



2N Sample Identification for VeriDose® CNV Analysis

ABSTRACT

One of the main challenges in analyzing genes with copy number variations is finding a suitable “control” sample to be used for data normalization. When running the Agena PGx Reporter, this 2N sample is used to identify the 2N cluster in each assay and is what all other samples in that run are compared to. Therefore, the control cannot contain any gene deletions, duplications or hybrid alleles. The sample can have SNPs in CYP2D6 that aren't associated with CNVs or hybrids, but ideally the sample will be *1/*1.

The Agena VeriDose CNV panel contains 22 assays, targeting seven regions of the CYP2D6 gene. Because the assays span the entire gene, we are able to detect not only copy number variations, but also hybrid alleles, where a portion of the CYPD6 gene is swapped for the highly homologous CYP2D7 gene. These hybrid alleles are important since their presence confers the same functional outcome as a gene deletion.

Because each of these 22 assays is unique and has its own performance, the 2N control sample needs to be carefully chosen to be as “normal” as possible. Agena has identified a commercially available gDNA sample (Roche #11691112001) that clusters as closely as possible with the 2N samples that were used during panel validation. If no other 2N samples are available in your lab, this can be used as a control sample. However, we have seen some instances where DNA extraction kit can impact clustering and can cause the Roche gDNA sample to fall outside of the 2N group.

Therefore, it is Agena's recommendation that every lab running clinical samples identify an in-house 2N control whose performance most closely mimics that of the 2N samples in each assay. Following are two methods that can be used to identify a suitable 2N control.

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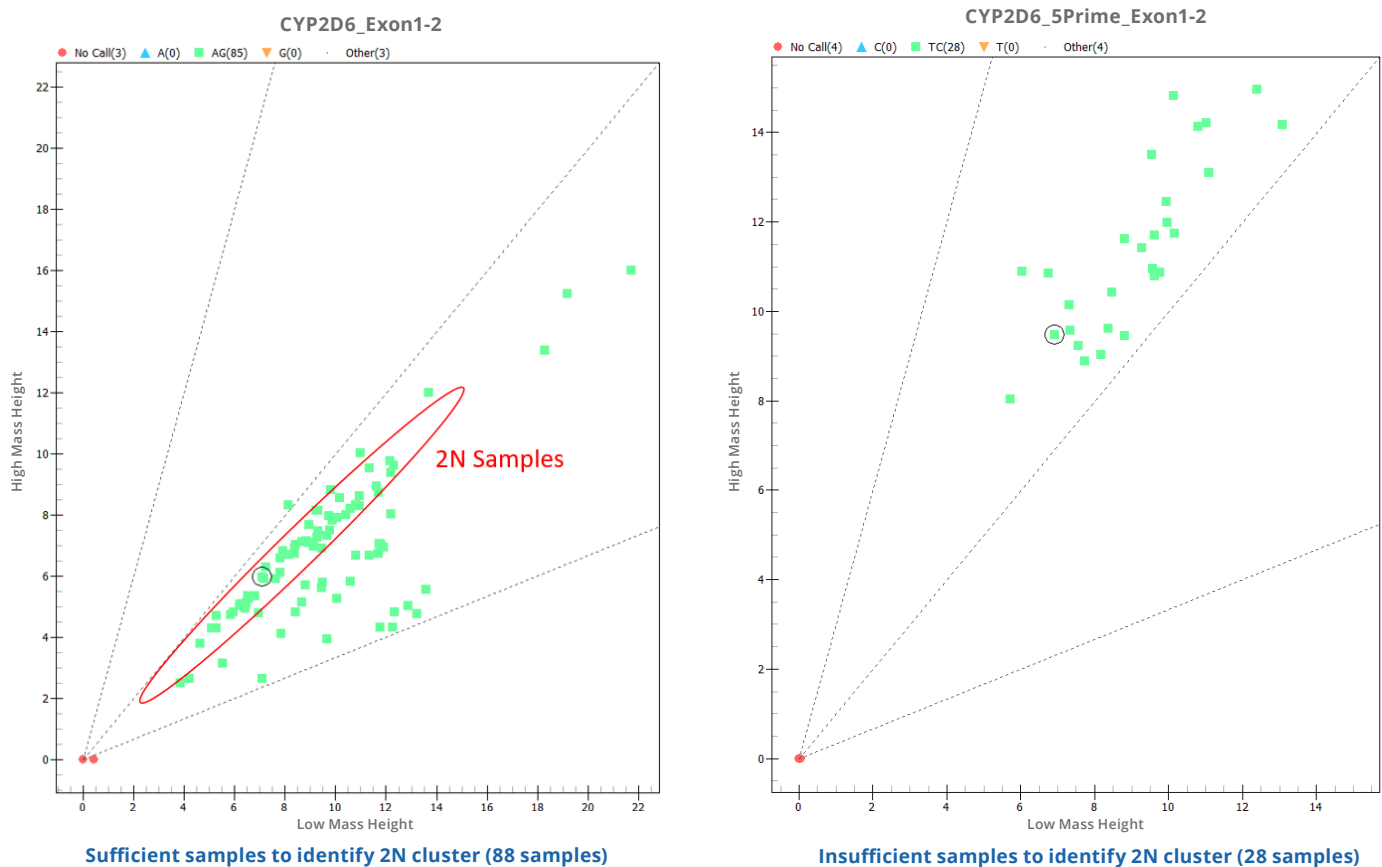
STEPS FOR IDENTIFYING A SUITABLE 2N SAMPLE

