Internal Validation Guide of qPCR Systems for Forensic Laboratories

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1. Introduction

Before an established method or procedure can be employed in a forensic laboratory, an internal validation must be completed to show that the method performs as expected. This guide outlines experiments that confer conformance with validation guidelines outlined by both the Scientific Working Group on DNA Analysis Methods (SWGDAM, http://swgdam.org/SWGDAM_Validation_Guidelines_APPROVED_Dec_2012.pdf) and European Network of Forensic Science Institutes (ENFSI) DNA Working Group (http://www.enfsi.eu/sites/default/files/documents/minimum_validation_guidelines_in_dna_profiling_-_v2010_0.pdf). These experiments are designed to demonstrate the sensitivity and reliability of a real-time quantitative PCR (qPCR) system. Upon completion of these experiments, you will have sufficient data to determine the following: optimal DNA concentration for input into short tandem repeat (STR) amplification reactions, sensitivity to inhibitors and degraded DNA, and the limit of detection of the assay. The correlation between DNA quantification and STR results will be assessed, including determining when Y-STR analysis might be more appropriate than autosomal STR analysis for samples containing a mixture of male and female DNA as well as when a profile might exhibit signs of DNA degradation or PCR inhibition.

2. General Considerations

Prior to beginning any internal validation study, ensure that you have the required reagents and tools necessary to complete the study. Do not perform the experiments described within this guide using DNA derived from cell lines. We recommend that you perform these tests with sample types that are commonly encountered in your laboratory. Long-term storage and storage conditions

may affect the stability of genomic DNA and the ability to generate a quantification result. For all experiments, prepare dilutions of the DNA standard as specified for the particular qPCR system. Refer to Section 5.A of this manual for an example using the PowerQuant™ System.

Note: The PowerQuant™ Male gDNA Standard and Plexor® HY Male Genomic DNA Standard are mixtures of human male DNA and are not suitable for use in experiments designed to evaluate the correlation between DNA quantification and STR results.

3. SWGDAM-Recommended Studies

3.A. Known and Nonprobative Evidence Samples or Mock Evidence Samples

DNA quantification of known and nonprobative evidence samples or mock evidence samples allows you to compare your current DNA quantification method to the new system being validated. Select five to ten single-source known samples such as an STR kit positive control DNA, NIST SRM sample or staff DNA to be analyzed. Be sure to select at least two single-source male DNAs with expected concentrations ≥10ng/µl and two single-source female DNAs with expected concentrations ≥50ng/µl. These will be used for subsequent validation studies. If the qPCR kit will be used with casework samples, examine five to ten nonprobative evidence samples or mock evidence samples. Quantify each sample in duplicate.

Determining the Optimal Target Amount of DNA for STR Systems

If a new STR system will be validated in conjunction with the new qPCR system, perform the studies recommended in the *Internal Validation Guide of Autosomal STR Systems for Forensic Laboratories Reference Manual* #GE053 or *Internal Validation Guide for Y-STR Systems in Forensic Laboratories Reference Manual* #GE713 to determine the optimal target amount of DNA based on the quantification values obtained using the new qPCR system.

If the new qPCR system will be integrated with the laboratory's current STR system(s), perform the following calibration study to determine the amount of template DNA required to produce STR results comparable to the laboratory's current quantification methods.

Following the process described above in the Known and Nonprobative Evidence Samples study, quantify the same samples using your laboratory's current qPCR system.

Perform the calculations provided below to determine the optimal DNA concentration to be used for STR analysis based on the new qPCR quantification results.

Once you have performed the calculations, assemble two sets of amplifications for each STR system using known and nonprobative evidence samples; use the quantification values from the current qPCR system to assemble one set of amplification reactions and the target amount determined using the new qPCR system to assemble the second set of amplification reactions. Confirm that the autosomal and Y target DNA concentrations determined using the new qPCR system provide results consistent with the current qPCR system.

Determining the Optimal DNA Concentration Based on the Quantification Results from the New qPCR System.

