Rapid diagnostics

Assessment of impacts for relevant mutations on point-of-care SARS-CoV-2 test

Key messages

- SARS-CoV-2 variants have public health significance if there is potential for or evidence of impact on transmission, disease severity, or medical countermeasures such as diagnostic tests.
- The impact of mutations on a test's performance is influenced by several factors, including the sequence of the variant, the prevalence of the variant in the population, and the design of the test itself.
- The Accula SARS-CoV-2 Test performance is not impacted by known variants outlined here.

Introduction

As of August 2022, the global incidence of coronavirus disease 2019 (COVID-19), caused by the RNA virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has reached 591 million cases, with a death toll exceeding 6.4 million [1]. Mutations across the SARS-CoV-2 genome have risen rapidly with infection rates [2], as recently evidenced by the emergence of the Omicron variant (composed of multiple lineages or subvariants). First reported in November 2021, the Omicron variant has become the dominant strain globally as of August 2022. Mutations can potentially alter the accuracy of diagnostic tests and lead to false-negative results.

As an indispensable component of patient management, infection prevention, and the public health response, the reliability of diagnostic assays is paramount for timely, optimal decision-making. This technical note describes how relevant mutations are identified and assessed for diagnostic impact in real time through an ongoing pandemic, with a focus on the successful coverage of SARS-CoV-2 variants by the point-of-care Thermo Fisher Scientific™ Accula™ SARS-CoV-2 Test.

Definition and origins of SARS-CoV-2 variants

Mutations arise in all viruses over time. RNA viruses typically have higher mutation rates than DNA viruses due to the lack of sufficient proofreading activities during genome replication. Coronaviruses, however, make fewer mutations than most RNA viruses because they encode an enzyme that corrects some of the errors made during replication. SARS-CoV-2 accumulates 1–2 nucleotide changes in its genome per month, which is roughly half the rate of influenza virus and a quarter the rate of HIV [3].

A variant of a virus contains a mutation (e.g., nucleotide substitution, insertion, or deletion) or constellation of mutations inherited from a single ancestor and distinct from a reference genome. For SARS-CoV-2, commonly used reference genomes are Wuhan-Hu-1, the first genetic sequence identified, isolated from a patient in China, and USA-WA1/2020, the first sequence identified in the US.

Significance of SARS-CoV-2 variants

Most mutations do not have a meaningful impact on the virus's ability to cause infections and disease. Testing to identify which viral variant is present in a patient's specimen is not routine, and clinical care is independent of variant identification in most cases. Knowing if the patient is infected with SARS-CoV-2 or not is what drives clinical decision-making.

However, in the public health domain, SARS-CoV-2 variants become a concern when there is potential to impact COVID-19:

- Transmission
- Disease severity
- Medical countermeasures including:
  - Vaccine effectiveness
  - Treatment efficacy
  - Diagnostic testing

To monitor the potential impact, public health bodies routinely evaluate emerging genetic lineages by conducting systematic genomic sequencing, laboratory studies, and epidemiological investigations.

Tracking virus evolution

In the US, public health laboratories, universities, and commercial diagnostic laboratories sequence SARS-CoV-2 specimens and contribute data to the SARS-CoV-2 Sequencing for Public Health Emergency Response, Epidemiology and Surveillance (SPHERES) consortium of the Centers for Disease Control and Prevention (CDC), and viruses are also sent to the CDC for sequencing and further characterization as part of the National SARS-CoV-2 Strain Surveillance (NS3) program. The genetic sequence data generated by the CDC and partners are submitted to publicly accessible databases maintained by the National Center for Biotechnology Information (**NCBI**) and the Global Initiative on Sharing Avian Influenza Data (**GISAID**).

More than 200 countries contribute genomes to GISAID. As of December 2021, ~2.2% of reported cases have been sequenced globally; in the US, 3.7% of cases have been sequenced [4]. It has been estimated that sampling of 5% of all positive tests allows the detection of emerging variants [5]. Unexpected trends or signals from routine epidemiological surveillance can be an indication of a variant with the potential to influence transmissibility, pathogenicity, and medical countermeasures. National and state level variant proportions are available on the CDC's website: **CDC COVID Data Tracker**.

Variant classification

There is no universal approach to classifying virus genetic diversity below the level of species, but a dynamic nomenclature system called Pango was developed to name and track global transmission lineages of SARS-CoV-2 [6]. Circulating lineages are labeled based on genetic changes associated with important epidemiological and biological events.

