, saturation of the dye appeared to be achieved at the 5µM final concentration (**Figure 5**). Thus, this concentration was selected for use in the final integrated Quantifiler™ Trio HRM assay.

In an effort to further optimize the integrated Quantifiler™ Trio chemistry, while keeping the HRM and the Quantifiler™ Trio component concentrations close to desired values, an increased DNA input volume of 2µl was tested (16µl reaction condition) and compared to the

previously described reaction condition with an input DNA volume of 1µl (11µl reaction condition) (**Table 1**). To identify which integrated Quantifiler™ Trio HRM assay reaction condition produced the most accurate quantification values, resulting quantification values were compared to those obtained from the same samples tested using the standard Quantifiler™ Trio kit chemistry. Given the critical impact of the small autosomal quantification values in practical forensic casework amplification, these values were carefully examined for the 16µl and 11µl reaction conditions. The 16µl reaction condition produced small autosomal quantification values that were, on average, similar to those obtained when using the standard Quantifiler™ Trio chemistry and similar to the normal inter-run variation observed with Quantifiler™ Trio (8.43% vs. 6.70%, respectively) (**Table 3**). Much larger differences were noted with the 11µl (**Table 3**), thus, the 16µl reaction was the chosen reaction condition selected for the integrated Quantifiler™ Trio HRM assay.

While quantification values from the small autosomal target using each new integrated Quantifiler™ Trio HRM 16µl reaction condition were essentially within the normal range of variability, quantification values for the large autosomal and Y-targets were considerably higher than those observed with the standard assay (**Table 3**). To assess whether further modifications in data analysis settings could mitigate these issues, additional male, female, and standard DNA samples were tested using the 16µl Quantifiler™ Trio HRM reaction condition. Modification in data analysis settings were made based on observations in decreases in PCR efficiency (lower quantification curve slopes) which likely resulted in delayed amplification when the 16µl Quantifiler™ Trio HRM reaction was used. To address this delay and improve detection of all targets using this assay, increased Ct thresholds for the IPC target were evaluated along with decreased Ct thresholds for the other quantification targets and increased baseline end values.

Although many values were evaluated for these data settings (data not shown), the data analysis settings that produced the most accurate quantification values and most consistent IPC Ct values were thresholds of 0.4, 0.08, 0.1, and 0.1 for the IPC, large autosomal, small autosomal, and Y targets, respectively, baseline start value of 3, and baseline end value of 17. With these data analysis settings, 16µl Quantifiler™ Trio HRM reaction produced large autosomal and Y quantification values that were consistent with those obtained when the same samples were analyzed using standard Quantifiler™ Trio chemistry; further, the differences observed were comparable to the inter-run differences normally observed with the Quantifiler™ Trio chemistry (**Table 4**). Additionally, using these new data analysis settings, the small autosomal quantification values 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 4**). IPC amplification curves and standard curve quality control values were also closely examined for samples analyzed using the 16µl Quantifiler™ Trio HRM reaction and new data analysis settings. IPC values crossed the threshold more uniformly (as desired) and within the expected range using the new data analysis settings (**Figure 6**). Although the IPC amplification curves near the edge of exponential phase (**Figure 6**), given the stated purpose of IPC, the selected threshold results in unform curves across runs and between lots. Standard curves used to generate the quantification values of all samples produced Y-intercept values that were within the expected range for the standard Quantifiler™ Trio chemistry (**Table 5**). Standard curve slopes were consistent across runs but were marginally lower than expected for the standard Quantifiler™ Trio chemistry (**Table 5**). Lower slopes in the integrated Quantifiler™ Trio HRM assay (due to decrease in PCR efficiency) was likely a result of the added STR targets in the reaction chemistry and consequent competition for PCR products.

Further testing of the Final, Optimized Reaction for the Integrated Quantifiler™ Trio HRM assay

Finally, samples analyzed over two different runs were evaluated to determine the inter-run variation observed with the 16µl Quantifiler™ Trio HRM reaction and new data analysis settings. The observed values were less variable than those normally obtained using the standard Quantifiler™ Trio chemistry (**Table 6**). Given the success noted with the reaction conditions, amplification/melt cycle parameters, and data analysis settings used with these studies, the conditions, parameters, and settings were accepted as final and a formal protocol for the optimized integrated Quantifiler™ Trio HRM assay was documented.

By design, the Quantifiler™ Trio kit offers information beyond just quantification data. The assay's three quantification targets are designed to provide supplementary sample information to provide information about the DNA quality (degradation index) and the amount of DNA attributable to a male versus a female (male:female ratios). Given the significant modifications in the new assay's reaction chemistry and data analysis settings, it was critical to reevaluate these metrics, which add value to the kit chemistry. Degradation assessments revealed DI's that were less than one, as expected, when samples were tested with both the standard Quantifiler™ Trio reaction and the newly optimized Quantifiler™ Trio HRM assay (0.90 and 0.96, respectively) (**Table 7**). This data suggests that the modifications made to the kit chemistry and data settings did not alter the ability to of this assay to detect non-degraded DNA.

