Promega

REFERENCE MANUAL

Internal Validation Guide of Y-STR Systems for Forensic Laboratories

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1. Introduction

Prior to implementing a new method for use with forensic samples, a complete internal validation study must be performed. This guide lists a set of experiments for use or adaptation by laboratories performing an internal validation of a Y-STR amplification system. These studies are intended to demonstrate the sensitivity and reliability of the Y-STR system for development of Y-STR profiles. These experiments are sufficient to determine the following: optimal amount of male DNA input, the analytical threshold for your capillary electrophoresis (CE) instrument in combination with the amplification system, the lower limit of detection for reliable profile results, the effect of excessive amounts of female DNA on the efficiency of the amplification, and the impact of multiple males in a DNA profile.

2. General Considerations

Before beginning any internal validation study, ensure that you have the required reagents and tools to perform the study. Do not perform the experiments described within this guide using DNA samples derived from cell lines. Long-term storage, storage conditions and storage buffer of genomic DNA may affect the ability to develop Y-STR profiles. Use designated work areas and pipettes for pre- and post-amplification steps to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid from one run to the next. Wear gloves, and change them often, especially after handling high-concentration DNA samples. **Note:** Installing fresh polymer, a new capillary and spectral calibration prior to performing validation experiments will provide optimal run conditions.

2. General Considerations (continued)

The following experiments are in accordance with the SWGDAM recommended guidelines.

http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/jan2009/standards/2009_01_standards01.htm/.

3. Calculating Analytical Threshold

To determine the analytical threshold for your amplification system in combination with your capillary electrophoresis instruments, run samples from the sensitivity study as described in Section 4.C under the recommended amplification conditions. Make sure to include the amplification conditions and capillary electrophoresis parameters that you will be using in your laboratory. Analyze sample profiles from the sensitivity study that contain minimal pull-up peaks and are within the linear range of the CE instrument. Calculate the baseline by using peaks that are "noise" peaks and not part of the profile. To obtain peak heights for the baseline/noise, either use a low-calling threshold in the GeneMapper® software settings or determine values by viewing the y-axis value for a peak that has been chosen by placing the arrow over the highest point of the peak. Figure 1 is a zoomed-in image of a profile that was generated using 0.5ng of male genomic DNA. The peaks with stars above them are pull-up peaks; do not use them to calculate the baseline. Also, do not include any stutter peaks or known artifacts like the ones indicated by arrows in Figure 1.

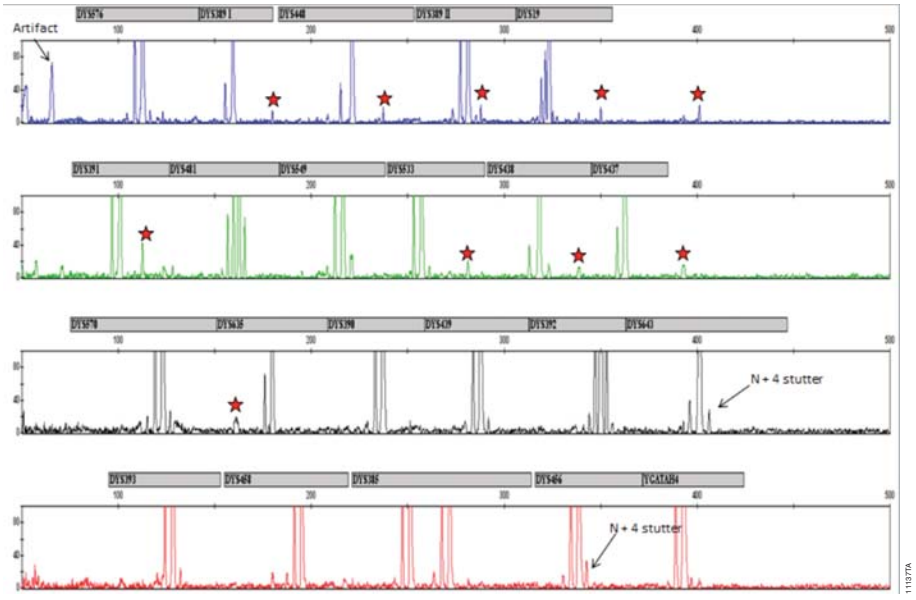

Figure 1. Image of a profile generated using 0.5ng of male DNA. Pull-up peaks are indicated with stars. Examples of stutter peaks and artifacts are indicated by arrows.

