Performance of Diagnovital SARS-CoV-2 Mutation Detection Kits (RUO)*

A Validation Study with a Unique Cohort

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AIM

Various mutations in different regions of SARS-CoV-2 genome have been reported since the beginning of the COVID-19 pandemic [1-3], thus resulting in occurence of new SARS-CoV-2 variants with increased transmissibility, disease severity and immune escape. The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) classified variants as variant of concern (VOC) or variant of interest (VOI) based on several criteria, such as their potential to affect clinical outcome [4, 5].

Identification of the mutations that have significant effect on the behaviour of the virus is crucial for the global control of the Covid-19. Reverse transcription real-time PCR (RT-qPCR) can be used as an alternative to gold standard, which is next-generation sequencing (NGS). The aim of this study is to assess that Diagnovital SARS-CoV-2 Mutation Detection Kits are capable of detecting the mutations in circulating SARS-CoV-2 variants by performing *in silico* analysis with data from GISAID database [6] and validating *in silico* results *in vitro* by sequencing assessment.

METHODS

We performed *in silico* and *in vitro* analyses to reveal the validity of the assays in detecting mutations in SARS-CoV-2 variants. We downloaded sequences of 46,546 SARS-CoV-2 specimens for 24 different variants that were classified as VOC or VOI by CDC or WHO, from the GISAID database [6]. Primer and probe sequences of the assays were aligned to these sequences and checked if there is any mismatches between the oligo sequences and the sample sequences.

A sample genome was assumed to be detected by the assay, in case all primers and probe sequences align to sample genome without a mismatch. Performances of the assays were calculated as the percent samples detected by the primer/probe set of the assay.

Validation study was performed by a unique cohort of SARS-CoV-2 specimens collected in Kocaeli University Hospital, Turkey. RT-qPCR experiments were performed according to the instructions explained in the user manual of DIAGNOVITAL SARS-CoV-2 Mutation Detection Kits. The samples were also sequenced by NGS for the whole S gene, including the receptor-binding domain.

RESULTS

1. *in silico* Results

In silico analyses of the DIAGNOVITAL SARS-CoV-2 Mutation Detection Assays showed that the assays have around 99% *in silico* detection rate (Table 1).

Table 1: *in silico* detection performance of Diagnovial Mutation Detection Kits

Mutation	# of Samples Detected	# of Samples not Detected	*in silico* Detection Rate
N501Y*	14828	22	99.9%
E484Q*	2410	5	99.8%
T478K*	12684	33	99.7%
P681R*	15016	68	99.5%
E484K*	13854	43	99.7%
K417N**	15848	161	99.0%
P681H*	5277	25	99.5%
L452R**	19137	58	99.8%
K417T*	5062	6	99.9%
HV69/70del*	2479	41	98.4%
T547K**	4266	3	99.9%

* Samples from Omicron variant were excluded, as there are Omicron-specific mutations in the target region

** Part of the DIAGNOVITAL SARS-CoV-2 High-Cov Multiplex Mutation Detection Assay, which is under development.

in silico detection rate of the assays were calculated based on the detection of mutant probe for the positive identification of mutant specimens. Note that the working principal of the DIAGNOVITAL SARS-CoV-2 Mutation Detection Assays is based on the dual probe approach (Figure 1) for each target mutation to detect both wild-type and mutant alleles.

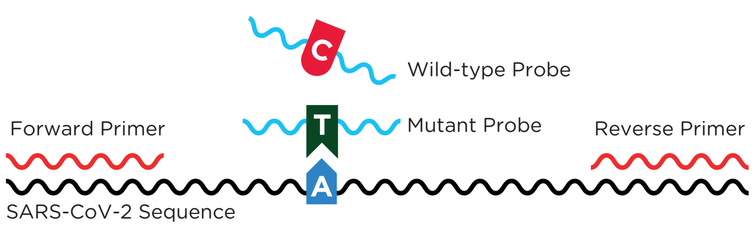

Figure 1: Molecular design of the DIAGNOVITAL SARS-CoV-2 Mutation Detection Assays.

Because the assays differentiate a single nucleotide difference within sample sequences, they are sensitive to mismatches between the oligos and the sample genome. Therefore, any other mutations in the regions targeted by the assays may affect the performance of the assays significantly. To eliminate this possibility, a holistic approach is used to design the oligos by evaluating the mutational profile of different SARS-CoV-2 variants at the same time. The oligos were aligned on to the reference SARS-CoV-2 genome together with the mutations present in any of the variants and designed such that there is no overlap between the mutations and the target regions (Figure 2).

