



REFERENCE MANUAL

Validation Guide for the Plexor[®] HY System



Validation Guide for the Plexor® HY System

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I. 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 that can be used or adapted for use by laboratories performing an internal validation of the Plexor® HY System(a–g). These studies are intended to show that this quantitation method is a sensitive and reliable method to estimate the quantity of DNA recovered upon extraction from a sample. In addition, these experiments will determine: 1) the optimal DNA concentration for input into short tandem repeat (STR) amplification reactions and 2) when it is more appropriate to perform Y-STR analysis rather than autosomal STR analysis for mixtures of male and female DNA. Laboratories also will be able to define the sensitivity of their STR amplification system and identify a quantitation “cut-off” value below which amplification is not expected to yield a DNA profile.

The developmental validation has been performed and published. For more information, see the Application Notes #AN157 *Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA*.

II. General Considerations

Experimental design is an important aspect of any validation study. Do not perform the experiments described within this manual using DNA samples that are derived from cell lines. The Plexor® HY Male Genomic DNA Standard is a mixture of human male DNA and is not suitable for experiments when a DNA profile will be generated using STR amplification systems. You may use previously extracted samples in some studies, but you should perform a new quantification with the laboratory's current system because DNA concentration can change during long-term storage due to degradation. Long-term storage also may affect the ability to develop STR profiles.

We recommend using designated work areas and pipettes for pre- and postamplification 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. Use of a dedicated set of pipettes can increase run-to-run consistency. Always use the same pipettes to dispense the DNA standards and unknown samples.

Before beginning any internal validation study, ensure that you have the required reagents and tools to perform the validation study. Refer to the *Plexor® HY System Technical Manual* specific to your laboratory's instrumentation for additional information regarding the appropriate reagents, tools and methods. These manuals, AN157 and the forensic release of the Plexor® Analysis Software can be found at: www.promega.com/plexorhy/

III. Reproducibility

III.A. Objectives

The first objective of this study is to demonstrate that the Plexor® HY System is capable of reproducibly quantifying an individual DNA sample. The second objective is to show that quantitation results are reproducible from assay to assay within your laboratory. These experiments will allow you to document the expected within-run and between-run variation.

III.B. Testing Conditions

1. Dilute the 50ng/μl Plexor® HY Male Genomic DNA Standard to 10ng/μl, 0.40ng/μl and 0.016ng/μl, and use this subset of the standard curve to determine the reproducibility of the system.
2. Demonstrate “within-plate” reproducibility by quantifying five replicates of the dilution series on a single plate. These samples represent high-, medium-, and low-concentration samples.
3. Demonstrate “between-plate” reproducibility by quantifying 3–5 replicates of the dilution series on two additional plates. Ideally, the same user will perform these analyses using the same equipment and lot numbers of materials. However, you should create new dilutions for each run.
4. Determine the mean, standard deviation and/or %CV of the autosomal and Y quantitation values for each DNA concentration (i.e., 10, 0.40 and 0.016ng/μl) for “within-plate” and “between-plate” results. Comparing the “within-plate” results allows you to determine the variability inherent to the Plexor® HY System, as compared to variability due to laboratory conditions. You may choose to perform this analysis using C_t values also.

IV. Calibration

IV.A. Objective

During validation of your laboratory's STR systems, you should have determined a target DNA quantity (or range) for STR amplification. With a change in quantitation methods, it is important to reassess the appropriate amount of template DNA to add to each of the STR systems used in your laboratory. The objective of this study is to determine the amount of template DNA, based on quantitation using the Plexor® HY System, required to produce STR results comparable to your laboratory's current methods.

IV.B. Testing Conditions

1. Extract DNA from 3–5 male and 3–5 female samples. Previously extracted samples may be used, but a new quantitation with the laboratory's current quantitation method is required.
2. Determine the concentration of DNA present in these samples using both the Plexor® HY System and the quantitation system currently being used in the laboratory. We recommend that samples be analyzed in duplicate and average quantitation values be used in subsequent steps.
3. Mathematically determine the target mass of DNA that should be used for STR amplifications following Plexor® HY quantitation.