In forensic DNA casework scenarios, a male-to-female ratio is often calculated for sexual assault samples or for any sample suspected to be a mixture of two or more contributors of different sexes. Male:female ratios which indicate 10 or more-fold more male than female DNA are typical of male major contributor(s) DNA. In these cases, a DNA analyst could be confident in pursuing Y STR analysis, if deemed appropriate. In our studies, both the standard Quantifiler™

Trio reaction and the newly optimized 16µl 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 7**).

Given the overlapping emission spectra of the intercalating dye used within the newly optimized integrated Quantifiler™ Trio HRM assay (SYTO™ 64) with the Quantifiler™ Trio's IPC target dye, (JUN™), it was important to determine if the IPC's ability to detect inhibition was altered. Thus, sample DNA spiked with either hematin or humic acid were tested using both the standard Quantifiler™ Trio chemistry and the optimized integrated Quantifiler™ Trio HRM protocol. First, quantification values were examined across the range of inhibitor tested using both protocols; inhibition was noted when quantification targets were "undetermined". For humic acid, inhibition was observed at the same concentrations for all three targets, regardless of the method used (**Table 8**). IPC flags were noted for all concentrations of humic acid tested using the standard method, while only the highest three concentrations of humic acid resulted in an IPC flag using the integrated Quantifiler™ Trio HRM assay (**Table 8**). This is not surprising given that the small autosomal and Y quantification values are accurate, and the IPC Ct is within normal range and only slightly delayed. For hematin, the standard Quantifiler™ Trio chemistry was able to detect the presence of the inhibitor at lower concentration values with the large autosomal target, as compared to the integrated Quantifiler™ Trio HRM assay. However, the integrated Quantifiler™ Trio HRM assay showed improved detection of the inhibitor with the small autosomal and Y targets. IPC flags were observed with all concentrations of hematin tested above 37.5µM, regardless of the method used. These results are inconsistent with those in the Quantifiler™ Trio validation reported by Green et al. 2016 (16), whose study displayed initial IPC flags at 75µM and higher hematin and 40ng/µl and higher humic acid. Higher sensitivity in

inhibition detection of both standard and integrated Quantifiler™ Trio assays may be a result of differences in instrumentation, as the Green et. al study conducted their analysis on the 7500 Fast Real-time PCR system (Applied Biosystems). Overall, the alterations to the Quantifiler™ Trio assay do not appreciably alter the ability of the chemistry to detect inhibition from common forensic inhibitors, though this seems to vary somewhat with specific inhibitors. Detection of humic acid was nearly identical between the two assays, while the presence of hematin was detected at the same concentration in both assays.

To evaluate the DNA profiles generated after PCR amplification between assays, samples were diluted according to quantification values obtained from the standard Quantifiler™ Trio reaction as well as the newly optimized 16µl 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 desired thresholds (**Table 9**) and all STR profiles were concordant across sets. Further, the number of loci flagged for heterozygote peak balance (<70%) was not significantly different (p=0.62) between the two sets of samples amplified and peak height ratios were also not significantly different (p=0.18) (**Table 9**). This data demonstrates that the minor differences in quantification values produced by the newly optimized Quantifiler™ Trio HRM assay show no appreciable differences in the resulting STR profiles, as compared to those obtained from the standard Quantifiler™ Trio chemistry,

Single Source vs. Mixture Prediction Accuracy Testing

The newly optimized 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. Three different algorithms were initially tested; for the D5S818 locus data,

sample genotypes were more accurately predicted using the SVM linear algorithm; however, for the D18S51, the SVM radial method was most accurate. Thus, all further prediction modeling used these algorithms; resulting confusion matrices were evaluated for calculation of prediction accuracy (**Table 10**). Using this approach, unknown "validation" single source samples were classified as the correct genotype only 35% and 16% of the time for D5S818 and D18S51 (data not shown), respectively. At the D5S818 locus, 86.76% of single source samples and 25% of mixtures were accurately predicted as such. At the D18S51 locus, 79.41% of single source samples and 62.5% of mixtures were accurately predicted as such (**Table 11**). As the HRM assay operates with two loci, to increase the power of discrimination in its predictions, a combined accuracy metric was used for overall prediction accuracies. This metric was used as a preventative measure; 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 more conservative approach is used. With both loci considered, 75% of single source samples and 68.75% of mixtures were correctly classified as such producing an overall accuracy of 73.8% in which 62 of the 84 samples tested were correctly classified.