Method 1

This method is recommended for customers that have a large sample set (>75 samples) that allows for clear identification of 1N, 2N, and 3N+ clusters.

- 1 Run samples on VeriDose CNV and view the Call Cluster Plots in Height View in Typer Analyzer. Identify the 2N cluster in the sample set (this will generally be where the majority of the samples cluster and should fall close to the 45° angle in most assays), see *Figure 1*.

Figure 1: Samples needed to identify 2N cluster

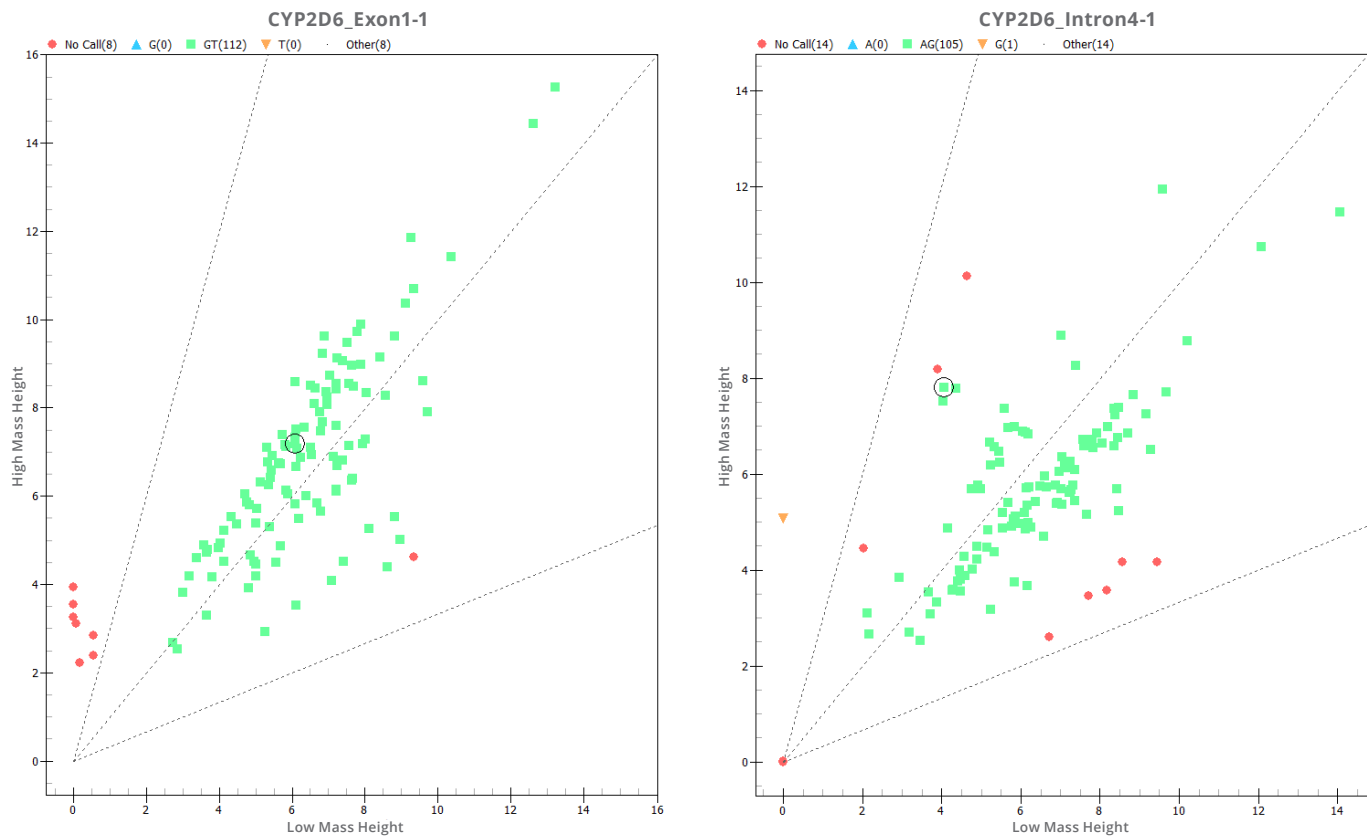


- 2 Identify a sample that falls as close as possible to the middle of the 2N cluster.
- 3 Check performance of that sample in other assays to ensure the sample falls in the 2N group in all assays. If a sample has a hybrid allele, it can look like a 2N in some assays, but will fall in either the 1N or 3N cluster in other assays. Below is an example of a sample that looks like 2N in CYP2D6_ Exon1-1 but actually contains a *68 hybrid allele and so falls in the 1N cluster in CYP2D6_ Intron4-1 (see *Figure 2*).



- Method 1 (continued)