- a. For each of the 6 to 10 samples quantified, calculate both the autosomal and Y difference ratio as follows:

$$\frac{[\text{Autosomal concentration, Plexor}^{\circledR} \text{HY}]}{[\text{Autosomal concentration, current system}]} = \text{autosomal difference ratio}$$

$$\frac{[\text{Y concentration, Plexor}^{\circledR} \text{HY}]}{[\text{Y concentration, current system}]} = \text{Y difference ratio}$$

- b. Determine the average autosomal and Y difference ratios for the 6 to 10 samples.
- c. The new target mass of DNA for amplification can be calculated as follows:

$$\text{Current autosomal target mass} \times \text{average autosomal difference ratio} = \text{new Plexor}^{\circledR} \text{HY quantitation autosomal target input}$$

$$\text{Current Y target mass} \times \text{average Y difference ratio} = \text{new Plexor}^{\circledR} \text{HY quantitation Y target input}$$

If desired, Steps 2 and 3 can be repeated and the results from replicate quantitations averaged.

4. To confirm the target mass of DNA for STR amplification identified in Step 3, amplify samples using all STR systems currently in use within the laboratory. Three sets of amplifications should be performed:
 - a. Amplify samples with each STR system using a quantity of target DNA based on the current quantitation system results.
 - b. Amplify samples with each autosomal STR system using the autosomal Plexor® HY quantitation results and the amount of target DNA identified in Step 3.

IV.B. Testing Conditions (continued)

- c. Amplify samples with each Y-STR system using the Y Plexor® HY quantitation results and the amount of target DNA identified in Step 3.

Note: For each STR system being compared, amplify and analyze the STR reactions using the same instrumentation and reagent lot numbers. When possible perform these steps within the same plate. This will reduce variability in results not associated with the Plexor® HY System.

5. Analyze the amplified samples. Use identical treatment (sample preparation and run conditions) for the samples amplified using each STR system. Comparison samples should be injected in the same run if possible.
6. Compare the STR profile results of the samples amplified based on the Plexor® HY quantitation values to those obtained using your current quantitation system. Confirm that the target quantity of template DNA, as determined by the Plexor® HY System, provides results consistent with your current quantitation system. It may be necessary to perform additional calibration amplifications at slightly above or below the mathematically derived target mass to optimize STR profile quality. We recommend that you identify a target quantity of template DNA that provides a well balanced profile with peaks that are neither saturating nor below the stochastic threshold.

V. Sensitivity

V.A. Objective

The objective of this study is to determine the quantity of template DNA below which amplification is not expected to yield a DNA profile.

V.B. Testing Conditions

1. For each of 2–3 male DNA samples selected from the calibration experiment (Section IV), prepare dilutions of 0.060ng/μl, 0.020ng/μl, 0.0067ng/μl, 0.0022ng/μl and 0.00070ng/μl using the autosomal Plexor® HY quantitation results (Table 1). Be sure to vortex each dilution before removing an aliquot to prepare the next dilution. Change pipette tips between each dilution in the series.

Table 1. Example Dilution of a DNA Sample.

	DNA Concentration	Volume of DNA	Volume of TE-4 Buffer
Dilution #1	0.060ng/μl	Varies ¹	to a final volume of 500μl
Dilution #2	0.020ng/μl	40μl	80μl
Dilution #3	0.0067ng/μl	40μl	80μl
Dilution #4	0.0022ng/μl	40μl	80μl
Dilution #5	0.00070ng/μl	40μl	80μl

¹The volume of DNA used to create the 0.060ng/μl dilution will depend on the initial DNA concentration of the sample. The equation $(C_1)(V_1) = (C_2)(V_2)$ can be used to determine the volume of DNA to add to create the 0.060ng/μl sample. C_1 is the concentration of your sample, V_1 is the unknown volume of that sample to be used, C_2 is the final concentration (0.060ng/μl) and V_2 is the final volume (500μl). Therefore, for a sample with a concentration of 2.0ng/μl the equation is $(2.0\text{ng}/\mu\text{l})(V_1) = (0.060\text{ng}/\mu\text{l})(500\mu\text{l})$, and $V_1 = 15\mu\text{l}$.

