



# Accurate and Efficient Chimerism Determination Using the SNP-Based Chimeric ID Panel from Agena Bioscience

## ABSTRACT

Recipients of allogeneic hematopoietic stem cell transplants (HSCTs) require clinical monitoring to allow for early diagnosis of post-transplant adverse events such as rejection, graft vs. host disease or malignancy relapse. Triaging of transplant recipients in a clinical setting is commonly achieved by Minimal Residual Disease (MRD) testing and performing chimerism analysis on post-transplant specimens to determine the genetic contribution from the transplant recipient and the donor. While MRD monitoring involves detection of malignancy-specific markers, measuring the chimerism can be achieved via general PCR-based techniques. The most commonly used methods for monitoring chimerism in post-transplant samples are based on analysis of short tandem repeats (STRs). However, assay setup and data analysis remain complicated and time-consuming processes.

The Chimeric ID Panel is a SNP-based panel designed to simplify chimerism testing. Automated reporting software analyzes data in seconds, eliminating the need for lengthy interpretation of STR data from pre- and post-transplant specimens. The panel was tested on samples with a range of chimerism levels and showed excellent accuracy (0.8% average variance from truth) and reproducibility (0.65% Standard Deviation). These results paired with streamlined data analysis and assay setup efficiencies make the Chimeric ID Panel a viable alternative to STR-based chimerism methods.

## INTRODUCTION

Recipients of allogeneic bone marrow engraftments require clinical monitoring to allow for early diagnosis of post-transplant adverse events such as rejection, graft vs. host disease or malignancy relapse. Triaging of transplant recipients in a clinical setting is commonly achieved by Minimal Residual Disease (MRD) testing and performing chimerism analysis on post-transplant specimens to determine the genetic contribution from the transplant recipient and the donor. Mixed chimerism is defined as presence of an additional genotype in the peripheral blood cells of the allograft recipient. This is attributed to the hematopoiesis of the recipient's native CD34+ derived cells despite the cytoreductive treatment prior to the allograft transplant. Several studies have shown strong positive correlation between the extent of mixed chimerism and the likelihood of patient hematologic relapse.<sup>1-3</sup> The most commonly used methods for monitoring chimerism in post-transplant samples are based on analysis of short tandem repeats (STRs). However, assay setup and data analysis remain complicated and time-consuming processes.

Approximately 20,000 bone marrow engraftments are performed in the United States every year<sup>4</sup>. In Europe, there are more than 40,000 annually<sup>5</sup>. Roughly 42% of these are allogeneic<sup>6</sup>. Due in part to advances in the field of HSCT and expanding indications, the number of transplants is expected to grow over the next 10 years<sup>7</sup>. The prevalence of HSCT survivors is also expected to increase considerably. A CIBMTR study estimates that the prevalence of survivors



will increase five-fold through 2030<sup>7</sup>. Growing transplant numbers and longer survival mean greater demand for bone marrow engraftment related chimerism testing. Lab resources will be strained by increasing sample volumes that must be processed using STR-based methods with burdensome assay setup and results analysis. A streamlined alternative is required to reduce the effort required to perform chimerism analysis.

The Chimeric ID Panel is a SNP-based panel designed to simplify chimerism testing by offering a user-friendly setup and automated results analysis. Several studies were performed to demonstrate the performance of this panel. These include tests to determine accuracy, reproducibility and performance compared to STR-based methods.

## METHODS

### Assay Design

The Chimeric ID panel is a highly multiplexed SNP-based chimerism determination panel developed by Agena Bioscience. The panel leverages the iPLEX Pro chemistry and is processed using the MassARRAY system. The panel consists of 92 independent (absence of linkage disequilibrium) SNPs with minor allele frequency (MAF) of 0.45-0.5 across major HapMap populations including ASW, CEU, CHB, GIH, JPN, and MEX. The 92 SNPs are multiplexed into 8 wells. The panel includes only A<>T and C<>T transitions as these result in the highest mass differences and highest quality data.