- Calculate the average quantification value for each sample with each qPCR system.
- For each sample, calculate the autosomal difference ratio between the two qPCR systems using the following equation:

$$\frac{\text{[Average autosomal quantitation value in new qPCR system]}}{\text{[Average autosomal quantitation value in current qPCR system]}} = \text{autosomal difference ratio}$$

- For each sample, calculate the Y difference ratio between the two qPCR systems using the following equation:

$$\frac{\text{[Average Y quantification value in new qPCR system]}}{\text{[Average Y quantification value in current qPCR system]}} = \text{Y difference ratio}$$

- Calculate the average autosomal and Y difference ratios across all samples.
- Calculate the new target DNA concentration for both autosomal STR and Y-STR systems using the following equations:

Current qPCR system autosomal target DNA concentration × average autosomal difference ratio = new qPCR system autosomal target DNA concentration

Current qPCR system Y target DNA concentration × average Y difference ratio = new qPCR system Y target DNA concentration

3.B. Sensitivity Studies

Sensitivity studies demonstrate the dynamic range of an assay. By testing a range of DNA concentrations, you can define the limit of detection as well as determine the quantity of template DNA below which you cannot expect to obtain an STR profile. Choose two male DNA samples from the Known Samples study (Section 3.A). Prepare DNA at concentrations of 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.6pg/µl, 0.4pg/µl and 0.1pg/µl. Each series should be quantified in triplicate. Refer to Section 5.B for an example of sample preparation.

Following DNA quantification, amplify the 0.1–100pg/µl samples in the following manner with each of your STR amplification systems:

Add the appropriate amount of the 100pg/µl sample to achieve the optimal target amount determined in the calibration study (Section 3.A, Determining the Optimal Target Amount of DNA for STR Systems). Add the maximum sample volume allowed for the STR kit for the 1pg/µl, 0.6pg/µl, 0.4pg/µl and 0.1pg/µl samples.

Following amplification, inject samples on your capillary electrophoresis (CE) instrument using the desired injection parameters.

3.C. Precision and Accuracy

Determine the precision and accuracy of the system by examining the quantification values for replicate quantifications of the DNA standard. Calculate the mean, standard deviation and/or coefficient of variance of the C_q values for each target (e.g., autosomal, Y, IPC and degradation).

To demonstrate the system's repeatability, the same operator should quantify six replicates of the DNA standard dilution series. To demonstrate the system's reproducibility, a different operator should quantify six replicates of the DNA standard dilution series in a separate plate.

3.D. Mixture Studies

Results from the mixture studies can be used to evaluate contamination in database laboratories and assist casework laboratories in establishing guidelines for downstream processing of mixtures of male and female DNA. For example, examining autosomal/Y ratios can allow you to define when autosomal STR analysis is likely to produce a clear male profile, when autosomal and Y-STR analysis in combination may provide complementary information, and when only Y-STR analysis is expected to provide a profile for the male contributor. Quantify a minimum of two male:female mixture sets in duplicate using the following ratios: 1:1, 1:5, 1:10, 1:15, 1:20, 1:25, 1:50 and 1:100. The amount of male template DNA per reaction should be the optimal target amount determined in the calibration study (Section 3.A, Determining the Optimal Target Amount of DNA for STR Systems). See Section 5.C for an example of sample setup for mixture studies.

Following DNA quantification, amplify each mixture sample using each of the laboratory's STR amplification systems. Use the quantification value that corresponds to the appropriate STR amplification system (i.e., autosomal quantification results for autosomal STR analysis, Y quantification results for Y-STR analysis). Target the optimal amount of template DNA determined in the calibration studies (Section 3.A, Determining the Optimal Target Amount of DNA for STR Systems). Inject samples on your CE instrument using the desired injection parameters.

Review the autosomal/Y quantification ratios in conjunction with the STR results to determine guidelines for downstream STR analysis.

