Guide

SARS-CoV-2 Variant ValuPanel assays – set-up guidance and data analysis

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BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Guide

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1. Introduction

SARS-CoV-2 Variant ValuPanel™ assays, from LGC Biosearch Technologies™, consist of separately delivered probes and primers that are designed for qualitative detection of specific SARS-CoV-2 mutations by reverse transcription polymerase chain reaction (RT-PCR)-based genotyping. Each SARS-CoV-2 Variant ValuPanel assay amplifies and discriminates between a specific mutation and the wild type SARS-CoV-2 sequence in respiratory tract samples that have previously tested positive for SARS-CoV-2 by diagnostic RT-PCR.

SARS-CoV-2 Variant ValuPanel assays are for research use only, and not intended for SARS-CoV-2 diagnosis. The Variant ValuPanel assays are intended for secondary, informational tests only, designed for screening samples that have previously tested positive for SARS-CoV-2 by diagnostic RT-PCR.

Full details of ordering information, storage conditions, sequence information and reaction set-up can be found in our SARS-CoV-2 Variant ValuPanel assay manual.

This document is to provide general guidance on running these assays and information on how to ensure the cluster plot data analysis is being performed correctly.

2. General set-up considerations

The SARS-CoV-2 Variant ValuPanel assays have been optimised to be run as end-point reactions, with the data analysed on a Cartesian plot (cluster plot). The total fluorescence of each of the fluorophores is plotted on a Cartesian plot, displaying two homozygous groups, one for the wild type samples (FAM), and one for the variant samples (CAL Fluor Orange 560).

Please note, in the event that the SARS-CoV-2 acquires proximal mutations that lie under the probe of the assay, such as the P681H and P681R mutations, it is possible that a distinct heterozygous cluster for new mutation is displayed.

For the cluster plot analysis software to assign each data point confidently and accurately within either homozygous group, we recommend the following reaction set-up conditions for each assay:

- **Include at least 18 to 22 positive samples.** These should ideally be a mixture of both the wild-type and variant. It is also possible to duplicate or triplicate any samples to reach the minimum 18 positive samples per assay. Running fewer than 18 samples will mean that the software may struggle to accurately call each sample, especially those with a slower amplification rate/lower starting cDNA concentration
- **Include positive controls for both wild-type and variant, if possible.** This will allow for the increased confidence in sample calling and will ensure that the assay is working optimally each time.

- **Include at least 2 non-template controls (NTCs).** An NTC is a sample well containing the reaction mix, but not genomic material, so it should not amplify during the reaction. Most qPCR instrumentation/cluster-analysis software allow the assignment of NTC samples, which aids the software to accurately plot the samples on the cluster-plot and controls for any contamination events during the PCR set-up.

3. Data analysis and software setting recommendations

Typically, the FAM fluorophore is plotted along the x-axis, and the CAL Fluor Orange 560 fluorophore is plotted along the y-axis. The position of the data points within each cluster will determine if the sample is designated as wild type or variant (figure 1).

