COVID-19 Variant Detection with RT-qPCR SARS-CoV-2 Mutation Assays

Matthew Moser[1], David Gauthier[2], Angelica Olcott[3], Steven Okino[3], Charlie Rzadkowolski[4], Jason Ma[3], Raf Podowski[3], Wei Vivian Fu[3], Alan Wu[1], Jeffrey Whitman[1]

1. University of California at San Francisco (UCSF). Department of Laboratory Medicine– San Francisco (United States)
2. Bio-Rad Laboratories, Clinical Diagnostics Group - Irvine (United States)
3. Bio-Rad Laboratories, Life Science Group – Hercules (United States)
4. Exact Diagnostics (a Bio-Rad Company) – Fort Worth (United States)

Introduction:

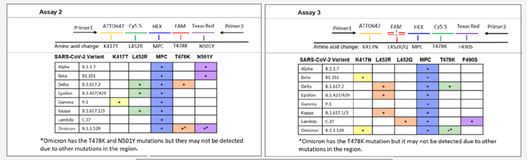
The COVID-19 pandemic has been our most significant global health crisis in modern times. Continuous molecular surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is necessary to understand how the virus is evolving over time in association with infectious characteristics. Efficient methods of identifying mutations are vital to public health efforts to detect when a new variant of interest or concern begins circulating.

The sequencing of SARS-CoV-2 variants is a costly and time-consuming operation that requires specialized expertise and methods. As such, sequencing is a major bottleneck for public health surveillance. Real-time quantitative polymerase chain reaction (RT-qPCR) has been utilized to overcome these limitations and has the potential to develop novel workflows for variant screening (Dikdan RJ, 2022).

To address this, Bio-Rad has developed a novel reverse algorithm RT-qPCR SARS-CoV-2 mutation assay called the "Multiple Mutation Assay" (MMA). This assay allows for the detection of eight of the most relevant SARS-CoV-2 mutations (Alpha/Beta/Gamma/Delta/Epsilon/Kappa/Lambda/Omicron). Single mutation assays have also been developed which target individual variant-associated mutations, as well as the Delta-Omicron variant discrimination assay which can distinguish between both the Delta and Omicron variants.

Here we report a study to verify the performance of these single and multiple mutation assays for research use, targeting variant-associated mutations found in the spike gene of key SARS-CoV-2 variants of concern (VOCs) in remnant clinical samples. The single and multiple mutation assays from Bio-Rad can identify up to four key SNPs and an internal reference gene in a single reaction, and both require <2 hours to perform on standard real-time PCR instrumentation.

Utility of the Multiple Mutation Assay Map



The Multiple Mutation Assay (MMA) is a novel technique for profiling SARS-CoV-2 variants based on their associated mutations. Multiplexed primer/probe combinations can detect up to 4 different mutations plus the MPC control in a single well. The use of the following reverse algorithm approach may aid in triaging samples for further sequencing by NGS. Results from each MMA for SARS-CoV-2 positive samples are compared to its mutation map to determine the probability that a known variant is present. If a SARS-CoV-2 positive sample does not correspond to any known mutation profile, then it may indicate a new emerging variant, and encourage sequencing by NGS.

Method:

Wet lab assay validation of specimens contrived in negative nasopharyngeal (NP) swab matrix with synthetic SARS-CoV-2 wildtype and variant (Alpha/Beta/Gamma/Delta/Epsilon/Kappa/Mu/Lambda/Omicron) RNAs, at high or low inputs (Cq=25.0, Cq=35.0, respectively), was first conducted at Bio-Rad to determine the accuracy of the assays. RNA extraction of the contrived sample was followed by testing 10 µL of sample using the manufacturer's procedure (see assay protocols). Following wet-lab validation, the assays were received at UCSF for the following performance evaluation study with remnant clinical samples.

RNA extracted from remnant NP swab specimens from the University of California, San Francisco Clinical Laboratory at Zuckerberg San Francisco General Hospital (ZSFG) were tested using Bio-Rad's RT-qPCR SARS-CoV-2 single- and multiple-mutation assays. The following assays were utilized: MMA 2, MMA 3, MMA 4 (to distinguish Delta/Omicron BA.1/Omicron BA.2), and a Delta-Omicron Single-Mutation Assay (Delta-Omicron Variant Discrimination Assay). Several cohorts of samples which varied based on the date of infection were analyzed using the single and multiple mutation assay types. The study included nucleic acid amplification test (NAAT) negative samples, NAAT positive samples, NAAT positive samples collected mid-2020 (European variant typed by PCR) and samples from fall and winter 2021 with epidemiological predominance of Delta and Omicron variants, respectively. All assay PCR plates included wells that were spiked with synthetic RNA positive controls corresponding to each of the eight key variants, provided by Bio-Rad.