Figure 2: Same sample showing different clustering in two assays



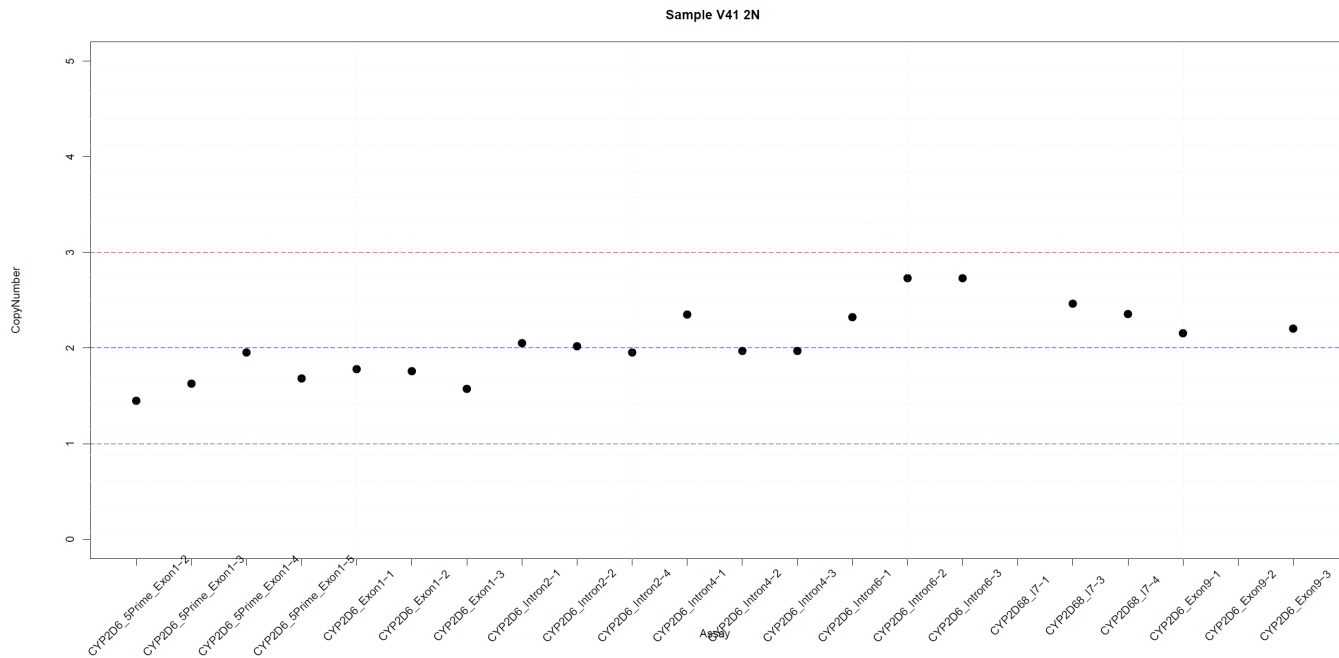
- 4 Once a suitable sample is identified, run the PGx report using the selected sample as the CYP2D6 2N control. It is recommended that a previously characterized sample set be used during the identification of a control sample so that the results of the Agena PGx report can be checked with the known CNV copy numbers of your samples. This will ensure the 2N that has been selected is suitable as a control sample.
- 5 Compare the PGx Report to the expected outcomes for each sample. If all samples give the correct CNV call, this is a suitable 2N control. If the results show discrepancies, select a different sample and re-run the report. Repeat until 100% concordance is achieved with the known dataset.



