

Detection of the Omicron variant mutation at position 26,270 in the SARS-CoV-2 E gene using the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit

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INTRODUCTION

Diagnostic testing and surveillance play a key role in the fight against COVID-19, particularly as the SARS-CoV-2 virus continues to evolve. At the time of writing, five different SARS-CoV-2 variants have been designated Variants of Concern (VOC) by the World Health Organization [1]. Omicron (B.1.1.529), the most recent variant designated on November 26, 2021, is of significant interest given that it contains roughly 60 sequence substitutions, deletions or insertions compared to the SARS-CoV-2 reference strain, some of which impact its transmissibility and antigenicity

[2,3]. These changes in the virus genome can also jeopardize molecular diagnostic testing and surveillance tools, as genetic variations may increase the rate of false negative or inconclusive results due to testing failures or reduced assay sensitivity. The ability to accurately detect SARS-CoV-2 infections in clinical samples is critical to controlling the spread of the disease, particularly as infections continue to occur, even among vaccinated individuals. The impact a particular SARS-CoV-2 variant may have on a test depends on several factors including: the prevalence of

the variant within the population; the location of mutations within its genome; and the design of the nucleic acid amplification test.

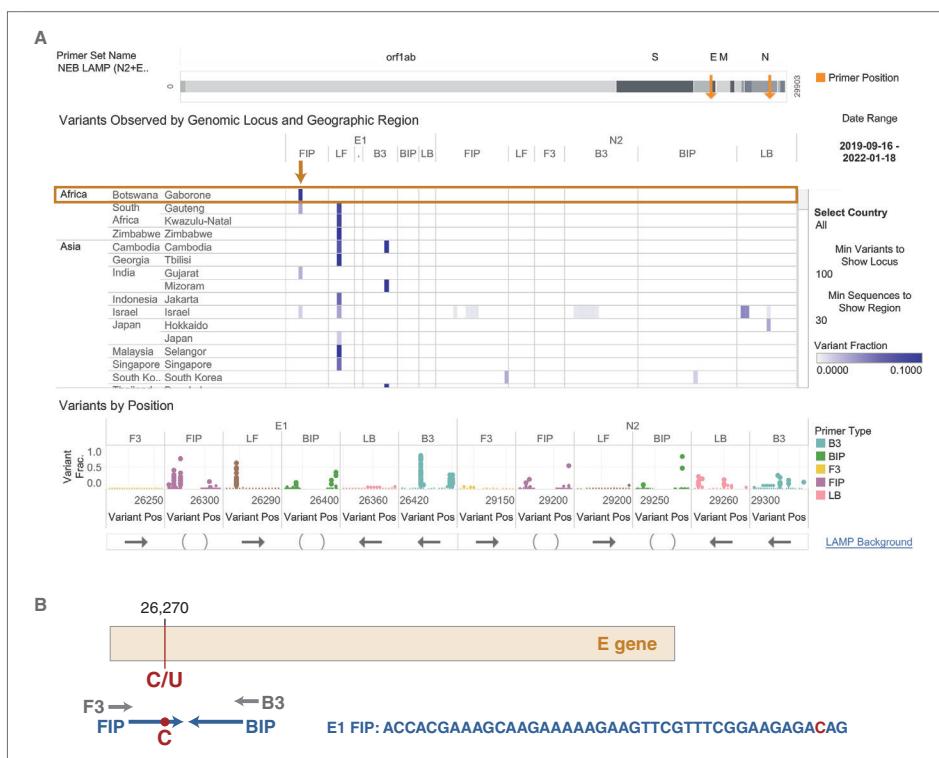
Given the importance of testing, the US Food and Drug Administration (FDA) routinely reports how virus mutations may impact COVID-19 tests in the United States that have previously been granted Emergency Use Authorization (EUA) to ensure continued accuracy and reliability [4].

Some diagnostic tests fail to detect Omicron and are no longer recommended for use by the FDA now that Omicron has become the dominant variant in the US population. The FDA also issued guidance for test developers on evaluating potential impact of viral mutations on assay performance [5].