Conclusion

The forensic DNA workflow proves inadequate in providing crucial sample information at an early stage. Integrated qPCR based HRM analysis is an economical and advantageous augmentation to the traditional system. Although guidelines exist for resolving low-template DNA samples and/or mixtures, these measures are often reactive rather than proactive and require substantial time and effort. The forensic community would greatly benefit from an HRM screening tool in which the challenges that accompany low-template samples can be mitigated

prior to PCR amplification. A proof-of-concept high-resolution melt (HRM) curve assay previously developed and integrated into the Investigator Quantiplex® qPCR kit was optimized for use on the QuantStudio™ 6 Flex qPCR platform and was able to accurately distinguish between single source and mixture samples 87.88% of the time. However, the Investigator Quantiplex® qPCR is not commonly used in the forensic community and provided limited sample information while the Quantifiler™ Trio is more robust and widely used. Due to spectral emission overlap of the HRM intercalating dye with Quantifiler™ Trio target dyes (and subsequent increase in quantification values), integration required redesign of the HRM assay encompassing inclusion and calibration of a new intercalating dye, optimization of the reaction chemistry, and an adjustment of data analysis settings for all targets. These modifications resulted in an assay that is as effective in producing reliable quantification values as the standard Quantifiler™ Trio assay. Additionally, the integrated Quantifiler™ Trio HRM assay was shown to produce similar M:F DNA ratios and degradation indices. Marginally lower quantification values of the small autosomal target in the integrated Quantifiler™ Trio HRM assay had no effect in downstream STR-amplification success, reporting 100% concurrent CE profiles with peak heights and heterozygote peak imbalance that were not statistically different from those generated with the standard Quantifiler™ Trio assay. Lastly, the presence of inhibitors in both the standard Quantifiler™ Trio and integrated Quantifiler™ Trio HRM assays were detected at the same or nearly the same degree and may be inhibitor dependent. Thus, it was concluded dye channel sharing of SYTO™ 64 with JUN™ did not affect inhibition detection by the IPC target. When assessed for its ability to distinguish between single source vs mixture samples, the assay was able to accurately identify 73.8% of all samples tested. Given that the probability of

accurately classifying one of the 16 genotypes tested for D5S818 and D18S51 is 6.25%, it had measurable success.

Overall, the integrated Quantifiler™ Trio HRM assay shows considerable promise as a mixture screening assay. Future directions include additional validation of the sensitivity of the assay. Given the increasing submission of touch DNA evidence to forensic laboratories, it is important to determine the lower limit of detection of the assay. Also, as all mixture testing was conducted with a 1:1 ratio of two-person mixtures, a range in DNA ratios of these 2-person mixtures will be evaluated. In addition to this, the number of contributors in the mixtures tested will be increased to reflect what is often seen in casework and to determine whether this has an impact on the accuracy of the assay. If validation proves successful, to be viable in a practitioner setting, the assay must be able to detect all possible genotypes for D5S818 and D18S51. Therefore, the current training set will be expanded with synthetic genotypes generated in R for both loci. Lastly, a user-friendly, downloadable interface will be created to allow practitioners to easily upload their melt curve data and receive classification reports.

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Appendix

Table 1. Reaction Condition Chemistries Tested for the Integrated Quantifiler™ Trio HRM assay.

11 µl Reaction Volume

Component	Volume per reaction (µl)
Quantifiler™ Trio Primer Mix	4.0 (1X)
Quantifiler™ THP PCR Reaction Mix	5.0 (1X)
D5 & D18 F+R Primers	0.11 each (x4 = 0.44) (1µM)
Intercalating dye	0.55
Sample/Standard	1.0
~Total Volume per well	11

16 µl Reaction Volume

Component	Volume per reaction (µl)
Quantifiler™ Trio Primer Mix	5.8 (1X)
Quantifiler™ THP PCR Reaction Mix	7.2 (1X)
D5 & D18 F+R Primers	0.10 each (x4 = 0.40) (0.625µM)
Intercalating dye	0.63
Sample/Standard	2.0
~Total Volume per well	16

