Primer Monitor: an online tool to track SARS-CoV-2 variants that may impact primers used in diagnostic assays

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Nucleic acid diagnostic tests for SARS-CoV-2, whether based on RT-qPCR, RT- LAMP or other amplification technologies, all depend on primers. While the SARS-CoV-2 genome seems to be less variable (1) than some other retroviral genomes, variants with potential effects on amplification efficiency have arisen and become prevalent in local areas. Some regions (e.g., Brazil; Madera County, CA, USA (2)) report greater than 15% of observed sequences with variants in genomic loci commonly used by diagnostic tests. We developed a streaming analysis method to identify variants that may affect specific primers, and an online tool (primer-monitor.neb.com) to allow interested users to register and track primer loci.

INTRODUCTION

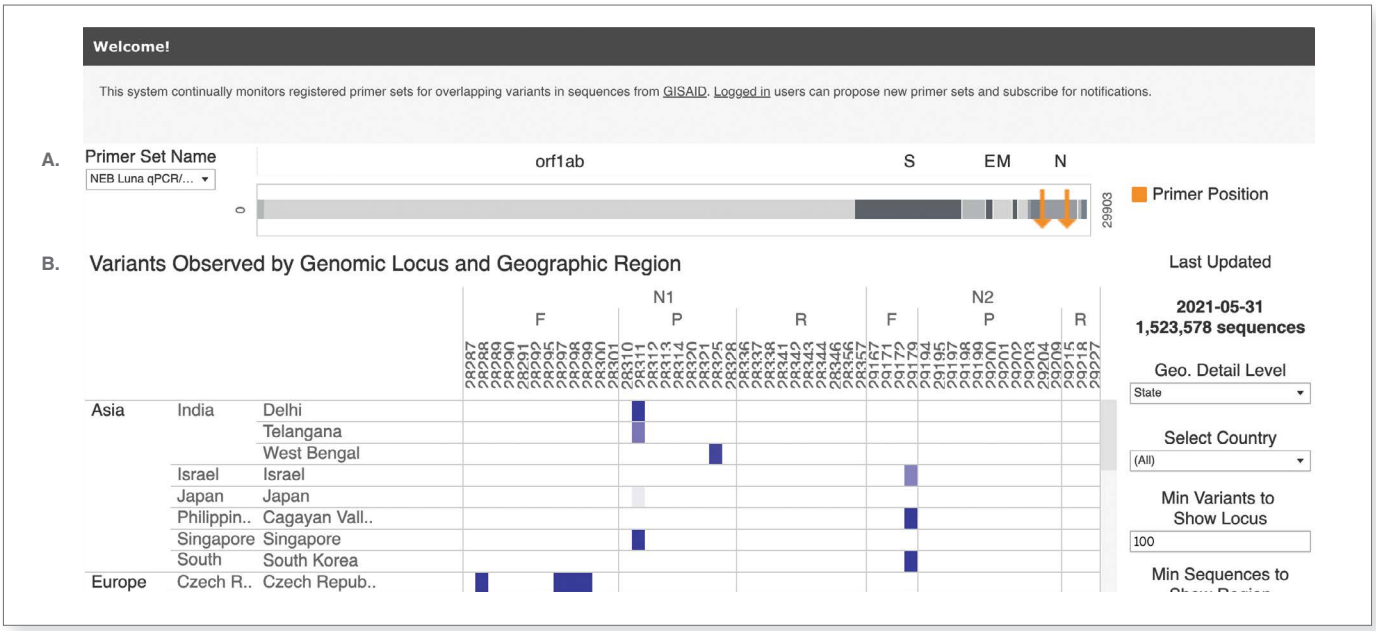
As SARS-CoV-2 variants continue to emerge across the world, diagnostic developers face increasing challenges to demonstrate that SARS-CoV-2 assays will continue to detect the virus variant that may be circulating in the population being tested. In the US, the FDA has issued a guidance document recommending that all test developers consider the impact of current and future variants on their COVID-19 assays during and post-development. To understand whether variants might impact assay performance, there is a need to first understand the nature of the variants that may be present in a given population. To that end, we have developed an online Primer Monitor tool to track SARS-CoV-2 variants as a function of geography and map those variants against user-defined and commonly used primer sets, such as those provided by the Centers for Disease Control and Prevention (CDC).