Method 2

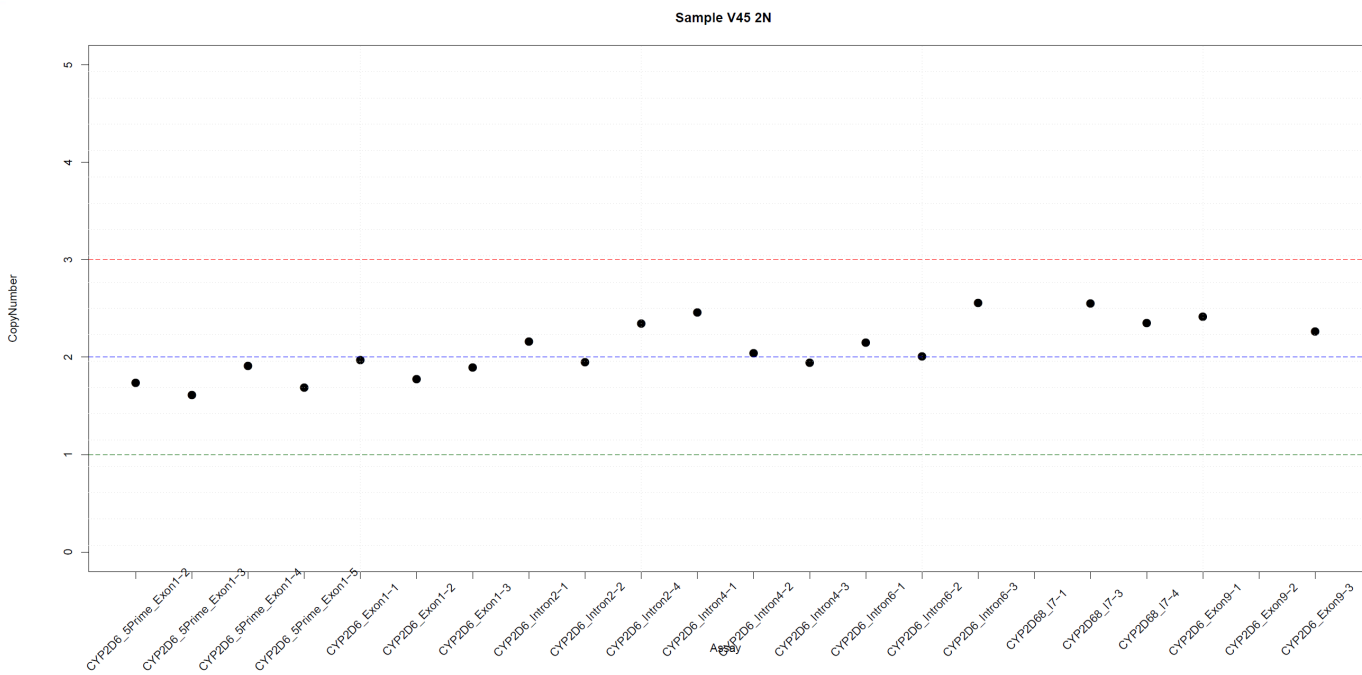
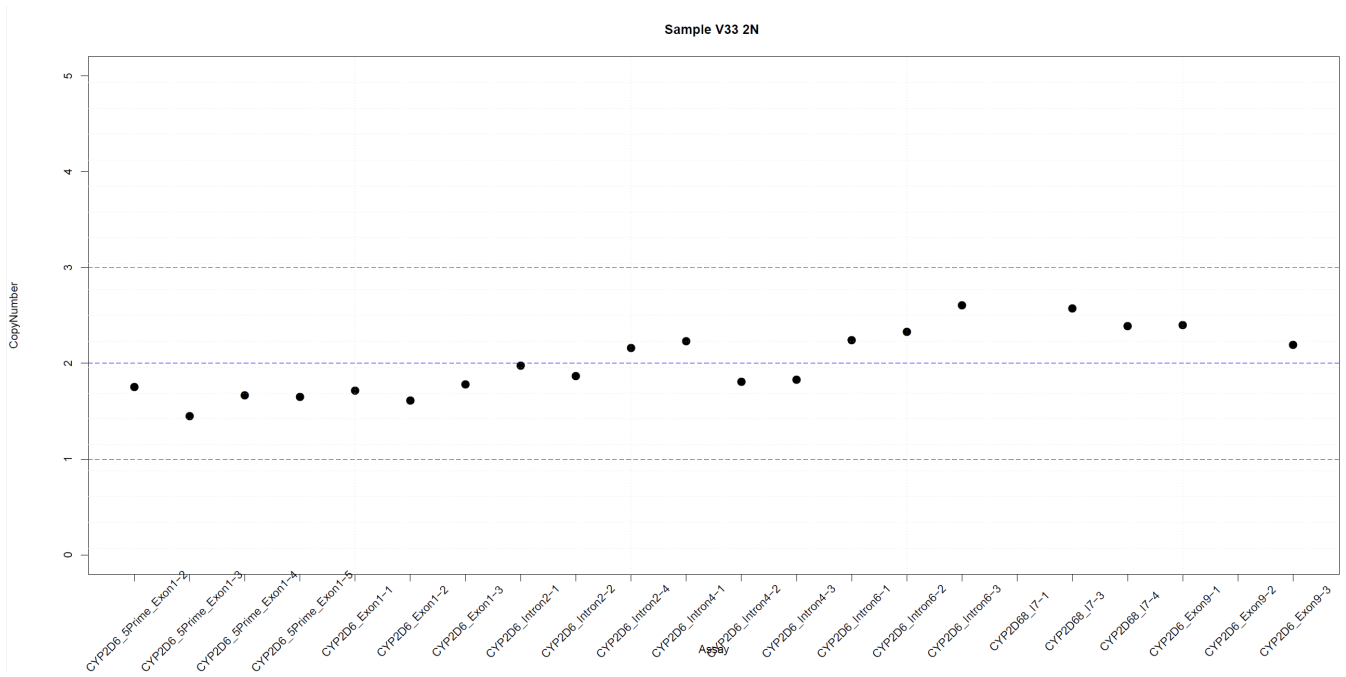
This method is recommended for customers that have a small data set that does not allow for identification of the 2N cluster in the Call Cluster Plot. This method requires that the Roche gDNA, mentioned above, be run on the same plate as your samples.