The informative SNPs will vary for different donor/recipient combinations. 92 SNP markers with high MAF provides the panel with sufficient power to compare related and unrelated individuals. (Figure 1).

### Software Design

The Chimeric ID Panel is accompanied by a reporting software that automatically analyzes recipient/donor pre-transplant profiles, determines which SNPs are informative, stores the profile for future reference and leverages the archived profile to calculate percent recipient/donor contribution in post-transplant follow-up specimens. By detecting peak height at each informative SNP, the algorithm calculates the composition of the sample and assigns a Z-score value which represents the confidence level in the call. These values are analyzed, and a final result is displayed in an easy to interpret report (Figure 2).

### Summary of key software features:

- Automatic analysis of recipient/donor pre-transplant profiles to identify informative SNPs
- Archive functionality saves pre-transplant profiles, so they only need to be run once
- Recipient/donor contribution in post-transplant follow-up specimens is calculated in seconds
- All results displayed in easy to interpret reports
- Historic results for a given recipient can be easily recalled and displayed in an intuitive report
- Multiple donor analysis

**Figure 1: Experimentally Determined Number of Informative Markers for Pairwise Comparison of Unique HapMap Samples**

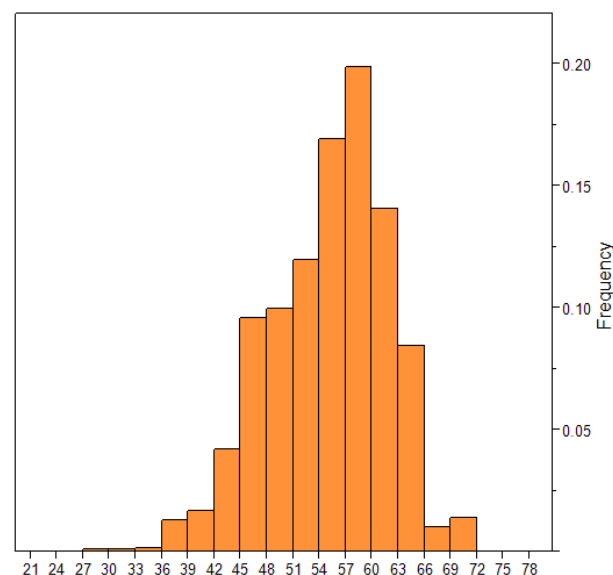
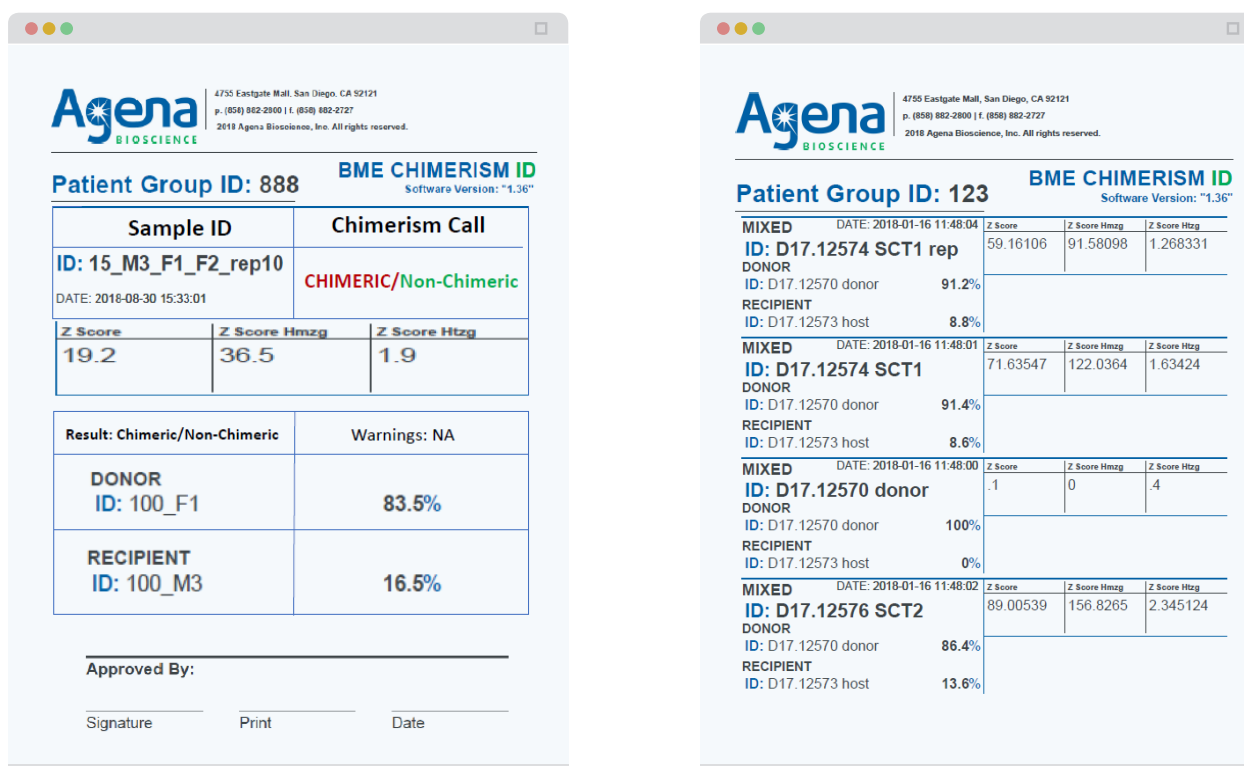