The earliest lineages in circulation were denoted as A or B. As they evolved, their descendants were marked by a series of numbers. For example, B.1 includes the outbreak in Europe in early 2020. The variant B.1.351 is its 351st descendant. When the names become too long, a new lineage begins under a different letter of the alphabet. For example, the variant that was first identified in Brazil is called P.1.

To rapidly characterize emerging variants and monitor their potential impact, the CDC, in partnership with the SARS-CoV-2 Interagency Group established by the U.S. Department of Health and Human Services, developed a classification scheme that defines four classes of SARS-CoV-2 variants.

- Variants Being Monitored (VBM) include variants for which there are data indicating potential or clear impact on virus characteristics and medical countermeasures but are circulating at low levels.
- Variant of Interest (VOI) is used to describe a newly emerging variant that contains mutations associated with changes to virus characteristics but for which the medical and public health importance is not yet known.
- When there is evidence of impact to virus features that impact public health, then a viral lineage is considered a Variant of Concern (VOC).
- A Variant of High Consequence (VOHC) has clear evidence for reduced effectiveness of medical countermeasures relative to other virus variants.
- As of April 2022, no VOHCs have been identified during the COVID-19 pandemic. Variant status may escalate or de-escalate, and further information on each class can be found at **SARS-CoV-2 Variant Classifications and Definitions**.

On a global scale, the World Health Organization (WHO) Virus Evolution Working Group monitors SARS-CoV-2 lineages for significant mutations that pose an increased risk to public health. A variant with mutations expected to affect virus characteristics, with unclear evidence or epidemiological impact, is classified as a Variant Under Monitoring (VUM). As with the CDC classification scheme, if mutations are predicted or known to impact transmission, disease severity, or medical countermeasures, and have caused significant community transmission, the lineage is considered a Variant of Interest. A Variant of Concern meets the definition for VOI with impact demonstrated to a degree of global public health significance. To assist with public discussions of variants, the WHO established easy-to-pronounce labels for VOIs and VOCs based on the Greek alphabet (e.g., variant BA.1 was labeled Omicron).

The WHO variant webpage, **Tracking SARS-CoV-2 variants**, maintains a list of latest classifications. Variants are reclassified through a critical expert assessment of several criteria, such as the observed incidence of variant detections among sequenced samples over time and between geographical locations.

Impact on diagnostic tests

COVID-19 tests include molecular and antigen tests that detect the SARS-CoV-2 virus and serology tests that detect antibodies to the virus. Viral mutations can potentially impact performance of all COVID-19 tests, but this will vary because of the inherent design differences of each test. While some investigations of the impact of variants on antigen [7] and serology [8] tests have been reported, the analysis is not as straightforward for molecular tests. Here we focus on the impact of mutations on molecular tests.

Mutations across sites targeted by molecular tests

To maintain assay sensitivity as new variants emerge, an optimal COVID-19 molecular test should target a genomic region with a low rate of mutation. However, many of the diagnostic tests in use today were developed early in the pandemic, when virus sequences were scarce and knowledge of the conserved genetic regions of SARS-CoV-2 was limited. Analysis of related pathogens helped to identify regions of the genome that are more likely to be highly conserved (expected to show low levels of variation), resulting in the selection of open reading frame 1ab (ORF1ab), the envelope gene (E), and the nucleocapsid gene (N) as common targets for diagnostic assays.

Over the course of the pandemic, genomic surveillance of SARS-CoV-2 variants has largely focused on changes in the spike protein, which mediates attachment to cells and is a major target of neutralizing antibodies (antibodies that bind virus and prevent it from infecting cells). There is also interest in whether mutations in the spike protein could potentially compromise vaccine effectiveness, since spike protein is the major viral antigen in the current vaccines. However, mutations arise across the ~30,000 base pair SARS-CoV-2 genome. Figure 1 shows a genetic diversity panel from Nextstrain [9], where the horizontal axis spans each nucleotide site in the genome, and the vertical axis indicates how much variability there is at each site. The heights of the bars in the panel reflect the relative level of change at a particular genomic location. Genomic sites with larger bars correspond to sites where more genetic variation has been observed. Smaller bars suggest that this position is more conserved across the genome.

To reduce the potential impact of mutations in test target regions, developers have employed multiple strategies. One approach is to target both a SARS-CoV-2–specific region to help ensure specificity and a region conserved among very closely related viruses, such as SARS-like coronaviruses, to help ensure sensitivity. Many commercial assays employ a multiplex approach, targeting two or more genes in combination, so that if one target fails, then that will not automatically produce a false-negative result.