DESCRIPTION AND USE OF THE PRIMER MONITOR TOOL

As shown in Figure 1, the main page of the tool (Primer Variant Summary tab) shows an overview of the primer set of interest within the context of the SARS-CoV-2 genome (Figure 1A), and a broad view of variants that may impact primer binding, as observed across geographic region (Figure 1B). Data is regularly uploaded (multiple times per week) to the tool directly from GISAID, an initiative that promotes the rapid

FIGURE 1: **Primer variant summary**

Users can choose from a variety of pre-loaded primer sets using a drop-down menu (A). The location of the chosen primer set is noted in the context of the SARS-CoV-2 genome by orange arrows. (B) shows an overview of variants that may impact primer binding across various geographic regions. Users can specify minimum thresholds using the right-hand panel. Hovering over shaded squares will reveal additional information, including total sequences deposited, to enable further evaluation of potential impact.



sharing of data from all influenza viruses and the coronavirus-causing COVID-19. To highlight emerging variants of interest, a variant fraction of at least 10% is depicted in dark blue. Note that only primer loci with variant fractions that meet user-defined minimum thresholds are shown in this panel. Registered users can subscribe to be notified when a variant overlapping a primer set reaches a threshold fraction of observed sequences in any geographic region.

To further investigate variant position as a function of primer location, a second visual is presented at the bottom of the main page (Figure 2). In this visual, variants are shown in the context of the full primer/probe sequences, enabling a complete assessment of potential impacts to primer/probe annealing dynamics. With qPCR primers, variants that occur closer to the 3´ end of primers/probes are typically more disruptive to assay performance than variants that occur closer to the 5´ end.

By evaluating both geographic and genomic regional variation, specific hotspots can be detected where primer assessment might be warranted. In the data above, a significant variant in South Korea with a mutation occurring near the 3´ end of the CDC N2 forward primer (position 29179) was detected. To further investigate the potential impact of this variant, RNA representing the N2 region from the wild-type SARS-CoV-2 sequence (Wuhan-Hu-1) and from the mutant S. Korean variant were assessed exper-

FIGURE 2: **Variants by position**

At the bottom of the main page, positions of the variants are shown in the context of the full primer/probe sequences (A). Dots indicate data points from the figure above and each dot reveals additional information upon hovering. A simple pictogram helps to orient the user to the forward and reverse primers and probe sequences (B). Data sharing, downloading, and further manipulation are all enabled using tools at the bottom right corner of the page.

