Internal Validation Guide of Autosomal STR Systems for Forensic Laboratories

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1. Introduction

Before an established method or procedure may be employed in a forensic laboratory, an internal validation must be completed to show that the method performs as expected. This guide outlines a set of experiments that confers 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 (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 an autosomal short tandem repeat (STR)-typing multiplex system. Upon completion of these experiments the laboratory will have sufficient data to determine the analytical and stochastic thresholds of the capillary electrophoresis (CE) instrument in combination with the amplification system, the impact of multiple contributors to a DNA sample and the limit of detection and heterozygote balance of the assay.

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. Long-term storage and the storage conditions may affect the stability of genomic DNA and the ability to develop an STR profile. Note that installing fresh polymer and a new capillary and performing a spectral calibration prior to performing validation experiments will provide optimal run conditions.

3. SWGDAM-Recommended Studies

3.A. Sensitivity and Stochastic Studies

Sensitivity studies are used to demonstrate the dynamic range of an assay. By testing a range of DNA concentrations, the laboratory can define the analytical threshold, dynamic range, optimal target range, heterozygote balance, stutter ratios and stochastic threshold. Using quantitation results from your laboratory's approved DNA quantitation method, amplify the following amounts from at least two different individuals: 1.0ng, 500pg, 250pg, 125pg, 62.5pg and 31.2pg. Selecting individuals with a high level of allele heterozygosity is desirable. Each series should be amplified in triplicate and injected on your CE instrument using the desired injection parameters. Refer to the appendix (Section 4.A) for an example of sample preparation.

Calculating Analytical Threshold

Signal-to-noise assessment of sensitivity data can help define an appropriate analytical threshold for the amplification system on the chosen CE instrument. The method described below is one of many scientific methods that can be used to analyze these data.

Select the sensitivity sample profiles that contain minimal pull-up and are within the linear range of the CE instrument. Examine the baseline for "noise" peaks—those that are not part of the profile, not stutter peaks and not known artifacts of the system. To obtain peak height values for these low-level "noise" peaks, ensure that the calling threshold in your analytical software system is set sufficiently low, such as 1RFU. Figure 1 shows an example of a profile highlighting peaks that should not be used in the calculation.

Once the "noise" peaks are identified, calculate the average peak height and standard deviation for these peaks. Use the average peak height plus X standard deviations as the analytical threshold, where X refers to the number of standard deviations needed to achieve the desired confidence level. Choose a confidence level that is appropriate for your laboratory. Table 1 displays the relationship between the number of standard deviations and confidence interval distribution.

Repeat this calculation for all injection parameters that the laboratory plans to use.

Table 1. Standard Deviation and Confidence Intervals.

Standard Deviation	Confidence Interval (%)
1	68.27%
2	95.45%
3	99.73%
4	99.99366%
5	99.99994%
6	99.9999998%
7	99.999999999%
10	99.9999999999%

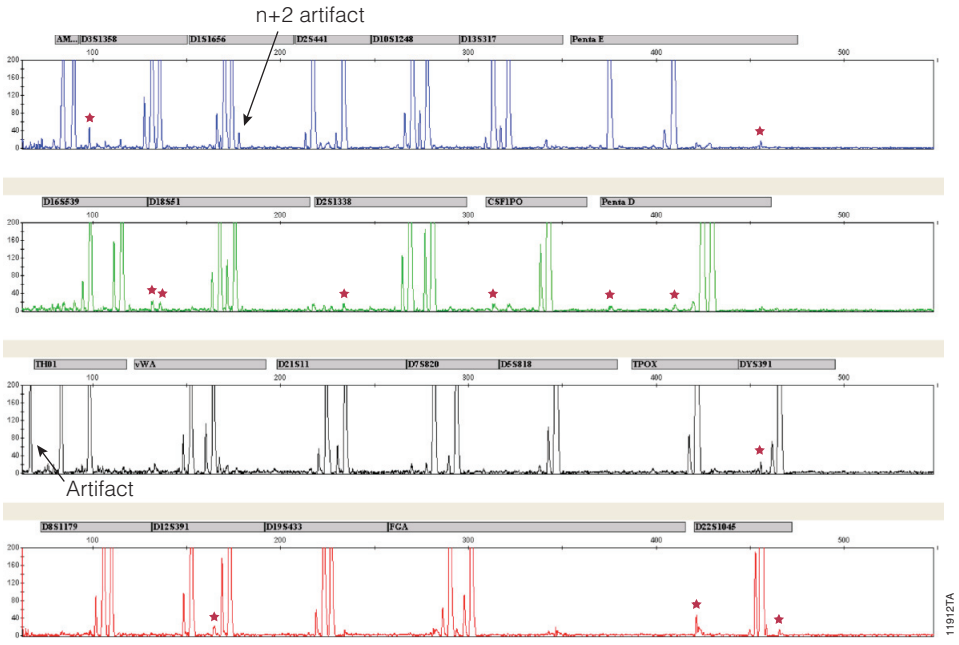

Figure 1. A profile generated using 0.5ng of DNA. Pull-up peaks are marked with stars. Artifacts are indicated by arrows.