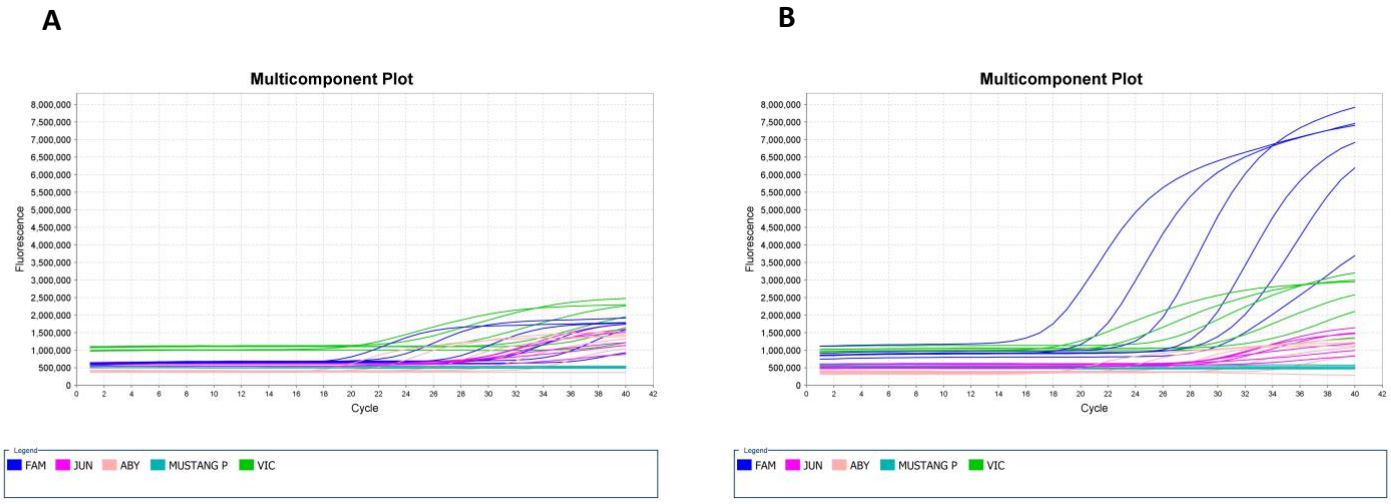

Figure 1. Multicomponent plots from initial testing of the Integrated Quantifiler™ Trio HRM assay with EvaGreen® dye. Multicomponent plot showing standard samples amplified using the standard Quantifiler™ Trio assay showing normal, expected fluorescence levels for all kit targets (A). Multicomponent plot showing the same samples amplified using the integrated Quantifiler™ Trio HRM assay with EvaGreen ® intercalating dye showing inflation of small autosomal (FAM™) and Y targets (VIC™) (B). This is likely due to spectral emission overlap between the EvaGreen® dye and FAM™/VIC™, respectively, dyes used in the standard Quantifiler™ Trio chemistry.

Table 2. Quantifiler™ Trio DNA standards initially tested with the standard Quantifiler™ Trio assay and the Integrated Quantifiler™ Trio HRM assay with EvaGreen® dye.

Samples *(n=5)*	Target	Standard Quantifiler™ Trio Assay	Integrated Quantifiler™ Trio with EvaGreen® dye
		Quantity (ng/µl)	Quantity (ng/µl)
Standard 1 (50ng/µl)	Large Autosomal	50	31.71
	Small Autosomal		**158.13**
	Y		**390.38**
Standard 2 (5ng/µl)	Large Autosomal	5	4.12
	Small Autosomal		**22.91**
	Y		**69.89**
Standard 3 (0.5ng/µl)	Large Autosomal	0.5	0.38
	Small Autosomal		**1.94**
	Y		**5.97**
Standard 4 (0.05ng/µl)	Large Autosomal	0.05	0.04
	Small Autosomal		**0.24**
	Y		**0.75**
Standard 5 (0.005ng/µl)	Large Autosomal	0.005	0.002
	Small Autosomal		**0.005**
	Y		**0.30**