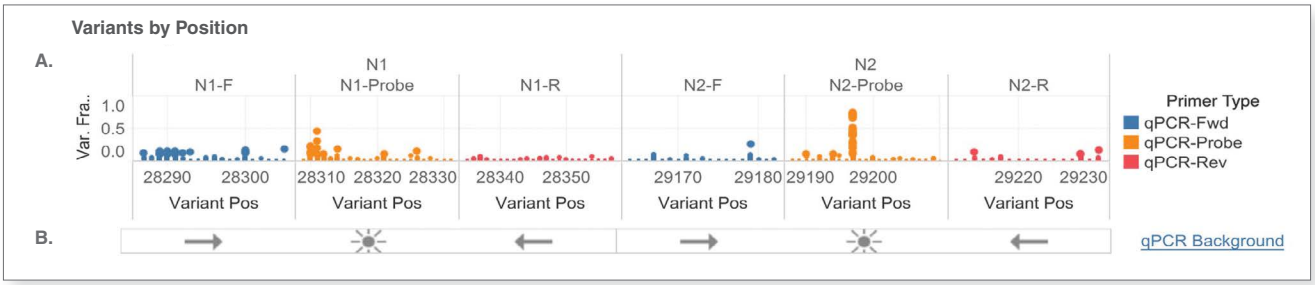
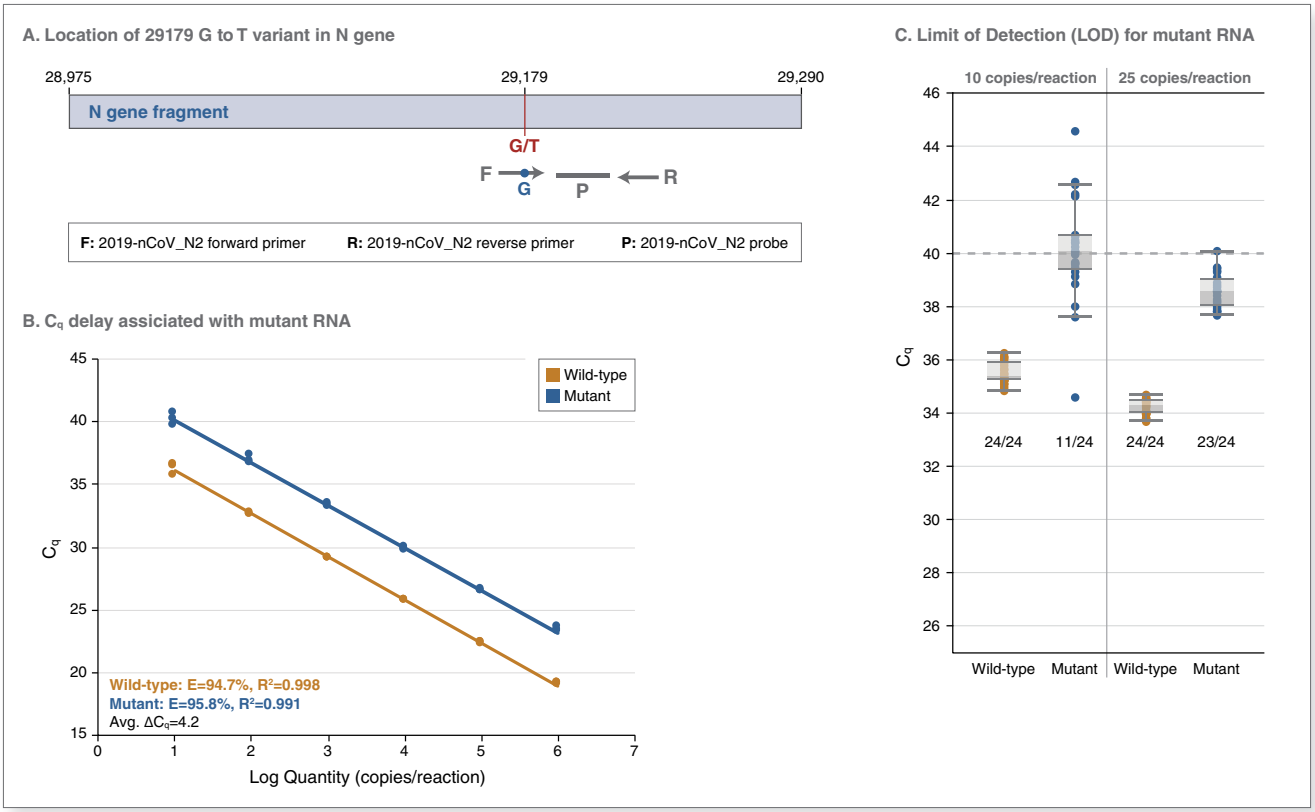


FIGURE 3: **Variant impact on N2 target detection**

The Primer Monitor tool identified a prominent variant from some countries with a SNP close to the 3´ end of the 2019-nCoV-2_N2 forward primer included in the Luna Kit, which detects the Centers for Disease Control and Prevention (CDC) SARS-CoV-2 N1, N2 targets and human RNase P gene with modifications (See details) (A). To evaluate the impact of this SNP on the N2 target detection, we prepared two N gene RNA fragments containing the wild type and mutant N2 targets, respectively, by *in vitro* transcription and quantitated them using the SARS-CoV-2 RNA Control 2 from Twist Bioscience. Using the primer/probe set included in the Luna kit, we observed an average 4.2 C_q delay for the mutant N2 target (B). The limit of detection (LOD) for the mutant N2 target was 25 copies/reaction. Though all the reactions containing 10 copies of the mutant RNA generated amplification signal, only 11 of 24 had a $C_q \leq 40$, the cut-off for detection (C).



imentally. Using the Luna SARS-CoV-2 Multiplex Assay Kit (NEB #E3019) based on the CDC N1 and N2 primer/probe sets described previously (FAQ), we observed a minor impact of the variant on assay sensitivity (from an LOD of 5 copies per reaction to 25 copies per reaction), and a consistent C_q delay across different RNA input amounts (Figure 3).