It is important to understand that tests detecting multiple SARS-CoV-2 genes do not necessarily perform better than those using a single SARS-CoV-2 target. In a July 2020 review of 150 EUA tests, over 25% of the tests were designed using a single viral target, and many of the most sensitive tests on the market only detect one viral target [10]. There is a perception that mutations would ultimately invalidate a single viral target test, but not a multi-target test. However, analyses of the known variability occurring in the SARS-CoV-2 population have shown minimal or no effect on the sensitivity of existing diagnostic tools for viral detection, including single-target tests [11,12]. Other studies showed mismatches in the primer/probe binding regions of SARS-CoV-2 diagnostic assays did not result in reduced assay performance and false-negative results [13,14]. The consequences of mutations in molecular assays are not straightforward because multiple factors impact test sensitivity and reliability.

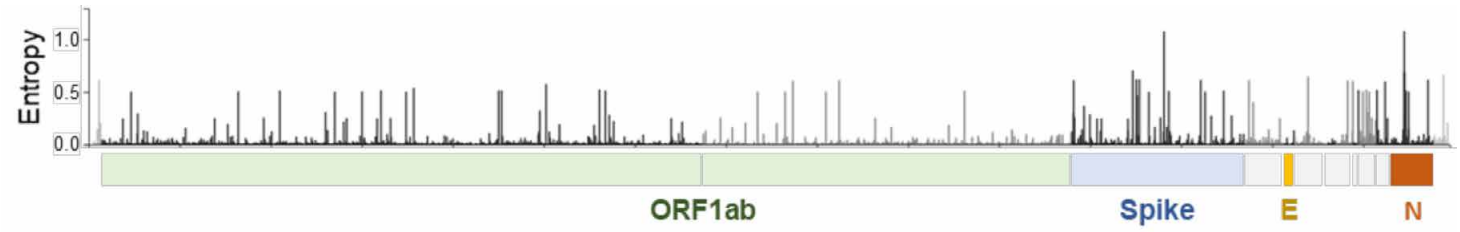

Figure 1. Genetic diversity across the SARS-CoV-2 genome.

How do mutations interfere with molecular testing?

Most molecular tests for SARS-CoV-2 progress through three stages: (1) reverse transcription of the RNA genome into complementary DNA (cDNA), (2) amplification of target(s) at isothermal or cycling temperature, and (3) probe-based detection, with each process requiring hybridization of assay oligonucleotides to a region of the viral genome. Mutations can result in primer or probe sequences that no longer perfectly complement the genetic region they target. Sequence mismatches in the primer and probe binding regions can have no, marginal, or a catastrophic effect on assay performance.

The effects of mutations are variable and depend on sequence context, nature of the mismatch, reaction conditions, polymerase, and primer length [15]. For example, mismatches located in the 3′ end region of a primer (defined as the last 5 nucleotides of the 3′ end) are more disruptive to assay performance than mutations in the 5′ end [16]. DNA polymerases catalyze the addition of nucleotides to the primer's 3′ end, so mismatches in that location can disrupt the enzyme active site (Figure 2).

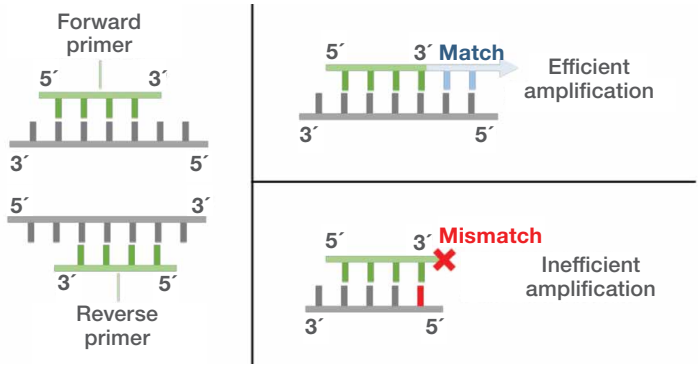

Figure 2. Impact of primer–target mismatches on amplification.

A few examples of mutations causing partial target-failure in multi-target SARS-CoV-2 assays, such as a 6-nucleotide deletion in the S gene of the B.1.1.7 (Alpha variant), have been reported [17-19]. As with any other diagnostic test, manufacturers must continually monitor and validate assay designs and reagents to ensure they remain fit for purpose. To support these efforts, the U.S. Food and Drug Administration (FDA) published a policy for test developers to assess the impact of SARS-CoV-2 mutations [20].