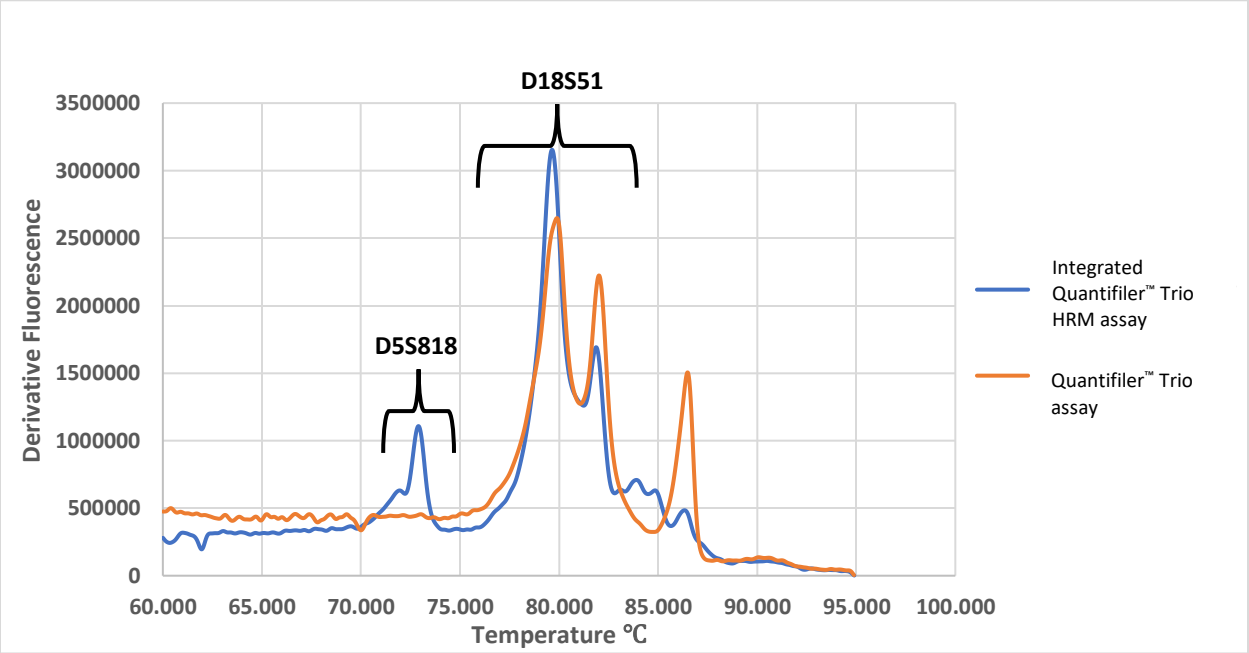

Figure 2. Melt curves from the Quantifiler™ Trio chemistry and the Integrated Quantifiler™ Trio HRM assay. Melt curves obtained from the integrated Quantifiler™ Trio HRM assay shows melt temperature ranges of 71 - 73° C for D5S818 and 77 - 83°C for D18S851 with EvaGreen® dye. Melt products from the Quantifiler™ Trio amplification targets (orange line) overlapped with the D18S51 melt peaks from the integrated Quantifiler™ Trio HRM assay (blue line).

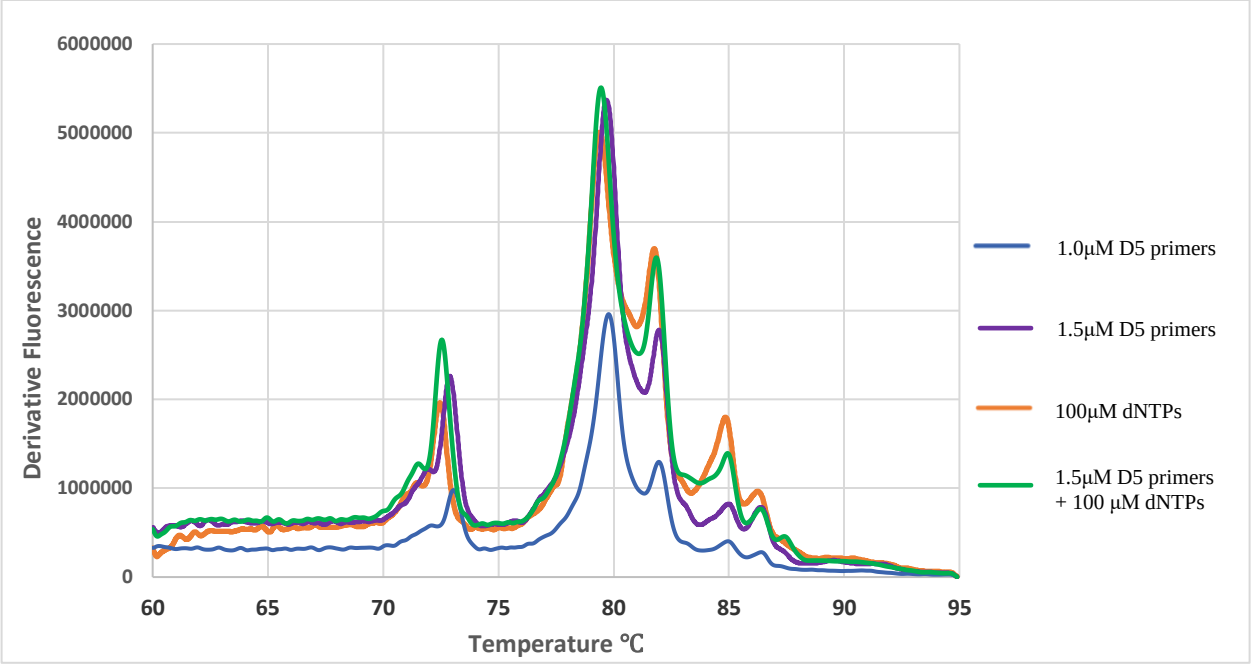

Figure 3. Integrated Quantifiler™ Trio HRM assay D5S818 Peak Optimization. Melt peak overlays from the integrated Quantifiler™ Trio HRM assay (with EvaGreen® intercalating dye) using the standard 1.0µM of D5S818 primers (blue line), an increased concentration of primers (1.5µM, purple line), additional dNTPs (100µM, orange line), and both (green line). To avoid added volume/reagent components in the assay, the 1.5µM D5S818 primer concentration was selected for further testing.