Figure 2: Example Chimerism Reports From The Chimeric ID Software



## Samples Tested

Analytical validation studies were performed across a wide range of contribution levels using contrived samples. These samples were created by extracting gDNA from whole blood drawn from male and female donors. Unmixed male and female DNA pairs were used to generate pre-transplant profiles. Mixtures were created to mimic post-transplant follow-up specimens at various contribution levels. Natural variability in extracted DNA concentration, fragmentation and dilution accuracy made it difficult to accurately create mixtures at the intended target contribution levels. To ensure that chimerism results were being compared to an accurate representation of the mixture composition, ddPCR X/Y chromosome analysis was performed to verify the percent recipient and donor contribution in each dilution.

## Experiments

Experiments were done to determine the Chimeric ID Panel's accuracy, reproducibility, limit of detection, tolerance to DNA input levels and performance compared to STR-based chimerism methods.

- **Accuracy** – How close are the Chimeric ID results to the experimentally determined "truth"?
- **Reproducibility** – Does the Chimeric ID Panel return similar results each time a sample is analyzed?
- **Limit of detection** – At which minor contribution levels is the Chimeric ID panel able to reliably distinguish between "pure" unmixed DNA and low-level contribution from the recipient/donor?
- **DNA input tolerance** – What is the panel's optimal DNA input range?
- **Comparison to STR** – Does the panel give similar results to STR-based methods?



## EXPERIMENTAL DESIGN AND RESULTS

### Accuracy

Two independent series of dilutions were created using gDNA from unique individuals. Dilutions were prepared targeting minor contribution levels of 3%, 5%, 10%, 15%, 25%, 30% and 45%. The actual minor contribution levels in the prepared dilutions were verified using X/Y chromosome analysis. This value was established as “truth”. Eight replicates of each dilution point were tested using the Chimeric ID panel. Results were analyzed to determine the variance from “truth”. All testing was done at the same location.

The data set generated an average variance from “truth” of 0.8% and, a median variance from truth of 0.48%. The data shows a strong linear relationship with a slope of 1.03 and R2 of 0.99 (Figure 3).

A similar study was performed at multiple sites. Three independent labs with unique instruments and operators tested 3 dilution series with minor contribution levels of 3%, 5% and 20%. Thirteen total replicates were generated at each dilution point.