PCR plates were set up as described in the assay protocols for Bio-Rad's PrimePCR SARS-CoV-2 Multiple and Single Mutation Assays. To set up the RT-qPCR reaction, sample RNA was added to a master mix containing Bio-Rad's "Reliance One-Step Multiplex Supermix" and the primers/probes from the mutation assay, as described in the protocol. Samples were run on a CFX Opus Real-Time PCR System, alongside variant-specific positive controls and negative controls designed for research use only. Specimens were blinded to variant status at the time of testing. Reagents and method are intended for research use only.[1]

Studies on remnant clinical specimens were approved by the institutional review board at UCSF and ZSFG protocol approval.

Design of Mutation Assays:

Single Mutation Assays
Identifies presence of specific SARS-CoV-2 mutation: over 20 assays are available

- 3-plex assay includes reference gene and internal human RNAseP control

Variant Discrimination Assays
Discriminates between two SARS-CoV-2 variants

- Assay distinguishes between Delta and Omicron (BA.1 and BA.2)
- Assay that discriminates between Omicron BA.1 and Omicron BA.2

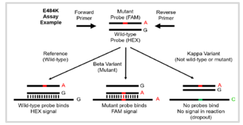
Multiple Mutation Assays
Multiplex assay contains internal control, detects four SARS-CoV-2 mutations in a single well*.

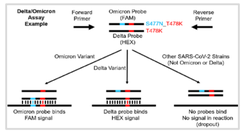
- Four Assays available
- Detect Pre-Delta, or distinguish between Delta/Omicron BA.1/Omicron BA1.1/Omicron BA.2

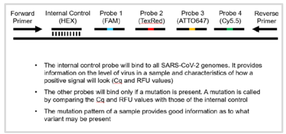
Assay Analysis

- Single mutation and variant discrimination assays can use allelic discrimination in CFX Maestro.
- Multiple mutation assays can use Cq and/or RFU analysis in CFX Maestro.

***Patent pending, Bio-rad Laboratories**







Performance of Single and Multiple Mutation Assays

Bio-Rad Wet-lab Validation of Multiple Mutation Assays
The results of wet lab validation for a series of PrimePCR SARS-CoV-2 single mutation assays for research use provided correct allelic discrimination calls when analyzing the wildtype reference, Alpha, Beta, Gamma and Epsilon variant RNAs. In total, 90 out of 90 low input and 328 out of 328 high input samples gave correct calls. Similarly, wet-lab validation of a multiple mutation assay showed that for all samples tested, 90 out of 90 high input and 90 out of 90 low input reactions identified the correct mutations.

	# In Assay	# Detected by MMA 2
SARS-CoV-2 Positive	20	20
SARS-CoV-2 Negative	7	7
Total Samples in Assay	27	27
Accuracy %		100

Table 1: European Variant Samples
Remnant NP samples (n=27) collected from April to May 2020 were analyzed by the MMA 2 assay. The set included 20 samples PCR positive for the European strain (mutation D641G) and 7 negative samples. The mutation assay was able to detect the SARS-CoV-2 virus in all positive samples but did not detect this variant mutation since the MMA assay lacks the D641G primer/probe set.

	# In Assay	# Detected by MMA 2	# Detected by MMA 3
SARS-CoV-2 Positive	0	0	1
SARS-CoV-2 Negative	20	20	19
Total Samples in Assay	20	20	20
Accuracy %		100	95

Table 2: Negative Sample Testing
NAAT Negative NP samples (n=20) were tested using MMA 2 and 3; MMA 2 did not show any "False-Positive" results. MMA 3 was borderline for a "Positive" indication (<0.1 Cq) just below the Cq assay cutoff of 40, and repeat runs indicated "Negative."

	# In Assay	MMA 2	MMA 3	MMA 4
SARS-CoV-2 Positive	29	29	29	29
SARS-CoV-2 Negative	11	11	11	11
Total Samples in Assay	40	40	40	40
Accuracy %		100	100	100

Table 3: Delta Variant Samples
NP samples (n=40) collected during fall of 2021 were analyzed using MMA 2, 3, and 4; All four assays were able to detect SARS-CoV-2 in the positive samples and type them as Delta variant, which is consistent with the predominant circulating variant at that time. No false positive results were found..