- 1 Run samples and Roche DNA on VeriDose CNV.
- 2 Run the PGx Report with Roche DNA selected as the CYP2D6 2N control.
- 3 Open the "AssaysPerSample.pdf" file that is generated in the PGx Report folder.
- 4 One diagram will be generated per sample, showing that samples' performance on all assays. Scroll through to find a sample that falls as close to the 2N line as possible, for all assays.
- 5 Example suitable samples:





- Method 2 (continued)



Note that not all assays will fall exactly on the 2N line. Some assays in the VeriDose CNV panel will consistently skew towards the 3N cutoff (ex. Intron6 and Intron7 assays).



- Method 2 (continued)

- 6 Once a suitable sample is identified, run the PGx report again, this time with the selected sample used as the CYP2D6 2N control. It is recommended that a previously characterized sample set be used during the identification of a control sample so that the results of the Agena PGx report can be checked with the known CNV copy numbers of your samples. This will ensure the 2N that has been selected is suitable as a control sample.
- 7 Compare the PGx Report to the expected outcomes for each sample. If all samples give the correct CNV call, this is a suitable 2N control. If the results show discrepancies, select a different sample and re-run the report. Repeat until 100% concordance is achieved with the known dataset.

OTHER CONSIDERATIONS

Unusually high number of 1N and 3N samples

If the number of samples with deletions or duplications is unexpectedly high and/or there are a large number of samples with hybrid calls, it's possible the 2N control that was used during reporting is not suitable. If it appears rare haplotypes are being reported too frequently, we recommend reviewing the 2N control sample and overall data quality using the following checks:

- Ensure that all samples show similar extension rates and peak heights in the cluster plots. For guidance, refer to the 'PCR inhibitors' section.
- Confirm that samples of similar concentrations have comparable peak heights. Large deviations from this expected pattern, due to low extension and yield, suggest a sample issue.
- Inspect the calculated copy number values to ensure that they are close to integer values, rather than between two copy number values (ex. 2.06 rather than 2.42).
- Review the confidence and coefficient of variation values for the predicted copy number calls. If a sample is listed as discordant, check cluster plots.
- Confirm that samples cluster into well-defined, well-separated copy number groups in the Typer Cluster Plot.

If comparing the data generated by Agena's VeriDose CNV panel with the Thermo Fisher Exon 9 TaqMan assay, be aware that TaqMan is not able to detect Exon 9 hybrid alleles (*4N and *36). This results in TaqMan underestimating copy number for samples containing hybrids.

DNA Normalization

- For optimal results, normalize DNA to the recommended 5-10 ng/uL concentration and use 2uL input for the PCR reaction.
- The DNA amount in each sample, and in each technical replicate on a single plate, should be similar (i.e. 10-20 ng).
- Sufficient genomic DNA input may be verified by alternate technologies (ex. Nanodrop).
- Check OD260/280 and OD260/230 for ratios over 1.7. Lower ratios may indicate the presence of PCR inhibitors.



Sample Types

- Different sample types and samples prepared by different extraction methods can amplify with different efficiencies.
- Samples analyzed together on the same plate should be of the same sample type and extraction method.
- If using an in house 2N control, it should be of the same sample type and extraction method as your clinical samples.

Note: a small number of control samples (e.g. a few Coriell samples on a 384-well plate) should not strongly impact analysis of buccal or blood derived samples)

PCR inhibitors

- If running samples extracted from blood, residual hemoglobin may cause samples to skew, resulting in copy number being reporter as either higher or lower than a 2N control that has no hemoglobin present.
- Other inhibitors may cause similar skewing.

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

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