2. Following quantitation, amplify the samples using all STR systems currently in use within the laboratory. Two sets of amplifications should be performed:
 - a. Amplify all five dilutions in duplicate with each autosomal STR system, adding 2 μ l of each dilution to each STR reaction. This approximates amplification of 120pg, 40pg, 13.4pg, 4.4pg and 1.4pg.
 - b. Amplify all five dilutions in duplicate with each Y-STR system, adding 2 μ l of each dilution to each STR reaction. This approximates amplification of 120pg, 40pg, 13.4pg, 4.4pg and 1.4pg.
3. Analyze the amplified samples. Use the same treatment (sample preparation and run conditions) for these samples that you would currently use for samples with low template amounts.
4. Determine the quantity of template at which you no longer generate STR results for each of the STR systems.
5. Your laboratory may choose to use this information to establish a quantitation “cut-off” value below which amplification is not expected to yield useful genetic information. Therefore, no further testing will be performed on samples below that value. One method to determine such a value is to identify the quantity of template at which you no longer generate an STR profile and select a value that is threefold less concentrated. For example, if no results were obtained by amplifying 0.060ng of DNA, the “cut-off” value would be 0.020ng.

VI. Male/Female Mixtures

VI.A. Objective

The objective of this study is to examine the quantitation results obtained for mixtures of male and female DNA using the Plexor® HY System. In addition, for mixed samples where the male profile is of interest, this study will allow you to define autosomal/Y ratios where:

- a. autosomal STR analysis is likely to produce a clear male profile.
- b. autosomal and Y-STR analysis in combination may provide complementary male contributor profile information.
- c. you can anticipate that only Y-STR analysis will provide a profile for the male contributor.

VI.B. Testing Conditions

1. Select two male and two female samples from the calibration study (Section IV) or another source. If the DNA samples are from another source, they must be quantitated using the Plexor® HY System, and the DNA profile must be determined using the laboratory's STR systems. Use one male and one female DNA to create one set of mixtures and the other male and female DNA to create a second set of mixtures. Create the two sets of mixtures with the following ratios: 1:1, 1:5, 1:10, 1:15, 1:20, 1:25, 1:50 and 1:100. The mixtures should contain 0.50ng/μl of male DNA with increasing amounts of female DNA. Another appropriate concentration of male DNA is the Y-STR target quantity determined in Section IV.B, Step 5, divided by 2.0μl. For example, if your new target quantity is 1.25ng, the male DNA concentration will be 0.625ng/μl. The male contribution should be based on Y chromosome Plexor® HY results and the female contribution on autosomal Plexor® HY results. Table 2 provides an example of how to create the mixture samples.

Table 2. Example of How to Create Mixture Samples.

Ratio		Stock DNA Concentration ¹		DNA Contribution in Final Sample ²			Final Sample Composition			
Female	Male	Female (ng/μl)	Male (ng/μl)	Female (ng/μl)	Male (ng/μl)	Total Human Sample (ng/μl)	Total Volume (μl)	Female (μl)	Male (μl)	TE ⁻⁴ Buffer (μl)
1	1	1.0	1.0	0.50	0.50	1.0	30.0	15.0	15.0	0
5	1	10	1.0	2.5	0.50	3.0	30.0	7.50	15.0	7.50
10	1	10	1.0	5.0	0.50	5.5	30.0	15.0	15.0	0
15	1	100	1.0	7.5	0.50	8.0	30.0	2.25	15.0	12.75
20	1	100	1.0	10.0	0.50	10.5	30.0	3.00	15.0	12.0
25	1	100	1.0	12.5	0.50	13.0	30.0	3.75	15.0	11.25
50	1	100	1.0	25	0.50	25.5	30.0	7.50	15.0	7.50
100	1	100	1.0	50	0.50	50.5	30.0	15.0	15.0	0

¹Starting with a female DNA sample at a concentration of 100ng/μl, a 1:10 dilution series was used to create the 10ng/μl and 1.0ng/μl samples (10μl of DNA plus 90μl of TE⁻⁴ buffer for each dilution). The male DNA sample was at a concentration of 1.0ng/μl.