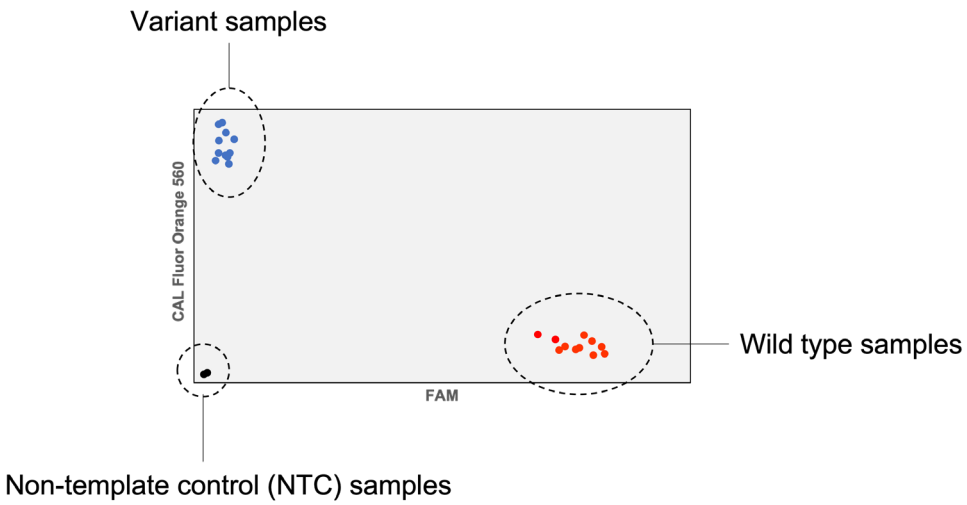

Figure 1. Schematic of a typical cluster plot. Wild type samples, detected via the FAM fluorophore, are shown in red. Variant samples, detected via the CAL Fluor Orange 560 fluorophore, are shown in blue. Non-template control samples (NTCs) are shown in black.

It is important that the cluster analysis is performed accurately, to ensure that the correct result is assigned to each data point. Below we have illustrated the most common analysis considerations.

3.1. Appropriate number of samples run per assay

Should too few samples be run per assay, the cluster analysis software may struggle to accurately assign the correct result to each sample. This is illustrated in figure 2 and figure 3.

Figure 2 illustrates a typical cluster plot with too few samples. The samples denoted in red appear to be wild type, whereas the sample denoted as a green triangle may be interpreted as heterozygous (e.g., a possible mixed sample). Also note, the *x*- and *y*-axis are not equally scaled.

Figure 3 illustrates a typical cluster plot with at least 18 samples per assay. The samples denoted in red remain as wild type, whereas they now cluster more tightly along the *x*-axis. Importantly, the sample previously denoted a green triangle has now moved into the red cluster, confirming that this sample is indeed wild type, and not heterozygous as suggested in figure 2. Also note that in this figure both the *x*- and *y*-axis are equally scaled.

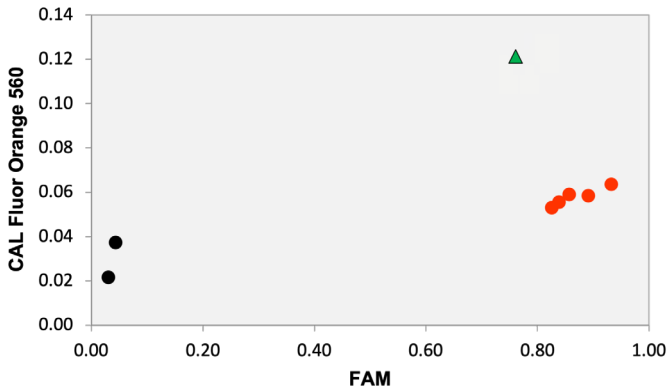

Figure 2. Cluster plot with too few samples per assay. Red circles are homozygous for wild type samples. The data point identified by the green triangle appears to be a heterozygous sample. Black circles denote NTC samples.

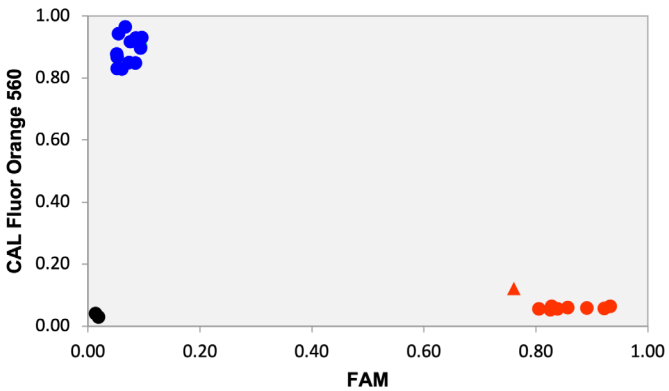

Figure 3. Cluster plot with appropriate number of samples per assay. Red circles remain as wild type samples. The data point identified by a triangle now clearly denotes a wild type sample. Blue circles denote variant samples. Black circles denote NTC samples.