	MMA 2	MMA 3	MMA 4	Delta-Omicron Assay
SARS-CoV-2 Positive; Delta Variant	3	3	3	3
SARS-CoV-2 Positive; Omicron Variant	28	28	28	27
SARS-CoV-2 Negative	1	1	1	2
Total Samples in Assay	32	32	32	32

Table 4: Delta/Omicron Variant Samples:
SARS-CoV-2 NAAT-positive samples (n=32) from late December 2021 were analyzed by MMA 2, 3, 4, and the Delta-Omicron variant discrimination assay. The MMA 2, 3, and 4 assays showed results consistent with the presence of Delta-associated mutations in 3 samples and results consistent with the Omicron variant in 28 samples. MMA 4 was able to distinguish the Omicron BA.1 sub-lineage; BA.2 was not detected, consistent with the time period. Omicron-consistent results were seen in 27 of the samples from the Delta-Omicron variant discrimination assay.

Results and Discussion:

In-house Bio-Rad wet lab assay validation using contrived samples with synthetic SARS-CoV-2 RNA showed that at both the low and high inputs, the single and multiple mutation assays were able to identify the correct mutation profile >99% of the time. The accuracy of these results encouraged the performance evaluation of these research use assays in a UCSF study using remnant NP samples. As described below, clinical samples analyzed using RT-qPCR to validate Bio-Rad's multiple mutation assays demonstrated >99% accuracy in detecting the variant-associated mutations of samples in known cohorts tested.

A collection of 20 SARS-CoV-2 European variant positive samples from April to May 2020 were run using MMA 2. The assay was able to correctly identify the presence of the virus in infected samples and did not show any false-positive results in the negative samples (Table 1). MMA 2 was not able to evaluate the specific variant as the European strain mutation D641G was not included in this assay. However, the ability to accurately determine the presence of the SARS-CoV-2 virus that does not match a variant of concern is important to demonstrate.

A small set of 20 SARS-CoV-2 negative samples were tested using MMA 2 and MMA 3. Both assays determined that all the samples were negative (Table 2). MMA 3 did have a borderline positive sample in one of the replicate tests however, this was by <0.1 CI of the cutoff.

A set of 40 specimens containing 29 NAAT positive samples collected between September and October of 2021 were evaluated using MMA 2, MMA 3, and MMA 4. Delta was the predominant circulating variant during this time period. All the assays were successful in detecting SARS-CoV-2 virus and typing as Delta variant. All negatives came back as expected with no false-positives being detected (Table 3).

The final evaluation focused on samples from late December 2021, a time period when both Delta and Omicron were circulating. All three of the multiple mutation assays and a Delta-Omicron variant discrimination assay were evaluated on a collection of thirty-two SARS-CoV-2 NAAT positive samples. The MMA 2, 3, and 4 assays showed results consistent with the presence of Delta-associated mutations in 3 samples, and results consistent with the presence of Omicron-associated mutations in 28 samples. MMA 4 was able to distinguish the Omicron BA.1 sub-lineage; BA.2 was not detected. Omicron-consistent results were seen in 27 of the samples from the Delta-Omicron variant discrimination assay (Table 4). One false negative result was observed for MMA 2, 3, 4 while 2 false negative results were seen by the Delta-Omicron assay. We are unable to determine if this is due to pre-analytical errors, such as specimen stability and freeze-thaw, or analytical sensitivity.

Overall, we conclude that there is excellent accuracy of these multiplexed assays for discrimination of SARS-CoV-2 positive and negative samples in addition to detection of variant-associated mutations in a single well.

Conclusion:

Streamlined RT-PCR SARS-CoV-2 variant assays have the potential to improve the speed and accessibility of variant screening (Dikdan RJ, 2022). Bio-Rad has developed a research use single mutation assay, variant discrimination assay and multiple mutation panel that have demonstrated excellent correlation with molecular testing performed in the clinical laboratory. Such assays allow for identifying SARS-CoV-2 specimens that do not type for known circulating variants of concern and triage them for sequencing, providing the potential to optimize workflows for new variant detection for public health surveillance.

References:

PrimePCR Single and Multiple Mutation Assay Protocols, bio-rad.com/SC2Variants
Exact Diagnostics SARS-CoV-2 Variant Controls Brochure Q-1684

Dikdan RJ, Marras SAE, Field AP, et al. Multiplex PCR Assays for Identifying all Major Severe Acute Respiratory Syndrome Coronavirus 2 Variants, The Journal of Molecular Diagnostics, volume 24, issue 4 (April 2022). https://doi.org/10.1016/j.jmoldx.2022.01.004