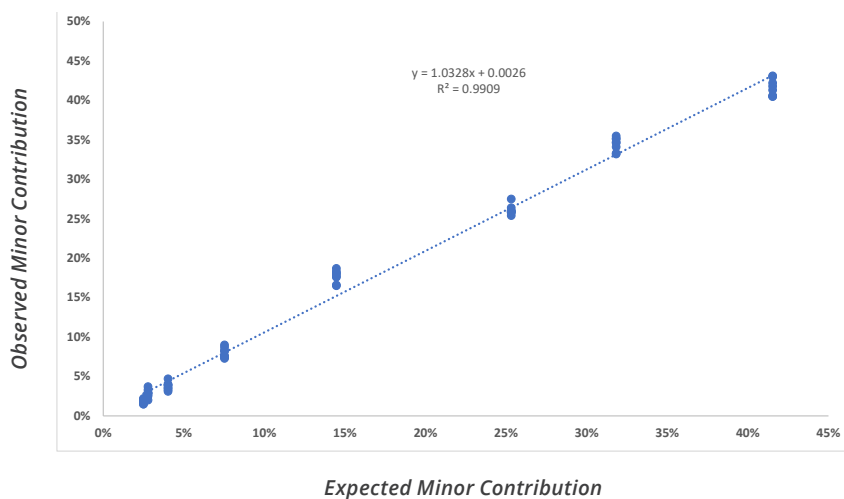
The data set generated an average variance from “truth” of 1.3% and showed a strong linear relationship (Figure 4).

### Reproducibility

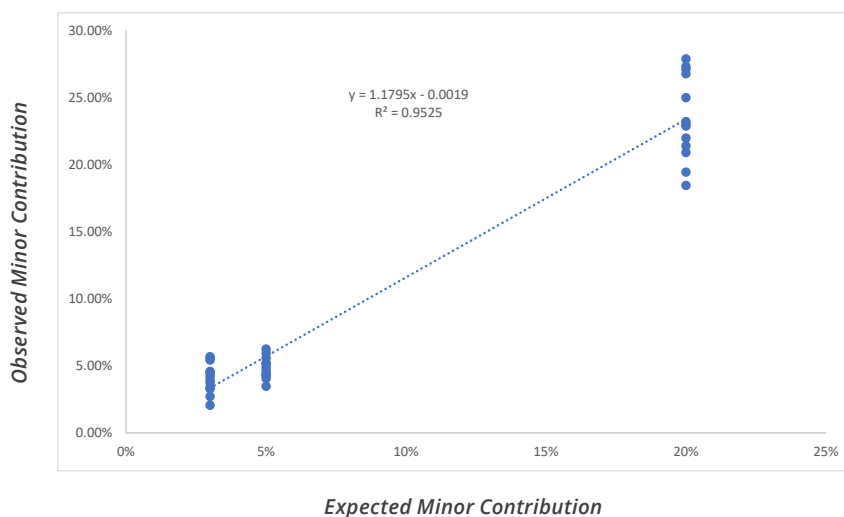
Two independent series of dilutions were created using gDNA from unique individuals. Dilutions were prepared targeting minor contribution levels between 3% and 50%. Actual minor contribution values in the dilutions were confirmed using X/Y chromosome analysis. This value was established as “truth”. Eight replicates of each dilution point were tested using the Chimeric ID panel. The minor contribution results from the replicates at each dilution point were analyzed to calculate the mean. Results were analyzed to determine how each replicate compared the others at that dilution level.

Overall standard deviation across the entire dynamic range was 0.65%. Figure 5 shows the data spread and standard deviation at each dilution point. The “% minor contribution” values have been normalized to account for variation during dilution. For example, if the 3% target dilution of series #1 was determined to be 3.3% and the same point in series #2 was determined to be 3.1%, both would be normalized to 3% in the graph. As the purpose of this study was to evaluate

**Figure 3: Single Site Accuracy Study Results**



**Figure 4: Multi-site Accuracy Study Results**





results variability of multiple replicates, displaying the absolute minor contribution level is of less importance.