²The amount of male DNA in the final sample is held constant at 0.50ng/μl, and the final volume of each sample also is constant at 30μl. Each row describes the female:male ratio of DNA, concentrations of stock male and female DNA solutions, male and female DNA contribution to the sample in ng/μl, and volumes of female DNA, male DNA and TE⁻⁴ buffer used to prepare the final sample.

2. Determine the amount of DNA present in the samples, as well as the [Auto]/[Y] value using Plexor® HY. The [Auto]/[Y] value for a 1:1 mixture is expected to be approximately 2.0. Some variation may be seen in ratio values; [Auto]/[Y] values in the range of 0.4 to 2 are commonly observed in single-source male samples, and this will impact the ratios determined for mixtures.
3. Following quantitation, amplify the mixture samples using all STR systems currently in use within the laboratory. Perform two sets of amplifications:
 - a. Amplify samples with each autosomal STR system using the target amount of DNA identified in the calibration study (Section IV). These quantities should be based on autosomal Plexor® HY quantitation results.
 - b. Amplify samples with each Y-STR system using the target amount of DNA identified in the calibration study (Section IV). These quantities should be based on Y chromosome Plexor® HY quantitation results.

4. Analyze the amplified samples. Use the same treatment (sample preparation and run conditions) for these samples that you currently use with similar sample types.
5. After reviewing the autosomal STR results, record the [AUTO]/[Y] ratio above which Y-STR analysis could be useful.
6. Record the maximum empirical [AUTO]/[Y] ratio that did not produce a useful partial male profile using autosomal STR analysis.
7. These results will assist you in determining what STR system(s) should be used for different empirical [AUTO]/[Y] values. Consider choosing:
 - a. a range of [AUTO]/[Y] ratios where autosomal testing will yield a male profile sufficient for comparison purposes.
 - b. a range where both autosomal and Y-STR analysis may be necessary.
 - c. a range where Y-STR analysis will be necessary to yield a male profile because autosomal STR analysis may not provide a profile that is sufficient for comparison.

Note: Creation of a figure similar to Figure 5, Panel B, of the *Developmental Validation of a Real-Time PCR Assay for the Simultaneous Quantification of Total Human and Male DNA #AN157* may be helpful in this process.

VII. Known and Nonprobative Samples

VII.A. Objective

The objective of this study is to compare the Plexor® HY System with current methods of quantitation and STR amplification. This study also examines the impact of laboratory extraction methods on quantitation using Plexor® HY.

VII.B. Testing Conditions

1. The following experiments should be performed with DNA extracted from 5–10 samples encompassing the different extraction protocols and sample types frequently tested in the laboratory. Previously extracted samples may be used and comparisons made to prior quantitation and STR results. However, long-term storage can impact the quality of results obtained from those samples. If nonconcordant results are obtained using stored samples, the samples must be requantified and amplified using the laboratory's current systems so that the results are useful for comparison in this study. In extreme cases it may be necessary to re-extract the sample.
2. Determine the amount of DNA present in the samples using both the laboratory's current quantitation system and Plexor® HY System.
3. Following DNA quantification, amplify the samples using all STR systems currently in use within the laboratory. Two sets of amplifications should be performed:
 - a. Amplify samples with each STR system using the current amount of target DNA based on the current quantitation system results.
 - b. Amplify samples with each STR system using the amount of target DNA determined in the calibration study (Section IV) based on the Plexor® HY System quantitation results.
4. Examine the DNA profiles obtained from all amplifications to ensure that the DNA profiles for Plexor® HY-quantified samples provide concordant results.



VIII. Related Products

Product	Size	Cat.#
Plexor® HY System*	200 reactions	DC1001
	800 reactions	DC1000
Plexor® Calibration Kit, Set A*	each	DC1500
Water, Amplification Grade**	5 × 1,250µl	DW0991

*Not for Medical Diagnostic Use.

**For Laboratory Use.

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