In response to the COVID-19 pandemic, NEB[®] launched the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit (NEB #E2019), a Research Use Only (RUO) product that enables visual, pH-based, binary detection (pink-to-yellow) of SARS-CoV-2 nucleic acid amplification in just 30 minutes at 65°C. The kit contains a dual primer mix (NEB #S1883) that recognizes the nucleocapsid (N) and envelope (E) regions of the SARS-CoV-2 genome simultaneously. Since primers for both target regions are present in the same reaction, amplification of either target can generate a positive signal. Omicron contains a point mutation in the E gene that overlaps the third position from the 3' end of the E1 FIP primer in the SARS-CoV-2 Colorimetric LAMP Kit (Figure 1). This mismatch was rapidly identified by the NEB Primer Monitor Tool (<https://primermonitor.neb.com>), which continually monitors registered primer and/or probe sets for overlapping variant mutations [6]. Although LAMP is tolerant to sequence variation and the dual N2/E1 primer mix reduces the risk of false negatives from mutations in either

FIGURE 1: The Omicron E gene contains a mutation targeted by the E1 FIP primer of the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit

- A. Visual depiction of the Omicron variant mutation within the E1 FIP primer site generated using the NEB Primer Monitor Tool. The Omicron variant from Southern Africa (Gaborone, Botswana) and the mutation are annotated with a box and an arrow, respectively.
- B. Schematic representation of four out of six E1 LAMP primers (F3, B3, FIP, BIP). The SARS-CoV-2 Omicron variant has a C to U mutation at position 26,270, which is within the E1 FIP target sequence. Not drawn to scale.



target, we sought to determine the performance impact of this mutation on detection of SARS-CoV-2 with the Colorimetric LAMP Kit [7]. We evaluated the effect on the assay by using *in vitro* transcribed (IVT) E gene RNA representing the wild-type (GenBank/GISAID ID MN908947.3) and Omicron variant (BA.1, GISAID ID EPI_ISL_6752027). The SARS-CoV-2 Colorimetric LAMP Kit was able to detect the Omicron variant with similar sensitivity as the wild-type sequence, suggesting that assays using this kit are unlikely to be impacted by the currently circulating Omicron variant.

RESULTS

SARS-CoV-2 E gene RNA containing the C to U mutation at position 26,270 was generated using site-directed mutagenesis (Q5® Site-Directed Mutagenesis Kit, NEB #E0554) using a plasmid containing a fragment of the SARS-CoV-2 genome (26188 to 26472, GenBank ID MN908947.3) that spanned the E gene. The mutation was confirmed by Sanger sequencing. The wild-type and mutant E gene RNA were subsequently synthesized by *in vitro* transcription (HiScribe® T7 High Yield Synthesis Kit, NEB #E2040) from linearized plasmids containing either wild-type or mutant E genes, respectively. The resulting RNA was purified using the

Monarch® RNA Cleanup Kit (NEB #E2050) and concentration measured with the Qubit® RNA BR Assay Kit (Thermo Fisher Scientific, Q33224). The RNA concentration and copy number were confirmed by RT-qPCR (Luna® Universal One-Step RT-qPCR Kit, NEB #E3005) using the E1 F3 (5'-TGAGTACGAACTTATGTACTCAT-3') and B3 (5'-TTCAGATTTAACACGA-GAGT-3') LAMP primers from the SARS-CoV-2 Colorimetric LAMP Kit that can function in PCR. The purity and quality of the plasmids and RNA were assessed on a 1.2% agarose gel. SARS-CoV-2 N gene RNA was also synthesized by IVT and characterized similarly.