## Limit of Detection

The Chimeric ID software allows users to adjust sensitivity settings to suit their needs. Chimerism Cutoff and Z-score Cutoff are the two parameters that can be adjusted. Increasing these values will make the panel less likely to detect low level minor contributions. Decreasing these settings will make the panel more likely to detect low level minor contributions. However, adjusting setting in either direction may impact specificity. For this experiment, the recommended default settings (Chimerism Cutoff: 1%, Z-Score Cutoff: 2) were used.

To determine panel sensitivity, dilutions were prepared with minor contribution limits of 1%, 2%, 3% and 5%. Minor contribution levels were verified using X/Y chromosome analysis. Twelve replicates of each dilution were tested using the Chimeric ID panel. “No Chimerism Detected” results indicate that the panel did not identify the replicate as a mixed sample. “Chimerism Detected” results indicate that the panel correctly distinguished the replicate as a mixed sample.

As sensitivity often has an inverse relationship with specificity, 48 unmixed “pure” gDNA samples were marked as post-transplant follow-up specimens and analyzed to determine specificity. “No Chimerism Detected” results indicate that the panel correctly identified these pure samples as unmixed DNA. “Chimerism Detected” results would indicate that the panel incorrectly determined that the pure sample was a mixture.

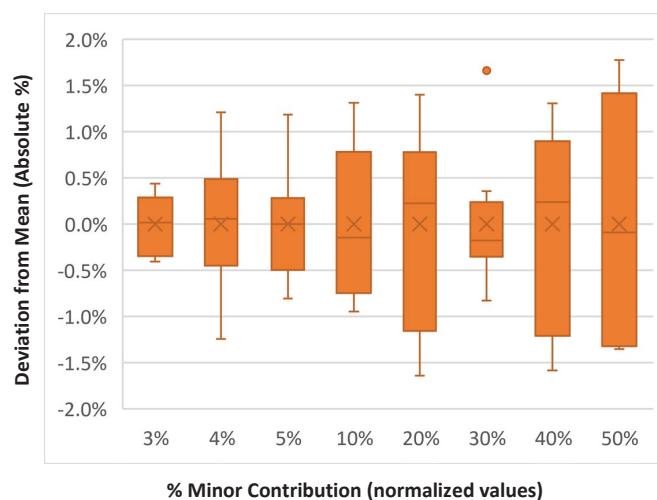
All pure samples were correctly called “No Chimerism Detected”. 100% of the 3% and 5% mixture replicates were correctly called “Chimerism Detected”. 83.3% of the 2% mixture replicates and 16.7% of the 1% mixture replicates were called “Chimerism Detected” (Figure 6).

## DNA Input Tolerance

Commercially available STR-based chimerism methods have DNA input recommendations. They warn that too much DNA can cause inaccurate results, off-scale data, spectral “pull-up” and other phenomenon. Too little DNA input can cause allelic dropout and result in a poor profile. Labs are often required to check the concentration of all DNA to be analyzed and prepare dilutions of samples with high concentration. DNA extracted from samples such as sorted cells can often have lower concentration. The purpose of this experiment was to confirm that the Chimeric ID panel is tolerant to both low and high DNA inputs; indicating that samples with low concentration will return results and samples with high concentrations will not have to be diluted.

Dilutions targeting 2%, 6% and 12% minor contribution level were prepared. Each dilution level had mixes created at DNA

**Figure 5: Reproducibility Study Results**



**Figure 6: Limit of Detection Study Results (Default Cutoff Settings)**

Sample	Replicates	No Chimerism Detected	Chimerism Detected	% Identified as Mixed
1%	12	10	2	16.7%
2%	12	2	10	83.3%
3%	13	0	13	100%
5%	13	0	13	100%
“Pure”	48	48	0	0%



concentrations of 1 ng/uL, 5 ng/uL, 50 ng/uL, and 100 ng/uL. Each sample was analyzed using the Chimeric ID panel to determine if varied DNA input affected chimerism results. 2uL were added to each reaction bringing the final DNA input of the replicates to 2ng, 10ng, 100ng and 200ng.