Evaluating the impact of variants on COVID-19 tests

In February 2021, the FDA issued the "Policy for Evaluating Impact of Viral Mutations on COVID-19 Tests" to provide recommendations on evaluating the potential impact of emerging and future mutations of SARS-CoV-2 on COVID-19 tests for the duration of the COVID-19 public health emergency, including considerations for test designs to minimize the impact of viral mutations and recommendations for ongoing monitoring [20]. The proposed evaluation process recommends regular evaluation of global sequences for identification of mutations in test target sites and a progressive assessment of identified issues to determine the impact on test performance (Figure 3).

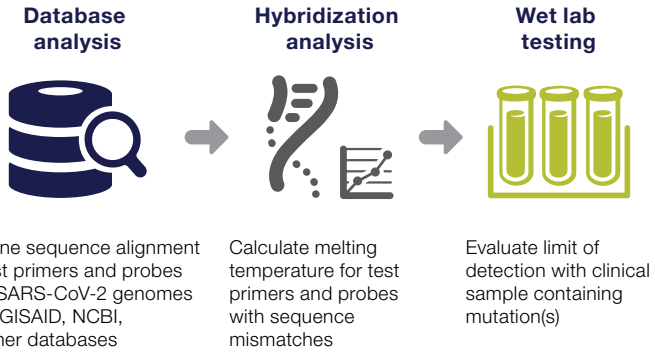

Figure 3. FDA guidance for evaluating the impact of viral mutations on COVID-19 tests.

Identification of mutations in test region

Periodic sequence alignment of primer/probe sequences with publicly available SARS-CoV-2 genomes, such as those in the GISAID database, can identify mutations in the test target region. The FDA currently considers mutation frequency of 5% to be significant (when considering at least 2,000 sequences over a recent period of time, such as the past week, month, or quarter). Genetic changes that may impact test performance—for example, based on sequence context, number of mutations, and prevalence in circulation—merit further investigation of impact to hybridization.

Assessment of hybridization impact

The thermodynamics of DNA hybridization can be predicted by calculating the melting temperature (T_m) of a primer or probe with target DNA. A single mismatch can cause a profound change to T_m. The identity of the mismatch, its position in the sequence, oligonucleotide concentration, and the reaction buffer all impact the degree of the mismatch impact. The FDA recommends performing this calculation using conditions reflective of the conditions of the test. A mismatch T_m that drops to or below the annealing temperature of the test may suggest a reduction in test performance and should be further investigated by wet testing.

Limit of detection analysis

Prediction of hybridization temperatures involving mismatches carries large uncertainties, and the most definitive approach to determine the impact of a potential mutation is to measure the limit of detection (LoD) of the test with a clinical sample containing the mutation. If clinical samples are not available, synthetic RNA constructs may be used to make these measurements. If a difference of ≥3-fold in the LoD is observed, the FDA recommends that test developers notify the FDA with an explanation of the potential risks and any necessary mitigations. The FDA will work with test developers on labeling changes and test modifications as necessary.

The FDA maintains a webpage (**SARS-CoV-2 Viral Mutations: Impact on COVID-19 Tests**) to provide information about certain tests for which the FDA has identified potential impacts on performance due to SARS-CoV-2 genetic mutations.

Demonstration of coverage of mutations by the Accula SARS-CoV-2 Test

The FDA variant webpage reports that Accula SARS-CoV-2 tests on samples that include the genetic variant at positions 28881–28883 (GGG to AAC) "may be impacted", while at the same time noting "the impact does not appear to be significant." The webpage also states there is potential impact on performance of the test due to a mutations at positions 28877–28878 (AG to TC) in patient samples. These mutations appear in the 5′ end of the Accula SARS-CoV-2 Test forward primer (Figure 4).

The Accula platform was designed to tolerate genetic variation, employing long primer sequences with very high melting temperatures far above annealing and extension temperatures. To demonstrate this resilience to mutations, the company supported the FDA analysis by conducting *in silico* database and melting temperature analyses and found no significant impact on the Accula SARS-CoV-2 Test performance. When compared to the LoD of SARS-CoV-2 virus without these mutations, there was no reduction in performance (Figure 4). The 28881–28883 (GGG to AAC) mutation has been circulating in SARS-CoV-2 sequences at varying frequencies since January 2020. The extensive characterization of resilient Accula performance on this mutation reinforces confidence in test performance when it pertains to emerging variants, as is the case for B.1.1.529 (Omicron).