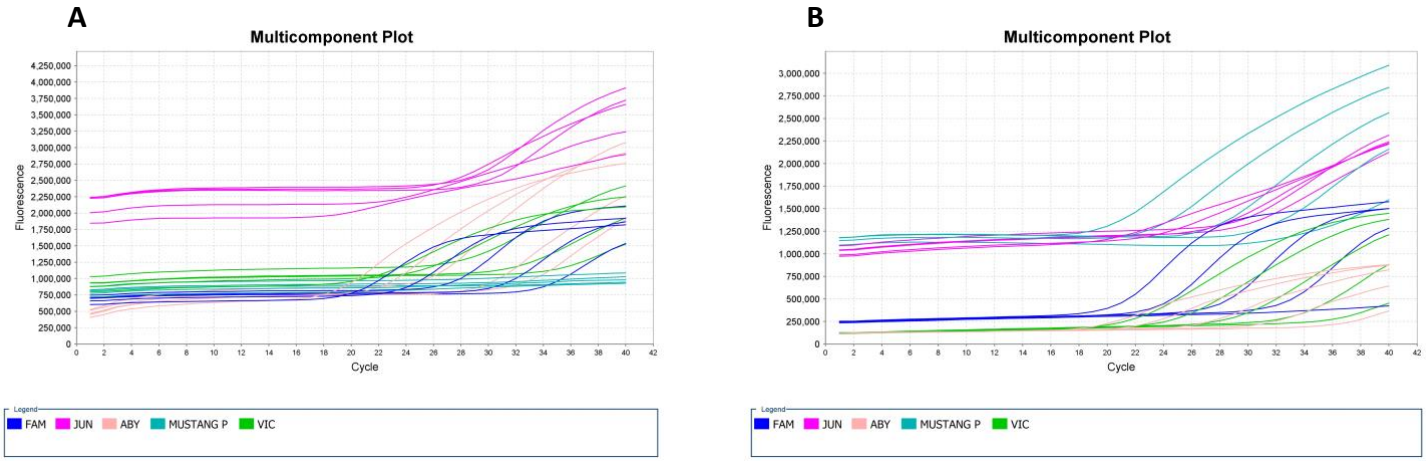

Figure 4. Multicomponent plots from samples tested with the Integrated Quantifiler™ Trio HRM assay using two different intercalating dyes. The IPC target (JUN™) and the large autosomal target (ABY™) were elevated when tested using SYTO™ 64 (A), while SYTO™ 17 caused inflation of the IPC target (JUN™) as well as the passive reference target (Mustang Purple™) (B).

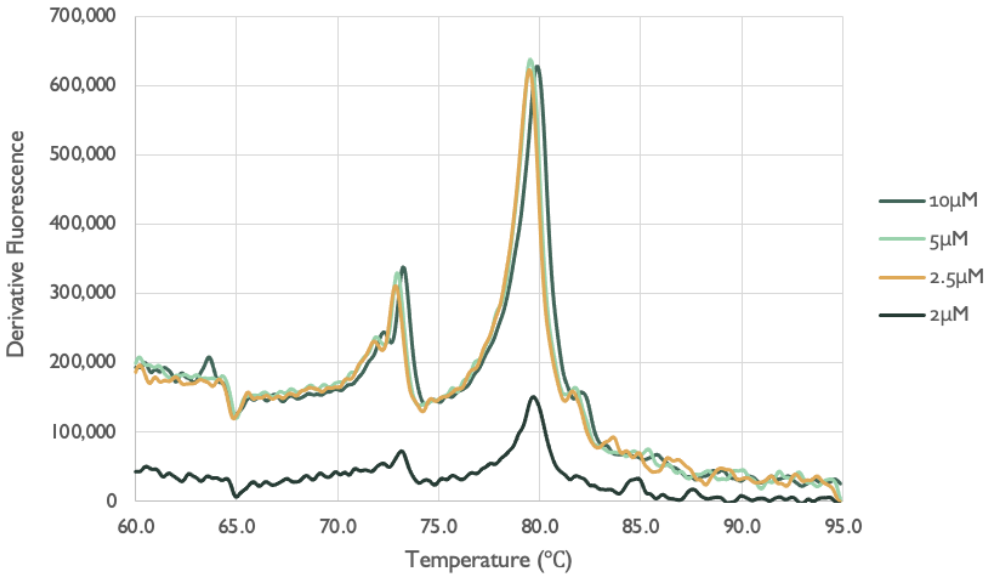

Figure 5. Testing of a SYTO™ 64 dye dilution series in the Integrated Quantifiler™ Trio HRM assay. Melt curve peaks with dye concentrations between 10µM and 2.5µM were comparable.

Table 3. Quantification accuracy of two Integrated Quantifiler™ Trio HRM assay reaction conditions.

	Large Autosomal *(n=5)*	**Small Autosomal** *(n=5)*	**Y** *(n=3)*
Standard Quantifiler™ Trio Inter-run Variation	9.53%	6.70%	8.05%
Integrated Quantifiler™ Trio HRM Assay with SYTO™ 64 dye - 11µl reaction	16.43%	21.08%	25.25%
Integrated Quantifiler™ Trio HRM Assay with SYTO™ 64 dye - 16µl reaction	18.97%	**8.43%**	22.61%

Table 4. Quantification accuracy of the integrated Quantifiler™ Trio HRM assay 16µl reaction with modified data analysis settings.