Results across the DNA concentration range were consistent with the panel accuracy and reproducibility observed in previous experiments. It was determined that the Chimeric ID panel is tolerant to a wide range of DNA input levels.

## Performance Compared to STR-Based Methods

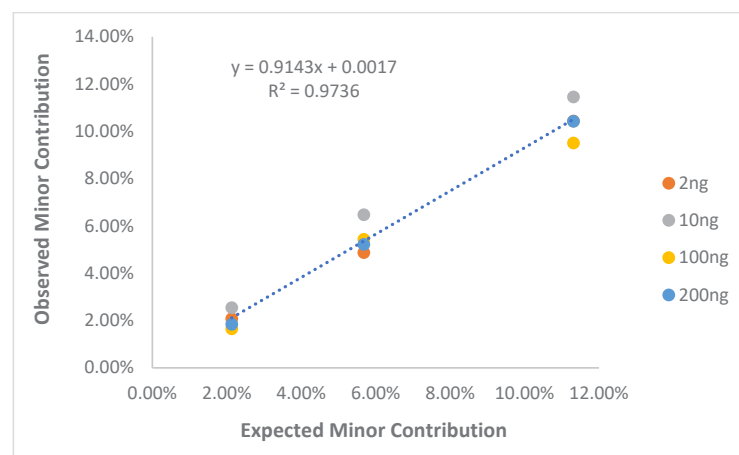
Two sets of samples were tested to determine how results obtained from the Chimeric ID Panel compared to STR-based chimerism methods. The first experiment tested the same dilution series using the Chimeric ID panel and the STR-based chimerism methods of three clinical laboratories who routinely perform chimerism analysis for bone marrow engraftment monitoring. For the second experiment, a set of proficiency samples were tested at a clinical laboratory using both the Chimeric ID panel and their STR-based chimerism method.

In the first experiment, a dilution series was prepared with targeted minor contribution levels of 5%, 10%, 15%, 20%, 25% and 30%. Minor contribution levels were verified with X/Y chromosome analysis. Aliquots of each dilution were created and sent to three clinical laboratories routinely performing chimerism analysis for bone marrow engraftment monitoring and to an Agena Bioscience laboratory for testing using the Chimeric ID panel. Each location tested two replicates of each dilution. The results were analyzed to compare the results of the Chimeric ID Panel and the STR-based methods.

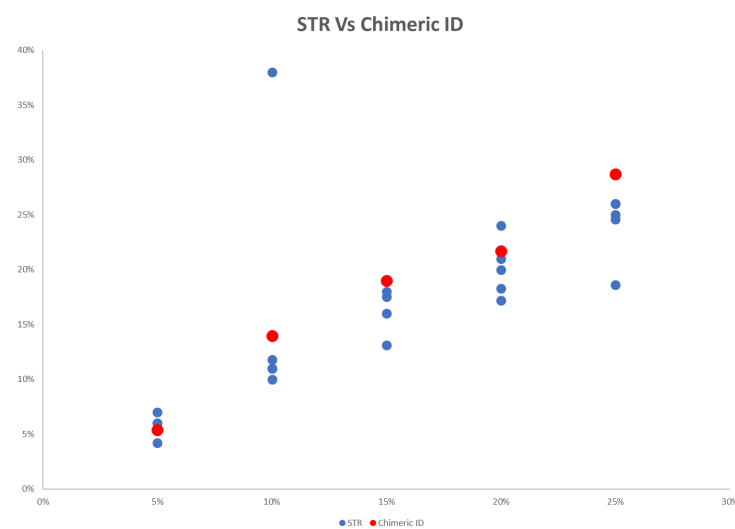
The results from the Chimeric ID panel were similar to those obtained by the STR-based methods. One STR-based method returned an outlier result at the 10% dilution point. This sample was later repeated and found to be closer to the “truth” established by X/Y chromosome analysis (*Figure 8*).