Additionally, an independent study carried out at the NIH investigated the accuracy of the Accula test for SARS-CoV-2 variants in clinical specimens [21]. Sixteen specimens collected between July 2020 and April 2021 were tested—11 of which contained the 28881–28883 (GGG to AAC) mutation—and included emerging variants R.1, P.2, B.1.526, B.1.1.7, and B.1.351. SARS-CoV-2 was detected by the Accula test in all specimens. A dilution series was generated from the specimens to approximate lower viral load samples and split for testing in parallel using the Accula SARS-CoV-2 Test and the Hologic Panther Fusion™ SARS-CoV-2 Assay. The Accula and Panther Fusion platforms showed nearly equivalent performance on the dilution series, with the exception of 2 samples missed by the Panther Fusion assay that were identified by the Accula test (estimated C_t value >38) and one sample not detected by the Accula test that was positive by the Panther Fusion assay at a C_t value of 38.4. The authors concluded that the 28881–28883 variant predicted by bioinformatics analysis to potentially reduce Accula test sensitivity did not impact performance in wet testing and underscored the need for *in vitro* studies to validate *in silico* predictions.

Accula test coverage of CDC and WHO variants

In addition to the ongoing analysis recommended by the FDA, Thermo Fisher Scientific works closely with governmental partners such as the NIH Rapid Acceleration of Diagnostics (RADx) initiative, to investigate the impact of emerging viral mutations on test performance. As part of variant testing through the RADx Variant Task Force, the sensitivity of the Accula test for SARS-CoV-2 variants was evaluated using clinical samples. Table 1 summarizes the results of multiple experiments where individual sequence-verified remnant clinical samples of SARS-CoV-2 or pools of 8–25 clinical remnants were diluted into negative nasal swab matrix to simulate a range of viral loads. An aliquot of each dilution was analyzed with a RT-PCR assay using the CDC EUA N2 gene primers/probe set, and the C_t values were used as a surrogate to estimate the viral load in the pool samples tested. The performance of the assay was assessed using a reference non-VOC/VOI substrain as a comparator in a test evaluation. In the blinded evaluation, the Accula Test detected all variants with equivalent sensitivity to the non-variant comparator (Table 1).

Forward primer 5′-A-G-T-A-G-G-G-3′ | | | | | | | Reference sequence 5′-A-G-T-A-G-G-G-3′	Forward primer 5′-A-G-T-A-G-G-G-3′ ≠ ≠ | | ≠ ≠ ≠ Mismatch sequence 5′-T-C-T-A-A-A-C-3′
Accula test LoD = 150 copies/mL	**Accula test LoD** = 114 copies/mL

Figure 4. Overview of mutations in the 5′ end of the Accula SARS-CoV-2 Test forward primer and corresponding LoD.

Table 1. Accula SARS-CoV-2 test results for patient samples of SARS-CoV-2 variants.