Calculate the average and standard deviation based on the chosen peaks. Use the average plus X standard deviations. The number of standard deviations will depend on the level of confidence that your laboratory is comfortable using. Refer to Table 1 for the relationship between the number of standard deviations and the confidence interval distribution.

Table 1. Standard Deviations.

Standard Deviation	Percentage
1	68.27%
2	95.45%
3	99.73%
4	99.99366%
5	99.99994%
6	99.9999998%
7	99.999999999%
10	99.9999999999%

4. SWGDAM Recommended Studies

4.A. Known and Non-Probative Evidence Samples

This set of samples will test the amplification kit's ability to amplify samples that are typically seen in the laboratory. The new Y-STR system should be tested with previously typed samples to verify the same profile is obtained with the new amplification kit. Test five to ten known samples representing all sample types as well as 10 to 15 non-probative/mock evidence samples, including the most common sample types.

4.B. Reproducibility and Precision

Reproducibility samples are used to demonstrate that the profile obtained in your laboratory is concordant with the profile observed in other laboratories. Testing of the NIST SRM 2391C samples, or the positive control included in the amplification kit, is recommended for the reproducibility study. To determine the precision of the internal lane standard and instrument, run five to ten allelic ladders across multiple injections and calculate the average base pair size and standard deviation for each of the alleles in the allelic ladder. Three times the standard deviation of each allele should be below 0.5bp.

4.C. Sensitivity Studies

Sensitivity samples are used to show the dynamic range of the amplification system. Test a variety of template amounts to determine the template amount that causes saturation on the CE instrument, the lower limit of the amplification yielding reliable results and the optimal template amount. We recommend testing a template range of 1ng, 500pg, 250pg, 125pg, 62.5pg and 31.25pg of male DNA in triplicate. Determine template amounts using the DNA quantitation method used in your laboratory. Testing three to five different male DNA samples will allow the laboratory to determine the variability between different individuals. Inject these samples on your CE instrument using the injection parameters being considered for use. These data can be used to determine the dynamic range and sensitivity for the Y-STR system in combination with the injection conditions for the CE instrument. Refer to the Appendix (Section 5.A, Table 2) for an example of sample preparation.

4.D. Mixture Studies

Male/Female Mixtures: The purpose of the male/female mixtures is to determine what effect female DNA has on the efficiency of the amplification. To assess this efficiency, we recommend two different types of experiments. In one experiment male DNA is held constant at the optimal template amount and female DNA is varied. In the other experiment the female DNA is held constant and the male DNA is varied. Refer to the Appendix (Section 5.A) for an example of sample preparation.

Male Variable/Female Constant: Mix 500pg, 250pg, 125pg, 62.5pg and 31.25pg of male DNA with 400ng of female DNA (Table 4). **Note:** Female DNA must be clean and free of male DNA.

Male Constant/Female Variable: Mix 0.5ng of male (or optimal template) with 10ng, 50ng, 100ng, 200ng or 400ng of female DNA (Table 5). **Note:** Female DNA must be clean and free of male DNA Tab.

Male/Male Mixtures: The purpose of the male/male mixtures is to determine at which ratio a clear major profile and minor profile can be determined. This experiment also aids in establishing the ratio for reproducible observation of the minor profile. We recommend using three mixture sets in the following ratios: 19:1, 9:1, 3:1, 1:1, 1:3, 1:9 and 1:19. The total amount of DNA per reaction should be the optimal template amount determined during the sensitivity study (Section 4.C). At Promega, we found 0.5ng of male DNA to be our optimal template. Each mixture set should be amplified in triplicate, and multiple males should be used in combination. Refer to the Appendix (Section 5.B) for an example of sample setup using samples from six males.

4.E. Contamination

The contamination study demonstrates that the laboratory's procedures for handling samples minimizes the risks for contamination. To check for possible contamination, random negative controls should be placed across the amplification plate to show that no unexpected peaks are seen. **Note:** When using robotic platforms more contamination studies should be included (i.e., zebra and checkerboard-patterned amplification plates).

5. Appendix

5.A. Setup for Sensitivity and Male/Female Mixture 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 of the *PowerPlex® Y23 Technical Manual* #TMD035 for composition of these buffers.

Table 2. Serial Dilution of Male Genomic DNA for Sensitivity and Male/Female Mixtures.