We analyzed mutational profiles of the variants to see if the combined evaluation of the assays may distinguish different

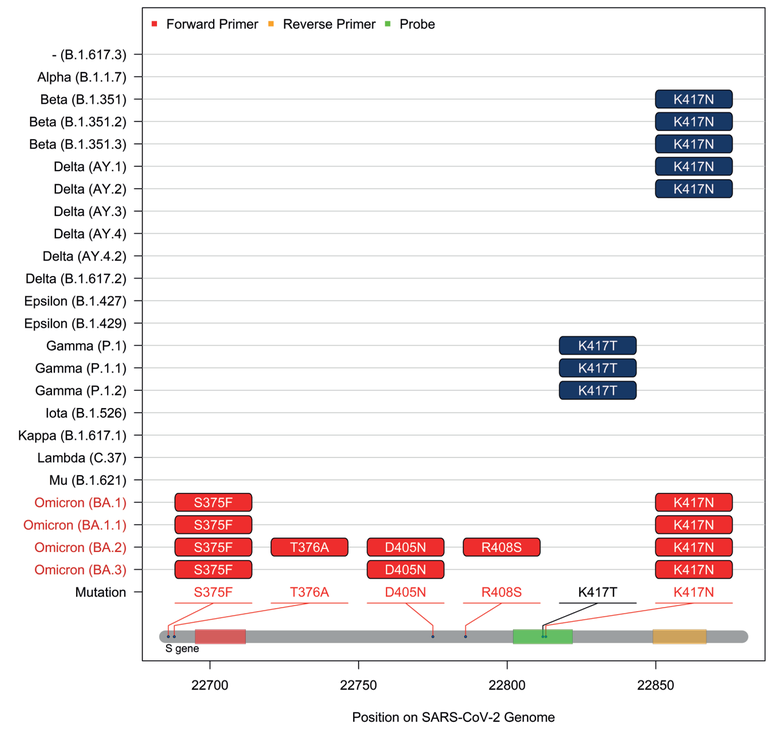

Figure 2: Variant specific mutations in K417N target region. Mutations are represented by the blue boxes for the variants in the y-axis. Position of the mutations on the genome indicated by the lines at the bottom of the figure. Red, green and yellow colors on the genome represent the regions targetted by forward primer, probe and reverse primer, respectively.