The SARS-CoV-2 Colorimetric LAMP Kit was previously shown to have an LOD of 50 target copies with synthetic SARS-CoV-2 RNA [8]. This sensitivity is due to the combined N2 and E1 primer mix, as each primer set individually has a higher LOD of approximately 100 target copies in colorimetric LAMP [9,10]. To evaluate the impact of the C26270U mutation on the LAMP kit, three input concentrations (5000, 500 and 50 copies/reaction) were evaluated for both the wild-type and the mutant E gene RNA in a background of Jurkat total RNA (BioChain, R1255815-50). The mutant and wild-type target RNA were subsequently amplified using the SARS-CoV-2 Colorimetric LAMP Kit at

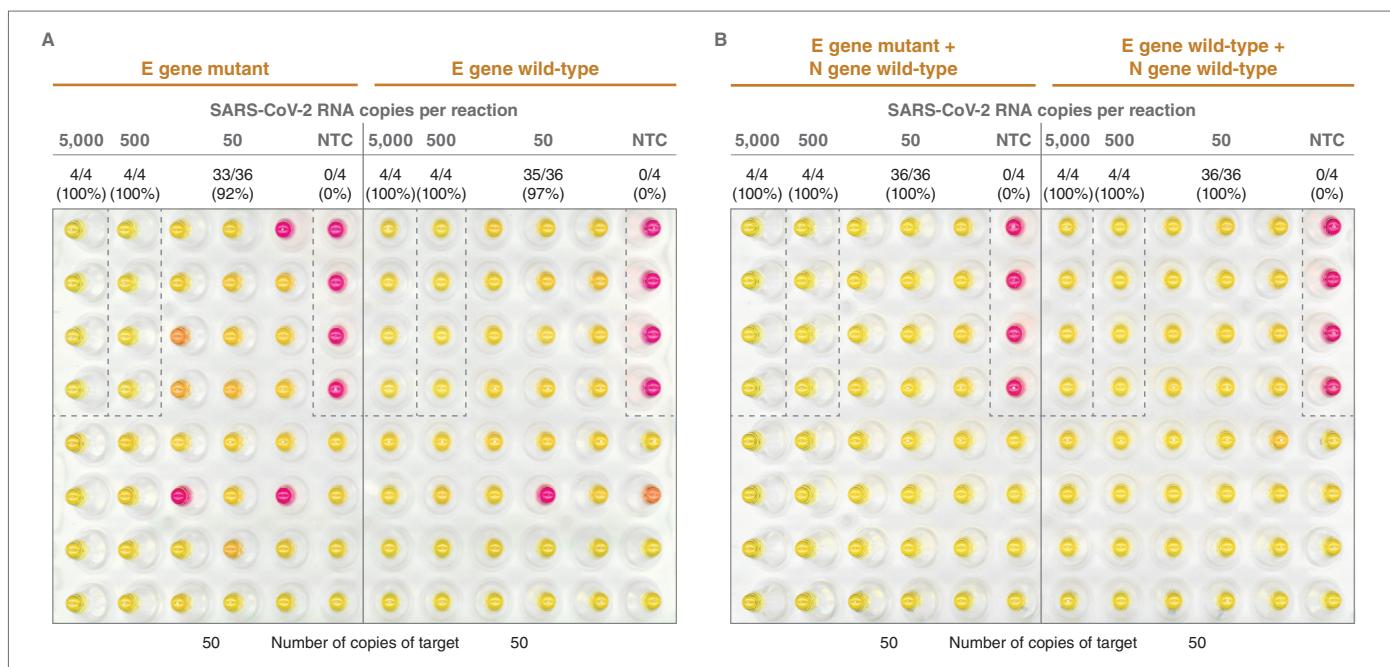
65°C for 30 minutes in the presence of 40 mM guanidine hydrochloride (25 µl reactions, 96-well plate). To investigate the impact on sensitivity, a total of 36 reactions containing 50 copies/reaction were analyzed. Identical experiments were also performed in the presence of IVT SARS-CoV-2 wild-type N gene RNA (5000, 500 and 50 copies/reaction) so that both N and E gene RNA targets were present together in the reaction, which more closely mimics a real sample. Mutant E gene detection was similar to wild-type E gene detection without (Figure 2A) or with (Figure 2B) N gene RNA, though the LOD improved with both targets present, consistent with previous findings [9]. These data suggest that the mutation in the E gene of Omicron does not impact SARS-CoV-2 detection or sensitivity of the SARS-CoV-2 Colorimetric LAMP Kit.