Pango lineage	WHO label	Sample type	N2 gene C_t (avg.)	Accula SARS-CoV-2 Test results		
				Result 1	Result 2	Result 3
B.1.1.7[a]	Alpha	Pool 1	24.32	POS	POS	POS
		Pool 2	27.53	POS	POS	POS
		Pool 3	**32.03**	**POS**	**NEG**	**POS**
		Pool 4	33.46	NEG	POS	NEG
B.1.351[a]	Beta	Pool 1	27.00	POS	POS	POS
		Pool 2	29.16	POS	POS	POS
		Pool 3	**31.53**	**POS**	**POS**	**POS**
		Pool 4	33.84	NEG	POS	NEG
P.1[b]	Gamma	Dilution 1	27.53	POS	POS	n.d.
		Dilution 2	28.52	POS	POS	n.d.
		Dilution 3	**29.79**	**POS**	**POS**	**n.d.**
		Dilution 4	30.62	NEG	POS	NEG
B.1.617.2[c]	Delta	Pool 1	26.03	NEG	POS	POS
		Pool 2	28.79	POS	POS	POS
		Pool 3	**31.40**	**POS**	**POS**	**NEG**
		Pool 4	33.94	NEG	NEG	NEG
B.1.427[a]	Epsilon	Pool 1	25.35	POS	POS	POS
		Pool 2	27.71	POS	POS	POS
		Pool 3	32.00	POS	POS	NEG
		Pool 4	**34.71**	**POS**	**POS**	**NEG**
B.1.429[a]	Epsilon	Pool 1	25.23	POS	POS	POS
		Pool 2	29.96	POS	POS	POS
		Pool 3	**33.15**	**NEG**	**POS**	**POS**
		Pool 4	34.69	NEG	NEG	NEG
P.2[a]	Zeta	Pool 1	26.74	POS	POS	POS
		Pool 2	30.64	POS	POS	POS
		Pool 3	**34.78**	**POS**	**POS**	**POS**
		Pool 4	36.51	NEG	NEG	NEG
B.1.525[a]	Eta	Pool 1	25.20	POS	POS	POS
		Pool 2	28.70	POS	POS	POS
		Pool 3	**31.21**	**POS**	**POS**	**POS**
		Pool 4	34.74	NEG	NEG	NEG
B.1.526[b]	Iota	Dilution 1	31.47	POS	NEG	NEG
		Dilution 2	32.32	POS	POS	n.d.
		Dilution 3	**33.54**	**POS**	**POS**	**n.d.**
		Dilution 4	34.20	NEG	NEG	n.d.
B.1.621[c]	Mu	Pool 1	22.18	POS	POS	POS
		Pool 2	25.56	POS	POS	POS
		Pool 3	**28.87**	**POS**	**POS**	**POS**
		Pool 4	32.18	NEG	NEG	NEG

a Pooled, heat-inactivated clinical samples diluted into negative matrix and 60 µL directly loaded into cassette. Highest detected pool N gene C_t for nonvariant comparator = 33.37.

b Individual, heat-inactivated clinical samples, diluted into negative matrix and 50 µL pipetted onto swab; swab eluted into Accula buffer and 60 µL loaded into cassette. Highest detected pool N gene C_t for nonvariant comparator = 31.5.

c Pooled, heat-inactivated clinical samples diluted into negative matrix and 60 µL directly loaded into cassette. Highest detected pool N gene C_t for nonvariant comparator = 30.65.

n.d.: not determined.

Shaded rows indicate highest C_t values with 2-of-3 or 3-of-3 positive results.

Thermo Fisher also evaluated performance for major Variants of Concern at concentrations near the reported LoD of the Accula SARS-CoV-2 Test as they have emerged over the course of the pandemic (Table 2).

Table 2. Inclusivity testing of SARS-CoV-2 Variants of Concern.

SARS-CoV-2 lineage[a]	Concentration (copies/mL)
USA-WA1/2020, reference strain (non-VOC/VOI)	150
USA/CA_CDC_5574/2020, B.1.1.7, Alpha	125
USA/PHC658/2021, B.1.617.2, Delta	150
USA/MDHP2087/2021, B.1.1.529, Omicron	200
USA/CO-CDPHE-2102544747/2021, BA.2, Omicron	200

a Dilutions of inactivated virus in clinical matrix were tested near the reported LoD (150 copies/mL). All evaluated variants had a positivity of at least 95% across 20 replicates. Each strain was tested independently.

Conclusions

Since the outbreak of COVID-19, clinical laboratories, regulators, and manufacturers have anticipated viral mutations would occur with the potential to impact diagnostic testing. The continual emergence of viral variants requires the vigilance of test developers to monitor global genomes for potential mismatches in primers and probes. However, the presence of mutations in a target region do not necessarily predict a deleterious effect on test performance and should be evaluated by a progressive assessment. The severity of impact of SARS-CoV-2 variants on test performance is moderated by the nature and frequency of mutations but also by the overall design of the test. The Accula SARS-CoV-2 Test has demonstrated resilience to mutations and robust coverage of emerging variants throughout the pandemic and serves as a reliable, sensitive test for SARS-CoV-2 detection at the point-of-care.*

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COL35358 0123 MKT-70063 Rev B

This test has not been FDA cleared or approved but has been authorized for emergency use by FDA for use by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. §263a, that meet requirements to perform high, moderate, or waived complexity tests. The test is authorized for use at the Point of Care (POC), i.e., in patient care settings operating under a CLIA Certificate of Waiver, Certificate of Compliance, or Certificate of Accreditation. This test has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens. The emergency use of this test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of *in vitro* diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the declaration is terminated or authorization is revoked sooner.

* The performance of this test was established based on the evaluation of a limited number of clinical specimens. Clinical performance has not been established with all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.