During the second experiment, samples from a national proficiency testing program were tested in a clinical laboratory using the Chimeric ID Panel and their STR-based chimerism method. The samples in this proficiency program are sent to over 100 lab participants who routinely perform chimerism testing for bone marrow engraftment monitoring. Results from all participants are collected, and a robust mean and standard deviation are generated. Passing results are defined as values falling within +/- 2.5 standard deviations from the robust mean of a given sample.

**Figure 7: DNA Input Results**



**Figure 8: Multi-Site Comparison of Chimeric ID and STR-Based Methods**





Both the Chimeric ID Panel and the STR-based method returned results within the passing criteria for each sample. The variance from the robust mean for both methods was also similar (Figure 9).

## CONCLUSIONS

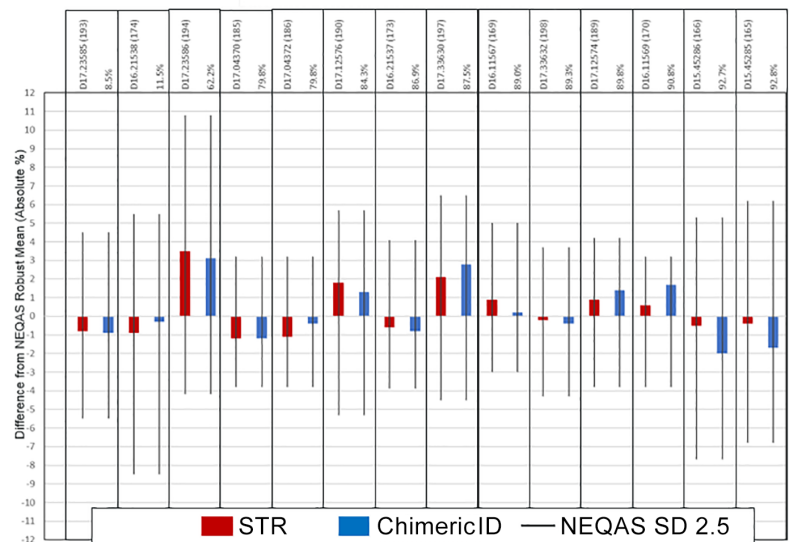
The Chimeric ID panel and STR-based chimerism methods have demonstrated similar performance for chimerism determination. The Chimeric ID panel has significant workflow and lab efficiency advantages including automated pre- and post-transplant sample analysis and the absence of DNA dilution or CE reinjection steps. Strong performance and increased lab efficiency make the Chimeric ID Panel a viable alternative to STR-based chimerism methods.

The Chimeric ID panel produces accurate and reproducible chimerism results across the entire range of minor contribution levels. Accuracy and reproducibility are preserved when testing is done with multiple sites, instruments and operators. Results obtained using the Chimeric ID panel and STR-based chimerism methods are similar. Therefore, re-testing of previously analyzed post-transplant follow-up specimens is not required when switching from STR-based methods to the Chimeric ID panel for chimerism testing. The Chimeric ID Panel has a similar sensitivity as that of STR-based methods as reported by the labs who use them and 100% specificity.

Consistent results across a wide range of DNA inputs show that users of the Chimeric ID panel will not be required to dilute DNA before PCR; reducing hands-on and setup time. The issue of off-scale or low capillary electrophoresis (CE) data is not a consideration with the Chimeric ID panel. Labs using Chimeric ID will eliminate the need to perform multiple CE reinjections to obtain on-scale data for samples within a run. Additionally, robust results can be obtained from DNA input as low as 1 ng/uL. DNA from sorted cells or other samples which routinely produce low DNA yield can be reliably analyzed.

Automated analysis of pre- and post-transplant samples with Chimeric ID significantly reduces the time required to generate chimerism results. Quantifying this impact will occur in later studies.

**Figure 9: Proficiency Sample Testing Results**



\* Solid bars indicate variance from robust mean. Black lines indicate +/- 2.5 SD.



## REFERENCES

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The MassARRAY System is for research use only. Not for use in diagnostic procedures.