	Large Autosomal % Difference *(n=10)*	Small Autosomal % Difference *(n=10)*	Y-Target % Difference *(n=5)*
Standard Quantifiler™ Trio Assay Inter-run Variation	**9.76**	**9.11**	**7.99**
Integrated Quantifiler™ Trio HRM Assay - 16µl reaction condition	**9.84**	**8.17**	**9.68**

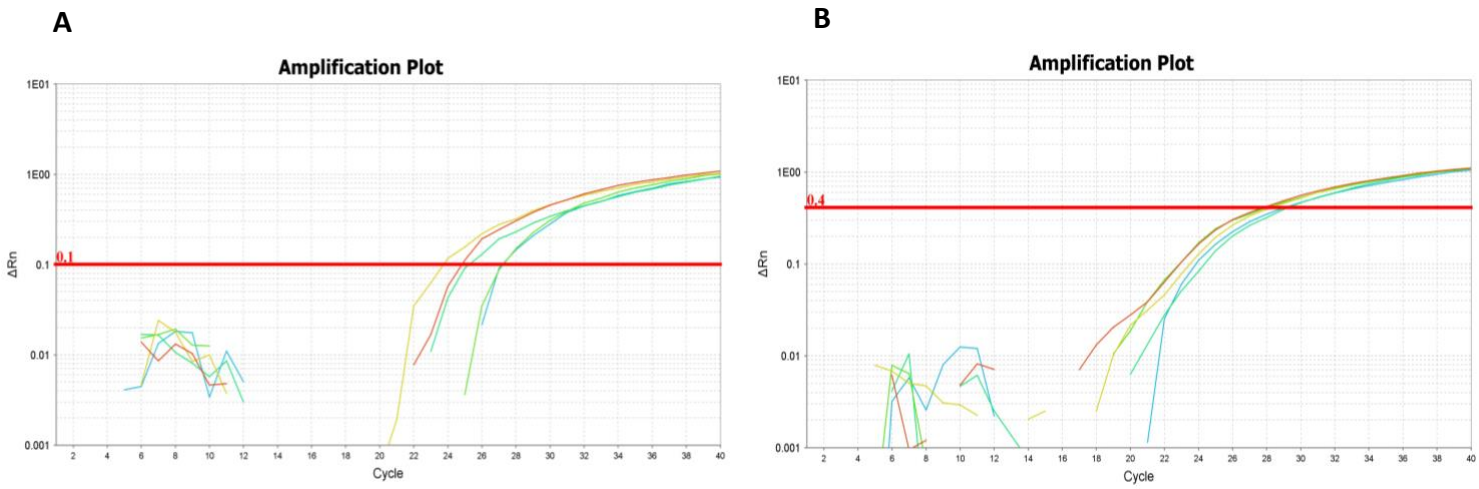

Figure 6. IPC amplification plots of the integrated Quantifiler™ Trio HRM assay with SYTO™ 64. Inflation of the IPC channel, due to emission overlap with SYTO™ 64, results in changes in the expected IPC Ct values displayed in its amplification plot when analyzed with manufacturer's analysis settings (A). Modifications of IPC analysis settings (Threshold: 0.4, Baseline: 3 to 17) resulted in IPC Ct values that were consistent across samples and within the expected Ct range of 27 to 31 (B). (n=5)

Table 5. Standard curve quality metrics of the standard Quantifiler™ Trio assay and the integrated Quantifiler™ Trio HRM assay with modified data analysis settings.

Target	Slope		Y-Intercept	
	Standard Quantifiler™ Trio Assay Manufacturer passing range	**Integrated Quantifiler™ Trio HRM Assay** ***(n=3)***	**Standard Quantifiler™ Trio Assay Lab validated range**	**Integrated Quantifiler™ Trio HRM Assay** ***(n=3)***
Large Autosomal ***(n=10)***	-3.1 – -3.7	-3.95 – -4.03	24.07 – 25.86	25.19 - 25.35
Small Autosomal ***(n=10)***	-3.0 – -3.6	-3.78 – -3.88	26.58 – 28.33	27.63 - 27.99
Y ***(n=5)***	-3.1 – -3.7	-3.60 – -3.73	25.52 – 27.17	26.06 - 26.24

Table 6. Inter-run quantification variation of the standard Quantifiler™ Trio assay and integrated Quantifiler™ Trio HRM assay.

	Standard Quantifiler™ Trio Assay	**Integrated Quantifiler™ Trio HRM Assay**
Average Difference between runs ***(n=10)***	0.54ng/µl	0.30ng/µl
% Variation between runs ***(n=10)***	9.1%	4.9%

Table 7. Degradation indices & Male:Female ratios observed for the standard Quantifiler™ Trio and Integrated Quantifiler™ Trio HRM assays.

Assay	Average DI *(n=10)*	Male:Female *(n=5)*
Quantifiler™ Trio Standard Assay - Manufacturers Analysis Settings	**0.90**	**17 : 1**
Integrated Quantifiler™ Trio HRM Assay	**0.96**	**26 : 1**

Table 8. Inhibition study comparing the standard Quantifiler™ Trio and Integrated Quantifiler™ Trio HRM assays.