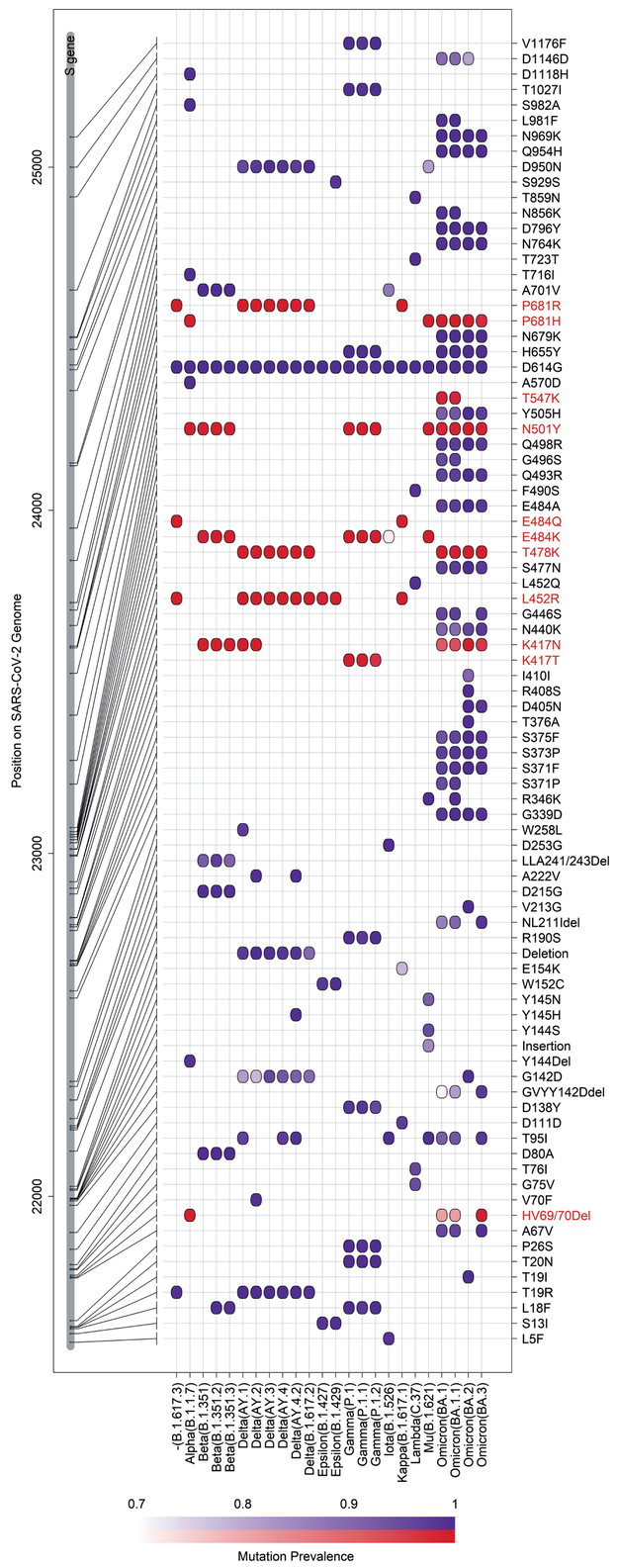

Figure 3: Mutation profiles of VOC and VOI variants in GISAID database. Each square represents the presence of a mutation in the right y-axis in a variant shown in the x-axis. The prevalence of the mutations in the variants is shown by the shades of purple/red color. The more purple/red the square is, the more prevalent the mutation is. The genomic positions of the mutations are shown in the left y-axis. The vertical gray line represents the S gene in the reference genome. The reference genome used is MN908947.3 [7].

variants (Figure 3). Alpha, Beta and Gamma variants can be identified by N501Y, K417N, E484K and HV69/70Del assays, as Delta, Kappa and Epsilon variants can be identified by E484Q, E484K, L452R and K417N assays. The Omicron variant can be distinguished from the Delta variant by the means of K417N and L452R mutations. It is also possible to distinguish the Omicron subvariants, BA.1 and BA.2 by using the K417N and the HV69/70del mutations (Figure 3).

2. *in vitro* Validation Results

In line with the *in silico* results, DIAGNOVITAL SARS-CoV-2 Mutation Detection Kits showed a great concordance with NGS in clinical samples. All the clinical samples were genotyped correctly for all the mutations, except for two samples that were undefined by N501Y and HV69/70del assays (Table 2). Those two samples did not display any amplification for any of the mutant and wild-type assays of N501Y and HV69/70Del mutations, because there were other minor mutations in the target regions on the genome of those samples (data not shown).

Table 2: in vitro detection performance of Diagnovial Mutation Detection Kits in the unique cohort.

	Mutant in NGS			WT in NGS				
RT-qPCR:	Mut	WT	Und*	Mut	WT	Und*	Sens.	Spec.
N501Y	109	0	0	0	58	1	100%	98.31%
HV69/70 deletion	95	0	1	0	71	0	98.96%	100%
E484K	10	0	0	0	158	0	100%	100%
L452R	28	0	0	0	247	0	100%	100%
K417N	100	0	0	0	15	0	100%	100%
E484Q	2	0	0	0	166	0	100%	100%
T547K	82	0	0	0	16	0	100%	100%

Mut: Mutant, WT: Wild-type, Und: Undefined, Sens: Sensitivity, Spec: Specificity

*Inconclusive due to another mutation in the target region

We performed another validation study for the new DIAGNOVITAL High-Cov Multiplex Mutation Detection Assay that targets T547K, K417N and L452R mutations together with a SARS-CoV-2-specific Orf1ab sequence and a human endogenous control in five channels. The study showed that the multiplex assay could detect the mutations in the Omicron clinical samples (Table 3A) and the presence of the virus (Table 3B). The assay can be used to differentiate between BA.1 and BA.2 Omicron subvariants, as T547K mutation is BA.1 specific (Table 3C). Another validation study with the Delta samples is to be performed to assess the capability of the assay to distinguish the Delta and the Omicron variants.

Table 3: in vitro detection performance of Diagnovial SARS-CoV-2 High-Cov Multiplex Mutation Detection Assay in the unique cohort.

A

	Mutant in NGS		WT in NGS			
RT-qPCR:	Mut	NoA	Mut	NoA	Sens.	Spec.
T547K	56	0	0	10	**100%**	**100%**
K417N	65	0	0	0	**100%**	NA
L452R	0	0	0	65	NA	**100%**

B

RT-qPCR (Orf1ab)	Samples
Det	66
NDet	0
%Det	**100%**

C

	NextStrain Clade (based on NGS sequences)			
	21K (BA.1)		21L (BA.2)	
T547K:	Mut	NoA	Mut	NoA
	55	0	1	10

Mut: Mutant, WT: Wild-type, NoA: Not Amplified, Und: Undefined, Sens: Sensitivity, Spec: Specificity, Det: Detected, NDet: Not Detected, %Det: Percent Detected.

CONCLUSIONS

The performance of DIAGNOVITAL SARS-CoV-2 Mutation Detection Assays were assesed with the data collected from GISAID database and validated with a clinical sample set. Although the kits have high performance in detecting targeted mutations, other mutations in the target region may prevent effective detection of mutations. The new DIAGNOVITAL SARS-CoV-2 High-CoV Multiplex Mutation Detection Assay is capable of distinguishing subvariants of the Omicron. These results suggest that real-time qPCR is an efficient method for the detection of SARS-CoV-2 mutations despite its limitations.

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