Given that colorimetric LAMP gives a binary readout, these assays were also evaluated by spiking in 1X LAMP Fluorescent Dye (NEB #B1700) to monitor reactions in real-time to determine any impact on time to detection. Reactions were run on a Bio-Rad® CFX96 Touch™ real-time PCR instrument with data collection every 15 seconds in the SYBR®/FAM™ channel. Interestingly, a loss of sensitivity and slight increase in time to detection for all template concentrations were observed in the presence of the mutant E gene RNA alone



FIGURE 2: The SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit efficiently detects E gene IVT RNA carrying the Omicron mutation

Three logs of mutant or wild-type IVT E gene RNA were amplified using the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit in the absence (A) or presence (B) of wild-type N gene RNA. Mutant E gene detection was nearly identical to wild-type E gene detection regardless if the wild-type N gene was present. Reactions that were orange rather than pink or yellow were counted as positive (A).



when compared to the wild-type E gene RNA (Figure 3A), while addition of wild-type N gene RNA to the reaction restored the performance (Figure 3B). The change in performance lead us to hypothesize that the DMSO carried into the reaction from the NEB LAMP Fluorescent Dye (2% v/v DMSO at 1X) may impact mutant E gene detection. To investigate this further, a

reaction mix cocktail was created using only the components of the SARS-CoV-2 Colorimetric LAMP Kit (i.e., no LAMP dye). The mix cocktail was subsequently divided and 2% DMSO was added to one half of the reaction mix. Forty reaction replicates containing 50 copies of mutant E gene RNA were then amplified using the cocktails with or without DMSO. Spiking in

2% DMSO into the SARS-CoV-2 LAMP assay carrying the mutant E-gene RNA alone reduced the detection sensitivity (Figure 4, page 4). DMSO only impacted reactions with the mutant E gene alone, as reactions in the presence of both the mutant E gene and wild-type N gene were essentially unchanged without (Figure 2B) or with (Figure 3B) the fluorescent dye.



FIGURE 3: Addition of 1X LAMP Fluorescent Dye to the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit impacts detection of Omicron E gene RNA

Three logs of mutant or wild-type IVT E gene RNA were amplified using the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit in the presence of 1X LAMP Fluorescent Dye (NEB #B1700) without (A) or with (B) wild-type N gene RNA. The right-hand panels highlight time to detection, where each dot represents the time at which the fluorescence signal for a single reaction crossed the instrument-defined threshold (dots frequently overlap). The average time to detection for amplified samples is indicated by the gray line. Lower sensitivity and a slight increase in time to detection was observed for mutant E gene RNA when compared to the wild-type E gene sequence in the absence of N gene RNA template (A). Addition of wild-type N gene RNA to the reaction containing the mutant E gene sequence restored low input detection such that the LOD was 50 copies/reaction and also corrected the speed of detection (B).