3.2. Equal scaling of the *x*- and *y*-axis

It is important to ensure that both the x- and y-axis are equally scaled, as incorrect scaling can cause either the analysis software being unable to assign calls to any sample types (figure 4 and figure 5) or returning an inaccurate result due to the location of the clusters on the plot (figure 6 and figure 7).

3.2.1. Equal scaling of axes – example 1

Figure 4 illustrates a cluster plot where the axes are unequal (x-axis maximum = 1.00, *y*-axis maximum = 0.16), resulting in the software being unable to confidently assign a sample type call to any of the data points. By adjusting the both the *x*- and *y*-axis so they are equally scaled, the software can assign a sample type to each data point (figure 5).

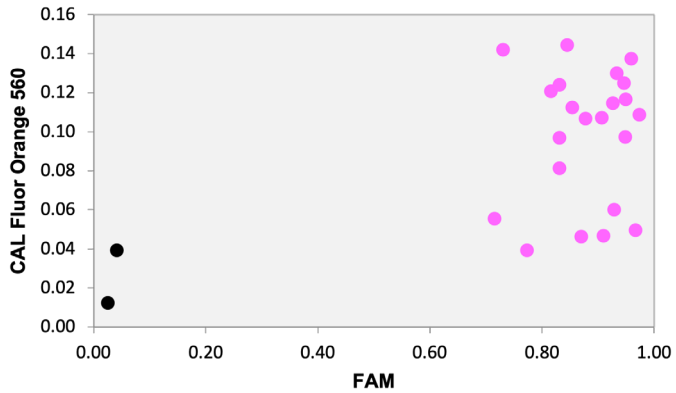

Figure 4. Cluster plot with unequal axes. Pink circles have an undetermined sample type. Black circles denote NTC samples.

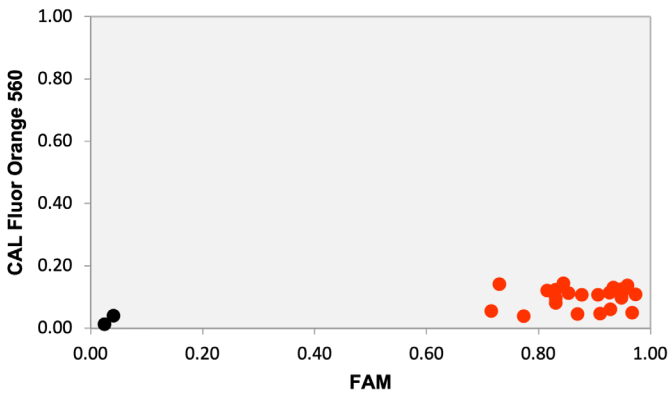

Figure 5. Cluster plot with same samples as illustrated in Figure 4, with equally scaled axes. Circles are now confirmed as homozygous for wild type samples (red). Black circles denote NTC samples.

3.2.2. Equal scaling of axes – example 2

Figure 6 illustrates a second example of a cluster plot where the axes are unequal (*x*-axis maximum = 2.00, *y*-axis maximum = 1.00), resulting in the software assigning a heterozygous sample type call to one of the clusters. By ensuring that both the *x*- and *y*-axes are equal, the software can confirm that the samples previously designated heterozygous in figure 6, are in fact homozygous for wild type samples (figure 7).