A

Sample	Inhibitor Concentration	Standard Quantifiler™ Trio HRM Assay (ng/µl)			IPC CT	IPC Flag
		SA	LA	Y		
Control 2800M (0.1ng/µl)	0	0.104	0.119	0.107	30.697	N
HA - A1	25ng/µl	0.127	0.002	0.115	35.944	Y
HA - B1	62.5ng/µl	0.108	Undeter.	0.083	Undeter.	Y
HA - C1	93.75ng/µl	0.076	Undeter.	0.018	Undeter.	Y
HA - D1	125ng/µl	Undeter.	Undeter.	Undeter.	Undeter.	Y
HA - E1	156.25ng/µl	Undeter.	Undeter.	Undeter.	Undeter.	Y
HEM - A1	25µM	0.085	0.119	0.101	31.195	N
HEM - B1	37.5µM	0.066	Undeter.	0.07	Undeter.	Y
HEM - C1	50µM	0.031	Undeter.	0.014	Undeter.	Y
HEM - D1	75µM	Undeter.	Undeter.	Undeter.	Undeter.	Y
HEM - E1	100µM	Undeter.	Undeter.	Undeter.	Undeter.	Y

B

Sample	Inhibitor Concentration	Integrated Quantifiler™ Trio HRM Assay (ng/µl)			IPC CT	IPC Flag
		SA	LA	Y		
Control 2800M (0.1ng/µl)	0	0.107	0.102	0.124	30.49	N
HA - A2	25ng/µl	0.112	0.025	0.127	31.523	N
HA - B2	62.5ng/µl	0.122	Undeter.	0.124	37.085	Y
HA - C2	93.75ng/µl	0.100	Undeter.	0.048	Undeter.	Y
HA - D2	125ng/µl	Undeter.	Undeter.	Undeter.	Undeter.	Y
HA - E2	156.25ng/µl	Undeter.	Undeter.	Undeter.	Undeter.	Y
HEM - A2	25µM	0.086	0.118	0.12	29.837	N
HEM - B2	37.5µM	0.094	0.018	0.103	35.602	Y
HEM - C2	50µM	Undeter.	Undeter.	Undeter.	Undeter.	y
HEM - D2	75µM	Undeter.	Undeter.	Undeter.	Undeter.	Y
HEM - E2	100µM	Undeter.	Undeter.	Undeter.	Undeter.	Y

HA = humic acid
HEM = hematin

Table 9. STR profile comparisons between the standard Quantifiler™ Trio and Integrated Quantifiler™ Trio HRM assays.

Assay	% STR Alleles Detected	Mean Allele Peak Height (rfu)*	# of Loci Flagged for Heterozygote Peak Imbalance *(n=115)*^
Standard Quantifiler™ Trio Assay ***(n=5)***	100%	1040.07 ± 588.90	22
Integrated Quantifiler™ Trio HRM assay ***(n=5)***	100%	962.29 ± 563.30	25

*p=0.18 ^p=0.62

Table 10. Confusion matrices generated for D5S18 and D18S51 loci to predict single source genotypes or mixture samples using the Integrated Quantifiler™ Trio HRM assay.

A

D5S818 Actual \ D5S818 Predicted								
SVM Linear	1011	1111	1112	1113	1212	1213	1313	Mix
1011	4	0	2	1	0	0	2	0
1111	2	3	1	0	0	0	1	1
1112	4	1	2	1	1	1	1	5
1113	0	0	0	4	1	2	0	0
1212	3	1	0	0	3	2	1	2
1213	1	0	0	1	1	6	0	1
1313	1	1	1	0	1	1	1	0
Mix	1	0	1	7	1	2	0	4
Single Source Prediction Accuracy: 86.76%								
Mixture Prediction Accuracy: 25%								

B

D18S51 Actual \ D18S51 Predicted								
SVM Radial	1213	1214	1215	1216	1314	1316	1415	Mix
1213	1	2	1	0	0	0	2	2
1214	2	1	2	1	0	2	1	2
1215	1	1	0	0	0	5	3	2
1216	1	2	0	0	0	3	0	3
1314	1	6	0	0	0	3	0	2
1316	2	0	0	0	1	2	1	2
1415	0	1	0	1	1	1	3	1
Mix	3	0	0	0	0	3	0	10
Single Source Prediction Accuracy: 79.41%								
Mixture Prediction Accuracy: 62.5%								

Table 11. Combined accuracy of single source and mixture predictions using the Integrated Quantifiler™ Trio HRM assay.

	D5S818	D18S51	Combined Accuracy
Single Source ***(n=68)***	86.76%	79.41%	75%
Mixtures ***(n=16)***	25%	62.5%	68.75%
	Overall Accuracy:		73.8%

Vita

Dayanara Torres was born in 1995 in Rochester, NY where she lived and grew up. In high school she attended School of the Arts where she majored in dance. As a first generation college student, she entered the State University of New York, Brockport in 2014 and graduated in 2020 with a Bachelor of Science having double majored in Criminal Justice and Biology with a minor in Forensic Science. In 2020, she entered the Forensic Science graduate program at Virginia Commonwealth University where she was awarded a Graduate Teaching Assistantship. In the Spring of 2021, she began her directed research in Dr. Tracey Dawson Green's Forensic Molecular Biology laboratory working on the labs HRM project.