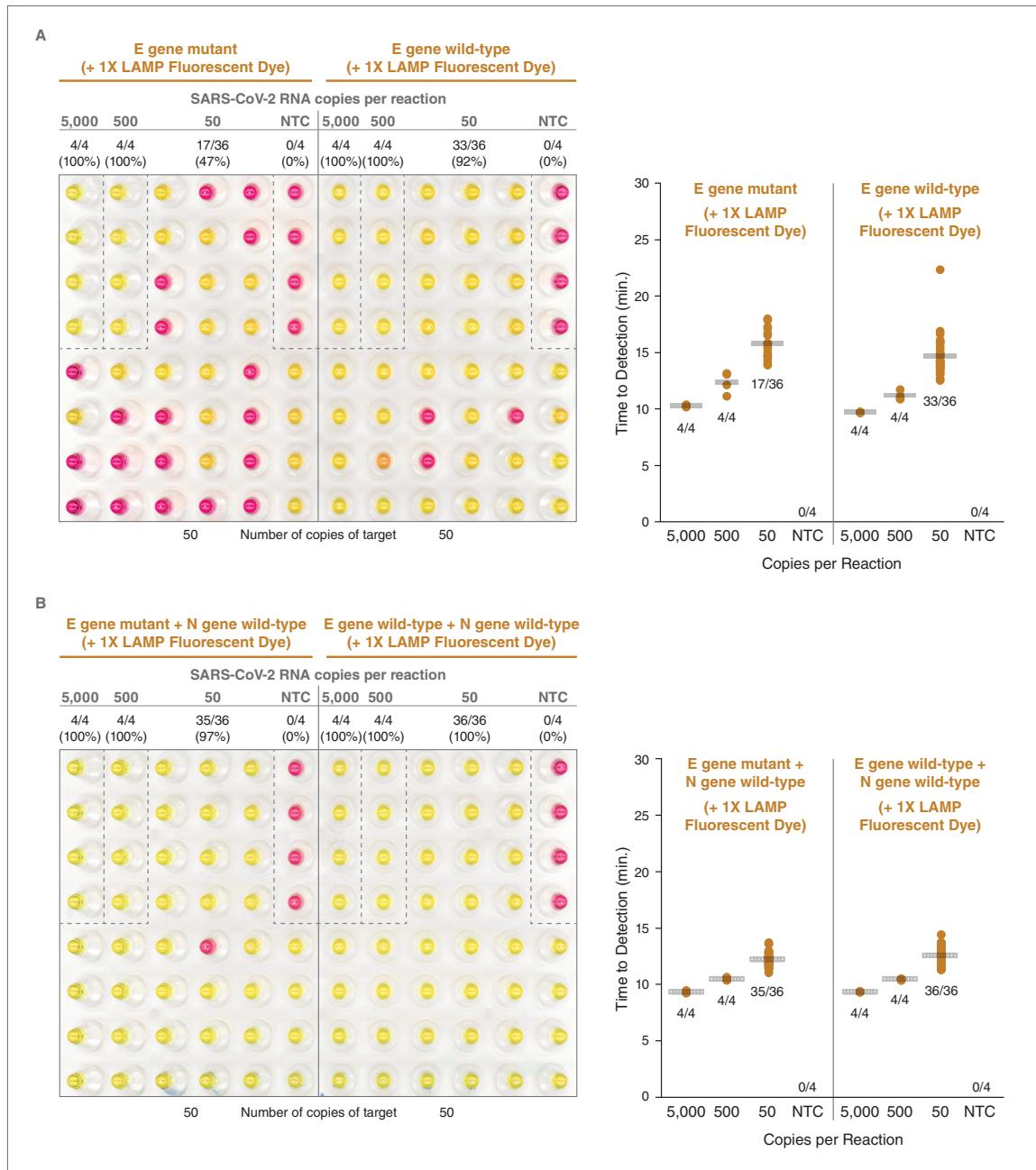
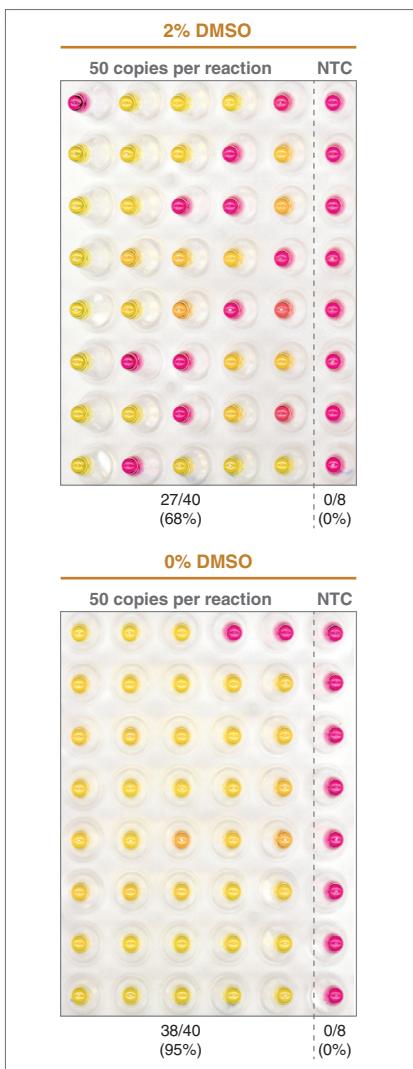