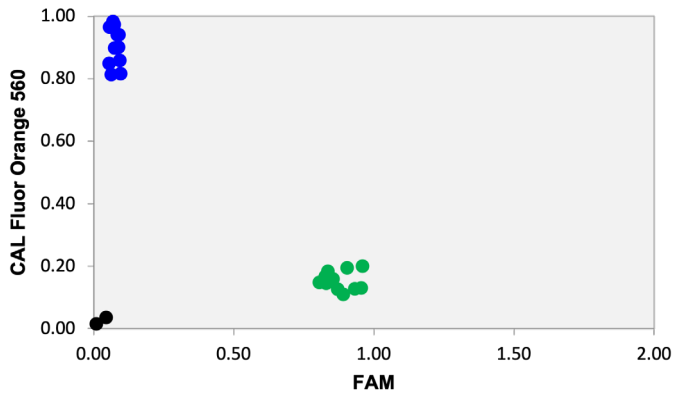

Figure 6. Cluster plot with unequal axis. Green circles have been denoted as heterozygous samples. Blue circles are denoted as variant samples. Black circles denote NTC samples.

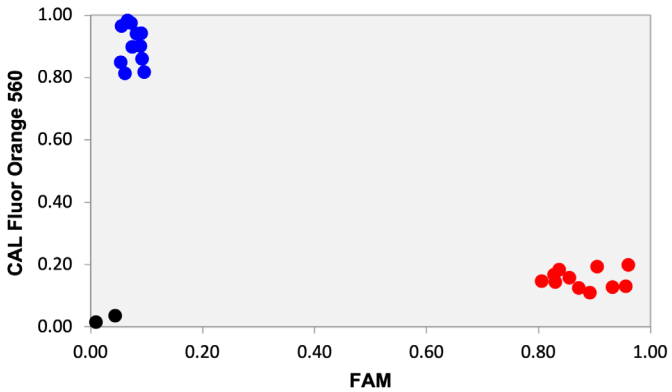

Figure 7. Cluster plot with the same samples as illustrated in Figure 6, with equally scaled axes. Red circles are denoted as wild type samples. Blue circles are denoted as variant samples. Black circles denote NTC samples.

3.3. Reliance of auto-calling and importance of positive controls

Over-reliance on the in-built auto-calling functionality of the cluster analysis software may lead to an incorrect assignment of the sample types. Therefore, it is important to ensure that the nature of samples and performance of the assay is fully understood, and know that overriding of the auto-calling function may be required. To confirm the results assigned by the software, it is important to include positive controls in each experimental run (figure 8 and figure 9).

Figure 8 illustrates a cluster plot in which a large cluster has been assigned a heterozygous sample type by the auto-calling function of the software. The sequence and functionality of some assays can result in the clusters pulling in towards the centre of the cluster plot. Figure 9 illustrates the same samples, but now with the inclusion of positive controls for both wild type and variant. Inclusion of these positive controls verifies that the previously identified heterozygous samples are in fact variant samples. The correct sample assignment can now be made manually.

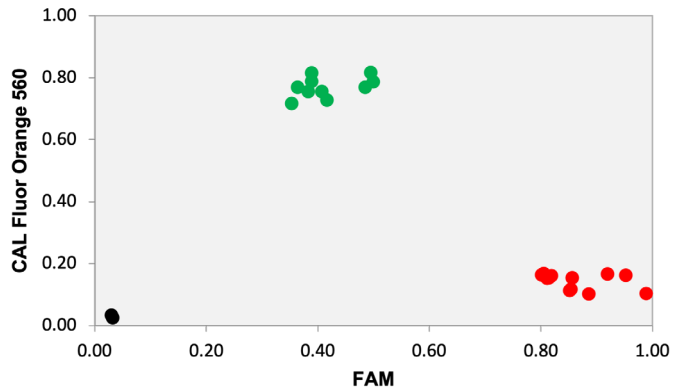

Figure 8. Cluster plot where the auto-calling function has denoted the red circles to be wild type samples and the green circles to be heterozygous samples. Black circles denote NTC samples.

