Executive Summary

Title: Validation of the Quantifiler™ Trio DNA quantification kit

Purpose: To evaluate the use of Quantifiler Trio for quantification of male and human DNA, and to characterize the relationship between Trio values and Globalfiler amplification results.

Investigators: Redacted

Initiation date: August 29, 2017

End date: November 8, 2019

Background:

Currently, the FBI Laboratory's DNA Casework Unit (DCU) and Scientific & Biometrics Analysis Unit (SBAU) use the Life Technologies (ThermoFisher) Quantifiler Duo realtime PCR kit to quantify the concentration of human genomic DNA and the male component in extracts before nuclear DNA analysis is performed. The Quantifiler Trio kit, also by Life Technologies, introduces new reporter dyes designed to increase spectral resolution and sensitivity. It also targets two genomic amplification fragments of differing lengths, allowing an assessment of DNA degradation, which will not be used by the FBI due to its previously established unreliability when analyzing low quantity samples. This validation assessed the Quantifiler Trio kit in accordance with the SWGDAM guidelines for internal validation. In addition to identifying the sensitivity and limitations of the kit, this study served to determine the acceptable operating parameters of the standard curve, to characterize the relationship between Trio quantification and Globalfiler amplification, and to ensure that the use of automated systems for reaction setup is reliable and repeatable. Finally, the data collected here were used to evaluate the feasibility of a 'zero is zero' processing policy in which no amplification is attempted if a zero quant is obtained.

Supporting Documentation: Associated documentation and data files are located on the DSU\Projects\Trio folder

Validation summary:

Accuracy and precision were first tested by pipetting standards across a plate and analyzing them as samples. As standards are designed to be of a known quantity, they should yield an expected measurement when analyzed as samples. A high **accuracy** of the kit was confirmed with a percent error (actual value compared to expected value) of only 1.53 when standards were measured as samples. The **precision** of the kit was tested by quantifying a collection of 24 known samples in triplicate, yielding a coefficient of variation (variation about the mean) of only 0.04, meaning that on average, the standard deviation was only 4% of the mean.

Contamination was evaluated by three experiments in which plates were prepared on DCU Tecans. The first plate, which was designed to detect environmental contamination, contained 83 blanks and yielded quantifiable signal ≤0.0004 ng/ul in 11 wells. However, when amplified using Globalfiler and analyzed with a PAT of 50 RFU, no alleles were detected. The second and third tests consisted of zebra and checkboard plates, respectively, and were designed to test for sample carryover on the tips of the Tecan. Nine blanks yielded signal on the zebra plate and 3 yielded signal on the checkerboard, none of which were greater than 0.0009 ng/ul. Again, when amplified using Globalfiler, no alleles were detected. The extreme sensitivity of Trio appears to result in sporadic signal, but in practice such signal does not result in data when amplified.

Sensitivity of the Trio kit was evaluated through the quantification of two male samples and a female sample across a dilution range from 10 ng/ul to 0.0001 ng/ul. The results of the experiment showed that Trio was capable of consistently detecting DNA at the lowest levels tested. Quantifiler Duo, in comparison, appeared to have a lower limit of detection of 0.001 ng/ul. When amplified, it became clear that Trio is far more sensitive than Globalfiler. When amplifying samples below 0.001 ng/ul, no alleles were detected when analyzed with a PAT of 150 RFU. When samples above 0.01 ng/ul were amplified, full profiles were obtained. The results of this study support the implementation of a 'zero is zero' policy and indicate that an even higher quantification limit (such as 0.0005) may be appropriate with further analysis of casework-derived data. It is important to note that this determination is based on an amplification input volume of 10 ul. If future protocols incorporate an option to amplify 15 ul of input volume, a new sensitivity study will be required.

Testing of **case-type samples** yielded data in agreement with the sensitivity study, showing that samples that quantify below 0.001 ng/ul did not yield data when amplified with Globalfiler. Unlike the sensitivity study however, a measurement of 0.02 ng/ul was required before a full profile was obtained with Globalfiler. Low level samples were collected from personal items and showed that Trio was capable of detecting a male minor contributor when Duo was not.

Mixtures were generated using two males and a female, and covered a range of ratios from 1:1 to 1:200, with each male being represented as either the major or minor contributor, for a total of four sets. The results showed that in samples less than 1 ng/ul, Trio is capable of detecting a male minor contributor as low as 1:200 (1:183 program-calculated), while Duo failed to detect one male below 1:20 and the other below 1:50. When amplified, Globalfiler was unable to detect the male minor at ratios greater than 1:100, again demonstrating that Trio is more sensitive than Globalfiler.

Analysis of 30 standard curves generated over the course of the validation allowed for the establishment of acceptable operating parameters. For the human small target, the slope should be between -3.435 and -3.115, the Y-intercept should be between 25.678 and 27.716 and R^2 should be ≥0.996. For the human large target, the slope should be between -3.664 and -3.185, the Y-intercept should be between 23.496 and 25.892 and R^2 should be ≥0.997. For the male target, the slope should be between -3.574 and -3.116, the Y-intercept should be between 24.697 and 27.296, and R^2 should be ≥0.995. A month-long **stability** study was conducted to determine how long a Tecan-prepared standard curve would meet these operating parameters and showed that prepared standards should be discarded after 5 days.

In order to evaluate the sensitivity of the kit to **inhibition**, and to compare its response to that of Globalfiler, five inhibitors commonly encountered in case-type samples were introduced at varying

concentrations into the quant/amp workflow. With the possible exception of the blue dye indigo, the results showed that Trio was equally or less susceptible to inhibition than Globalfiler.

Site specific studies including assessments of contamination, sensitivity, and precision were performed in Huntsville and all results fell within the expected ranges established during validation at Quantico.

Conclusion:

This validation study has shown the Quantifiler Trio kit to be extremely sensitive and highly reproducible. In fact, this kit is at least 10 times more sensitive than the currently used Quantifiler Duo kit and can detect a male minor contributor at a level four times lower. Because the kit is significantly more sensitive than the Globalfiler amplification kit, a casework processing policy of ‘zero is zero’ is considered valid based on the data generated here.