Calculating Stochastic Threshold

Data from the sensitivity studies also can be used to define a stochastic threshold. One method of calculating this threshold is to examine heterozygous loci where one sister allele has dropped below the previously established analytical threshold. Calculate the average peak height and standard deviation of the observed false homozygotes. The stochastic threshold will be the average peak height plus three standard deviations. Repeat this calculation for all injection parameters the laboratory plans to use.

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

Amplification of known and nonprobative evidence samples or mock evidence samples allows the laboratory to provide evidence of concordance using the new amplification system. Five to ten known samples such as the kit's positive control, NIST SRM samples and staff profiles should be analyzed to verify the correct type. In addition, if the kit will be used to examine casework samples, five to ten nonprobative evidence samples or mock evidence samples should be examined.

3.C. Precision and Accuracy

Data from samples that were run during other studies (e.g., sensitivity samples and known and nonprobative evidence samples) can be used to assess the system's precision and accuracy.

The precision of the system can be determined by examining five to ten allelic ladders across multiple injections. Calculate the average base pair size and standard deviation for each allele in the allelic ladder. Three times the standard deviation for each allele should be less than 0.5bp.

To demonstrate the system's repeatability, a minimum of one sample should be amplified and typed a minimum of five times by the same operator. To demonstrate the system's reproducibility, a minimum of one sample should be amplified and typed a minimum of five times by different operators.

3.D. Mixture Studies

Results from mixtures studies can be used to evaluate contamination in database laboratories and assist casework laboratories in establishing mixture interpretation guidelines. A minimum of two mixture sets should be tested using the following ratios: 19:1, 9:1, 3:1, 1:1, 1:3, 1:9 and 1:19. The total amount of template DNA per reaction should be the optimal target amount determined in the sensitivity studies (Section 3.A). See the appendix (Section 4.B) for an example of sample setup using six individuals.

3.E. Contamination Assessment

To ensure that the laboratory's sample-handling process minimizes the risk of contamination, evaluate negative controls for the presence of exogenous DNA in every amplification set up throughout the validation process.

4. Appendix

4.A. 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. The values shown in Table 2 were calculated by assuming the input volume required to achieve the target template amount is 2µl.

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 (10mM Tris-HCl [pH 8.0], 0.1mM EDTA, 20µg/ml glycogen). See the Composition of Buffers and Solutions section of any *PowerPlex® System Technical Manual* for instructions to prepare these buffers.

Table 2. Serial Dilution of Genomic DNA for Sensitivity Studies.

Tube	Template Amount per 2µl (per Reaction)	Volume of DNA	Volume of TE^{-4} Buffer	DNA Concentration
A	1.0ng	5µl of DNA (10ng/µl)	95µl	500pg/µl
B	500pg	50µl from Tube A	50µl	250pg/µl
C	250pg	50µl from Tube B	50µl	125pg/µl
D	125pg	50µl from Tube C	50µl	62.5pg/µl
E	62.5pg	50µl from Tube D	50µl	31.2pg/µl
F	31.2pg	50µl from Tube E	50µl	15.6pg/µl

4.B. Sample Setup for Mixture Studies

Table 3 shows an example of sample setup using six individuals. The values shown in Table 3 were calculated by assuming the input volume required to achieve the target template amount is 2µl. For example, if 500pg of DNA is the target amount of DNA, normalize all DNA samples for the mixture studies to 250pg/µl.

Table 3. Dilution of Genomic DNA Samples.

DNA Sample	Volume of DNA at 10ng/µl	Volume of TE^{-4} Buffer	DNA Concentration
Sample A	10µl	390µl	250pg/µl
Sample B	10µl	390µl	250pg/µl
Sample C	10µl	390µl	250pg/µl
Sample D	10µl	390µl	250pg/µl
Sample E	10µl	390µl	250pg/µl
Sample F	10µl	390µl	250pg/µl

To prepare mixture set 1, combine the DNA samples prepared in Table 3 at the volumes indicated in Table 4.

Table 4. Volume of Each Diluted DNA Sample Required to Prepare Mixture Set 1.

	Mixture Ratio						
	19:1	9:1	3:1	1:1	1:3	1:9	1:19
Sample A	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Sample B	5µl	10µl	25µl	50µl	75µl	90µl	95µl

To prepare mixture set 2, combine the DNA samples prepared in Table 3 at the volumes indicated in Table 5.

Table 5. Volume of Each Diluted DNA Sample Required to Prepare Mixture Set 2.

	Mixture Ratio						
	19:1	9:1	3:1	1:1	1:3	1:9	1:19
Sample C	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Sample D	5µl	10µl	25µl	50µl	75µl	90µl	95µl

To prepare mixture set 3, combine the DNA samples prepared in Table 3 at the volumes indicated in Table 6.

Table 6. Volume of Each Diluted DNA Sample Required to Prepare Mixture Set 3.

	Mixture Ratio						
	19:1	9:1	3:1	1:1	1:3	1:9	1:19
Sample E	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Sample F	5µl	10µl	25µl	50µl	75µl	90µl	95µl