For LAMP assays, most single point mutations are not disruptive enough to result in significant assay performance perturbations (3, 4), suggesting that this technique may offer additional benefits in the face of emerging SARS-CoV-2 variants. Additional information on NEB's LAMP-based SARS-CoV-2 assay can be found at www.neb.com/E2019.

PRIMER SETS

The tool is preloaded with commonly used primer sequences from SARS-CoV-2 qPCR and LAMP assays and ARTIC sequencing workflows (currently v3). Users who create a free account may also upload additional primer sets, which will become public and available for any/all to monitor after a simple review and mapping process. Users who subscribe will also be able to receive notifications if variations within a specific primer set region cross a specified threshold in a geographic region of interest.

NAMED VARIANTS OF INTEREST/CONCERN

Numerous agencies have been tracking specific SARS-CoV-2 variants that have been recently classified by the CDC as Variants of Interest or Concern. These include named variants with mutations that may impact receptor binding, susceptibility to current treatments or vaccines, or represent an increased likelihood of transmission (e.g., B.1.1.7, P.1, etc). On the Lineage Variants page of the Primer Monitor Tool, static visuals representing many of these variants are depicted along with the genomic location of the mutations present within these named variants and potential overlap with commonly used primer sets (Figure 4). Specific genomic regions of interest are also presented in additional figures on the page to enable further investigation.

CONCLUSION

This tool provides diagnostic assay developers with additional resources to evaluate SARS-CoV-2 assay effectiveness by providing up-to-date data highlighting potential issues around primer/probe binding. It has been released quickly in response to ongoing concerns and needs in the scientific and diagnostic community in the hopes that this task will be made a bit easier for all. It is under active development at GitHub and additional features including time-course assessment, and primer-centric scoring are in progress or planned. Visualization is enabled by Tableau. Contributions, problem reports, and feature requests are welcome and requested.

Acknowledgements

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References

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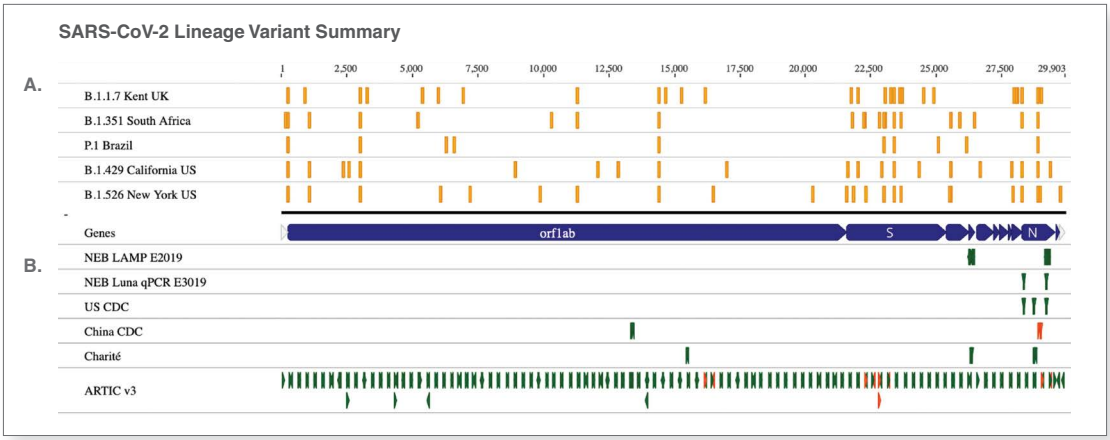
4. Tanner, N., Personal communication

FIGURE 4: **Named variants of interest/concern**

Commonly discussed variants of interest or concern are depicted along with specific mutational loci (A). Below the reference SARS-CoV-2 genome (blue), commonly used primer sets that overlap variants of interest/concern are highlighted in orange.



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