3.E. Contamination Assessment

To ensure that the laboratory's sample-handling process minimizes the risk of contamination, include a minimum of one no-template control for every set of qPCRs throughout the process. Evaluate each no-template control for the presence of exogenous DNA at the quantification and amplification stages of the process.

4. Additional Studies

4.A. Assessment of Degradation Target

Examining the autosomal/degradation ratio can assist you in defining when STR analysis is likely to produce a degraded profile (e.g., ski slope). Choose two DNA samples from the Known Samples study (Section 3.A). Expose separate aliquots of each sample to the following amount of UV radiation: 0mJ, 50mJ, 100mJ or 300mJ. Alternatively, samples may be heat-treated at 95°C for increasing lengths of time: 0 minutes, 30 minutes, 60 minutes, 90 minutes or 120 minutes. Quantify each treated sample in duplicate.

Following DNA quantification, amplify each sample using each of the laboratory's STR amplification systems, targeting the optimal amount of template DNA determined in the calibration studies (Section 3.A, Determining the Optimal Target Amount of DNA for STR Systems). Inject samples on your CE instrument using the desired injection parameters.

Review the autosomal/degradation ratios in conjunction with the STR results to determine the ratio above which you are likely to observe a degraded profile.

4.B. Assessment of IPC in the Presence of Inhibitors

Examining the C_q shift of the IPC can help you define when STR analysis is likely to produce a profile with symptoms of PCR inhibition (e.g., locus-specific imbalance). Choose two DNA samples from the Known Samples study (Section 3.A). Prepare separate aliquots of each sample, holding the amount of DNA constant while increasing the amount of inhibitors. Ensure the amount of DNA is sufficiently low to require the maximum input volume for the laboratory's STR amplification system (e.g., 15µl for PowerPlex® Fusion). See Section 5.D for examples of sample preparation using humic acid and hematin. Quantify each treated sample in duplicate.

Following DNA quantification, amplify each sample using each of the laboratory's STR amplification systems. Inject samples on your CE instrument using the desired injection parameters.

Review the C_q shift of the IPC in conjunction with the STR results to determine the shift above which you are likely to observe an inhibited profile.

4.C. Assessment of the Standard Curve

Examining the variation in the slope, Y-intercept and R^2 values of the standard curve for the autosomal, Y and degradation targets can help you establish guidelines for evaluating acceptable standard curve values. Using data from a minimum of five plates, calculate the average and standard deviation for the slope, Y-intercept and R^2 values for each of the targets: autosomal, Y and degradation. Use the average ± three standard deviations to determine the range of these values. Compare these values to those stated by the manufacturer of the qPCR kit, and choose the range that is appropriate for your laboratory.

5. Appendix

5.A. Standard Curve Preparation

Table 1 shows an example of standard curve preparation following the guidelines for the PowerQuant™ System.

Table 1. Standard Curve Preparation for the PowerQuant™ System.

DNA Concentration	Volume of DNA	Volume of TE^{-4} Buffer (µl)
50ng/µl	Use undiluted PowerQuant™ Male gDNA Standard	0µl
2ng/µl	Use 4µl of 50ng/µl	96µl
0.08ng/µl	Use 4µl of 2ng/µl	96µl
0.0032ng/µl	Use 4µl of 0.08ng/µl	96µl

5.B. Sample Setup for Sensitivity Studies

Table 2 shows an example of how to dilute genomic DNA at an initial concentration of 10ng/µl for use in sensitivity studies. Prepare these dilutions using at least two different male DNA samples.

Note: Store DNA templates in TE^{-4} buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA) or TE^{-4} buffer with 20µg/ml glycogen. See the Composition of Buffers and Solutions section in any of the PowerPlex® STR System Technical Manuals for the composition of these buffers.

Table 2. Dilution of Genomic DNA for Sensitivity Studies.