Tube	Template Amount per 2µl (per Reaction)	Volume of Male DNA	Volume of TE^{-4} Buffer	DNA Concentration
A	1ng	5µl of male DNA (10ng/µl)	95µl	500pg/µl
B	500pg	50µl of Tube A	50µl	250pg/µl
C	250pg	50µl of Tube B	50µl	125pg/µl
D	125pg	50µl of Tube C	50µl	62.5pg/µl
E	62.5pg	50µl of Tube D	50µl	31.25pg/µl
F	31.25pg	50µl of Tube E	50µl	15.6pg/µl

Table 3. Serial Dilution of Female Genomic DNA for Sensitivity and Male/Female Mixtures.

Tube	Template Amount per 2µl (per Reaction)	Volume of Female DNA	Volume of TE^{-4} Buffer	DNA Concentration
G	400ng	40µl of Female Genomic DNA (200ng/µl)	0µl	200ng/µl
H	200ng	15µl of Tube G	15µl	100ng/µl
I	100ng	15µl of Tube H	15µl	50ng/µl
J	50ng	15µl of Tube I	15µl	25ng/µl
K	10ng	3µl of Tube J	12µl	5ng/µl

Tables 4 and 5 illustrate one possible way of adding DNA for the male/female mixture samples. This setup uses 2µl of the male DNA template and 2µl of the female DNA template in the amplification reaction for a total of 4µl of template per reaction.

Table 4. Samples to be Used for the Variable Male with Constant Female Mixture Amplification Reactions.

Sample	Volume of Normalized Male DNA	Volume of Normalized Female DNA
500pg male plus 400ng female	2µl of Tube B	2µl of Tube G
250pg male plus 400ng female	2µl of Tube C	2µl of Tube G
125pg male plus 400ng female	2µl of Tube D	2µl of Tube G
62.5pg male plus 400ng female	2µl of Tube E	2µl of Tube G
31.25pg male plus 400ng female	2µl of Tube F	2µl of Tube G

5.A. Setup for Sensitivity and Male/Female Mixture Samples (continued)

Table 5. Samples to be Used for the Constant Male with Variable Female Mixture Amplification Reactions.

Sample	Volume of Normalized Male DNA	Volume of Normalized Female DNA
500pg male plus 400ng female	2µl of Tube B	2µl of Tube G
500pg male plus 200ng female	2µl of Tube B	2µl of Tube H
500pg male plus 100ng female	2µl of Tube B	2µl of Tube I
500pg male plus 50ng female	2µl of Tube B	2µl of Tube J
500pg male plus 10ng female	2µl of Tube B	2µl of Tube K

5.B. Setup for Male/Male Mixture Samples

When preparing the male DNA samples, dilute the samples to the concentration that would be used if 2µl of template was going to be added to the amplification reaction. For example, if 500pg of male DNA is the target amount of DNA, normalize all male DNA samples for the male/male mixtures to 250pg/µl.

Table 6. Dilutions of Male Genomic DNA Samples.

Preparation of Diluted Male Samples to 0.25ng/µl	Volume Male Stock DNA at 10ng/µl	Volume of TE^{-4} Buffer
Male A	10µl	390µl
Male B	10µl	390µl
Male C	10µl	390µl
Male D	10µl	390µl
Male E	10µl	390µl
Male F	10µl	390µl

Table 7. Mixture Set 1. Ratios of male DNA samples using each diluted male sample from Table 6.

	Volume of Each Diluted Male DNA Sample						
Male Sample	**19:1 Ratio**	**9:1 Ratio**	**3:1 Ratio**	**1:1 Ratio**	**1:3 Ratio**	**1:9 Ratio**	**1:19 Ratio**
Male A	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Male B	5µl	10µl	25µl	50µl	75µl	90µl	95µl

Table 8. Mixture Set 2. Ratios of male DNA samples using each diluted male sample from Table 6.

	Volume of Each Diluted Male DNA Sample						
Male Sample	**19:1 Ratio**	**9:1 Ratio**	**3:1 Ratio**	**1:1 Ratio**	**1:3 Ratio**	**1:9 Ratio**	**1:19 Ratio**
Male C	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Male D	5µl	10µl	25µl	50µl	75µl	90µl	95µl

Table 9. Mixture Set 3. Ratios of male DNA samples using each diluted male sample from Table 6.

	Volume of Each Diluted Male DNA Sample						
Male Sample	**19:1 Ratio**	**9:1 Ratio**	**3:1 Ratio**	**1:1 Ratio**	**1:3 Ratio**	**1:9 Ratio**	**1:19 Ratio**
Male E	95µl	90µl	75µl	50µl	25µl	10µl	5µl
Male F	5µl	10µl	25µl	50µl	75µl	90µl	95µl

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