FIGURE 4: DMSO carried over into the reaction from addition of 1X LAMP Fluorescent Dye impacts the detection sensitivity for Omicron E gene RNA

Forty reaction replicates containing 50 copies of mutant E gene RNA were amplified in the presence or absence of 2% DMSO. The addition of DMSO decreases the sensitivity of the detection for mutant E gene RNA alone.



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CONCLUSION

It is essential for assay developers to determine the effectiveness of SARS-CoV-2 testing and screening tools to ensure robust detection of COVID-19 as the virus evolves. The fast rise of Omicron around the globe and its mismatch near the 3' end of the E gene FIP primer led us to investigate a potential performance impact to the SARS-CoV-2 Rapid Colorimetric LAMP Assay Kit (NEB #E2019). In RT-PCR, mismatches at the 3' end of the forward or reverse primer are more likely to impact assay performance, though the impact is often dependent on the position and sequence context of the mismatch, in addition to experimental conditions [11]. In one SARS-CoV-2 example, sensitivity of N gene detection decreased 67-fold when a single mismatch occurred four bases from the 3' end (16th of 20 bases; G29140U) of a forward N gene primer [12]. In contrast, a recent study in LAMP showed that SARS-CoV-2 detection was quite tolerant to mismatches [7]. Single point mutations at any position in the six N2 or E1 SARS-CoV-2 LAMP primers had little impact on detection of synthetic SARS-CoV-2 RNA, with the most common result being a 5-10% slowdown in detection, particularly for mutations at the 3' end of the FIP and BIP primers. No loss of sensitivity was observed when testing was conducted at 100 copies of input. While the Omicron E gene mismatch had no observable performance impact on the binary readout of SARS-CoV-2 detection using the dual N2/E1 primer mix in the SARS-CoV-2 Colorimetric LAMP Kit, we did identify a testing scenario that impacted sensitivity and time to detection. Addition of LAMP Fluorescent Dye to the SARS-CoV-2 Colorimetric LAMP Kit to monitor amplification in real time compromised the sensitivity of the assay when the mutant E gene RNA alone was used as template. There also was a slight increase in the average time to detection for all input concentrations (Figure 3A, right panel). The decrease in sensitivity in this scenario was due to addition of 2% DMSO in the reaction, which was carried into

the assay from the LAMP Fluorescent Dye given that the 50X stock is stored in 100% DMSO (Figure 4). We speculate that the DMSO impacts primer annealing due to the mismatch at the 3rd nucleotide from the 3' end of the E1 FIP primer. The addition of the wild-type N gene RNA to the mutant E gene reaction restored the LOD to 50 copies/reaction and corrected the detection time. This observation suggests there will be little to no impact on detection of Omicron for assays that add LAMP Fluorescent dye to the SARS-CoV-2 Colorimetric LAMP Kit, as both genes will be present for detection. However, assays containing DMSO that use the E1 primers alone may have a decreased sensitivity or increased detection time due to the mismatch. These data highlight how small changes in assay design and conditions can impact detection of SARS-CoV-2 variants while also demonstrating how the dual primer mix safeguards detection of SARS-CoV-2 regardless of sequence variation. Finally, although our evaluation has determined that the mutation in the E1 FIP primer does not diminish amplification performance using the SARS-CoV-2 Colorimetric LAMP Kit, empirically assessing the impact variants may have on testing workflows will be critical to ensure continued reliability of SARS-CoV-2 detection.

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