Tube	DNA Concentration	Volume of DNA	Volume of TE^{-4} Buffer
A	1ng/µl	12µl of DNA (10ng/µl)	108µl
B	100pg/µl	10µl from Tube A	90µl
C	10pg/µl	10µl from Tube B	90µl
D	1pg/µl	10µl from Tube C	90µl
E	0.6pg/µl	6µl from Tube C	94µl
F	0.4pg/µl	4µl from Tube C	96µl
G	0.1pg/µl	10µl from Tube D	90µl

5.C. Sample Setup for Mixture Studies

Table 3 shows an example of how to dilute female DNA samples at an initial concentration of 50ng/µl and male DNA samples at an initial concentration of 10ng/µl for use in the mixture studies. Prepare these dilutions using at least two different female and male DNA samples for a total of four samples.

Table 3. Dilution of Genomic DNA Samples.

DNA Sample	DNA Concentration	Volume of DNA	Volume of TE^{-4} Buffer
Female DNA			
H	50ng/µl	At least 50µl of female DNA (50ng/µl)	0µl
I	10ng/µl	10µl from Tube H	40µl
J	1ng/µl	10µl from Tube I	40µl
Male DNA			
K	1ng/µl	30µl of male DNA (10ng/µl)	270µl

Table 4. Mixture Sets. Ratios of DNA Samples Using Each Diluted Sample from Table 3.

Ratio		Diluted DNA Concentration (ng/µl)		Volume of Diluted DNA Required to Prepare Mixture Set (µl)			DNA Concentration in qPCR (ng/µl)		
Female	Male	Female	Male	Female	Male	TE^{-4} Buffer	Female	Male	Total Human
1	1	1.0	1.0	7.5	7.5	15	0.25	0.25	0.5
5	1	10	1.0	3.75	7.5	18.75	1.25	0.25	1.5
10	1	10	1.0	7.5	7.5	15	2.5	0.25	2.75
15	1	50	1.0	2.25	7.5	20.25	3.75	0.25	4
20	1	50	1.0	3.0	7.5	19.5	5.0	0.25	5.25
25	1	50	1.0	3.75	7.5	18.75	6.25	0.25	6.5
50	1	50	1.0	7.5	7.5	15	12.5	0.25	12.75
100	1	50	1.0	15	7.5	7.5	25	0.25	25.25

To prepare a mixture set, combine the DNA samples prepared in Table 3 at the volumes indicated in Table 4. Prepare this mixture set using two different female and male DNA samples for a total of two female:male mixture sets.

5.D. Sample Setup for Inhibitor Studies

To prepare an inhibitor study set, combine the volumes of 1ng/µl male DNA sample prepared in Table 3 (i.e., Sample I), inhibitor stock solution (5,000ng/µl humic acid or 2,000µM hematin) and water as indicated in Table 5. This setup assumes a maximum input volume of 15µl for STR analysis and an optimal template amount of 500pg. If the maximum input volume and optimal template amount differ from those used for your laboratory's STR system, adjust the volumes accordingly.

Table 5. Preparing the Inhibitor Study Samples.

Humic Acid

Sample	Volume of 5,000ng/µl Humic Acid Stock	Volume of Sample I	Volume of Water	Final Volume	10X Inhibitor Concentration	Final Inhibitor Concentration in qPCR[1]
L	19.2µl	16.0µl	444.8µl	480µl	200ng/µl	20ng/µl
M	38.4µl	16.0µl	425.6µl	480µl	400ng/µl	40ng/µl
N	57.6µl	16.0µl	406.4µl	480µl	600ng/µl	60ng/µl

Hematin

Sample	Volume of 2,000µM Hematin Stock	Volume of Sample I	Volume of Water	Final Volume	10X Inhibitor Concentration	Final Inhibitor Concentration in qPCR[1]
O	240.0µl	16.0µl	224.0µl	480µl	1,000µM	100µM
P	320.0µl	16.0µl	144.0µl	480µl	1,333µM	133µM
Q	400.0µl	16.0µl	64.0µl	480µl	1,667µM	167µM

[1]Assumes 2µl of the inhibitor study sample is added to each qPCR.