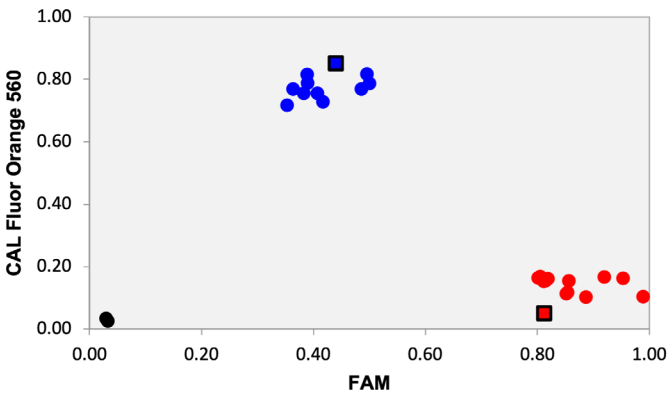

Figure 9. Cluster plot with the same samples illustrated in Figure 8, this time with the inclusion of positive controls, denoted by the square data points (red = wild type, blue = variant). Red circles are confirmed as wild-type samples and blue circles are now confirmed as variant samples. Black circles denote NTC samples.

4. Troubleshooting end-point cluster analysis assays

4.1. Poor or no signal

Possible cause	Recommended solutions
Incomplete activation of reverse transcriptase Without activation of the reverse transcriptase, conversion of RNA into cDNA, and then subsequent amplification of the cDNA during the PCR stage will not be possible.	Ensure the thermal cycling temperatures and times are correctly programmed.
Presence of inhibitors These are typically carried over from the extraction/purification stage. Common inhibitors include phenol, detergents, proteases and organic compounds from the primary biological sample.	Dilute isolated RNA to minimise effect of any inhibitors. Test for the presence of inhibitors using an Internal Quality Control (IQC). Repeat nucleic acid extraction/purification protocol using alternative methods.
Suboptimal reaction components or reaction set-up The reaction set-up may have been performed incorrectly, or the RNA and/or oligonucleotide concentration/sequences may not be optimal.	One or more of the reaction components was not added. Repeat reaction set-up, ensuring all components are added, at the correct volumes. Verify the sequences of the oligonucleotides against target sequence. Ensure instrument setup is correct for each of the fluorophores selected for the reaction. RNA concentration was suboptimal. Quantify RNA to ensure concentration falls within desired ranges.
No RNA present in sample There is no RNA template in the sample.	Include known positive External Quality Controls (EQC) on the run to validate true-negative samples. Enzymatic degradation of RNA has occurred (e.g., via RNases). Re-purify the RNA or repeat RNA isolation using alternative isolation method.

Table 1. Troubleshooting guidance for poor or no qPCR signal

4.2. Amplification evident in non-template control (NTC) samples

Possible cause	Recommended solutions
Reaction mix is contaminated with nucleic acid There has been some form of carryover of nucleic acid into the reaction mix, and/or surface contamination on the equipment. Random contamination is when several NTC wells show varying amplification efficiencies. A common (e.g., reagent) contamination is when all NTC wells show a similar amplification efficiency.	Ensure all workstations and equipment are thoroughly cleaned before and after use. Follow equipment manufacturers' recommendations for use of ethanol and UV-light for decontamination procedures. Use nuclease-free consumables (e.g., tubes, plates, pipette tips) and molecular-grade reagents (e.g., water). Use filter-tipped disposable tips to minimise aerosol production during pipetting. Set up all reactions following Good Laboratory Practices (GLP).
Primer-dimer formation Primers may anneal together, forming potential templates for non-target specific amplification.	Verify the sequences of the oligonucleotides against target sequence, checking for secondary-structure primer formation. Reduce primer concentration. Verify presence of primer-dimers via melt-curve analysis.

Table 2. Troubleshooting guidance for amplification evident in non-template control (NTC) samples

5. Further support

Full details of ordering information, storage conditions, sequence information and reaction set-up can be found in our SARS-CoV-2 Variant ValuPanel assay manual.

For any technical queries regarding the running or analysis of these assays, please contact techsupport@lgcgroup.com.

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