Rapid Methods for Early Detection of SARS-CoV-2 Variants

Subjects: Infectious Diseases | Health Care Sciences & Services

Contributor: Giulia Berno , Lavinia Fabeni , Giulia Matusali , Cesare Ernesto Maria Gruber , Martina Rueca , Emanuela Giombini , Anna Rosa Garbuglia

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) straightaway showed its tendency to mutate and adapt to the host, culminating in the emergence of variants; so it immediately became of crucial importance to be able to detect them quickly but also to be able to monitor in depth the changes on the whole genome to early identify the new possibly emerging variants.

SARS-CoV-2 COVID-19 variants characterization

1. Rapid Methods for Early Detection of SARS-CoV-2 Variants

Several methods have been developed for early detection of SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) variants (variants of concerns (VOCs), variants of interest (VOI) or variants under monitoring (VUM)). These methods for diagnostic screening mainly consist of nucleic acid amplification technique-based assays able to generate preliminary results in a few hours.

Many of these methods can also accurately identify the variants, while others will require subsequent verification/confirmation by sequencing [1]. The following contents introduce some of the most widely used in diagnostic routine commercially available and home-made methods for variants screening and identification.

2. Main Commercially Available Methods for SARS-CoV-2 Variants Identification

One of the commercially available methods for identifying variants of SARS-CoV-2 is “Coronavirus Disease-2019 (COVID-19) Variant Catcher” developed by Clonit S.r.l. This is a qualitative CE-IVD test, based on Real Time Reverse Transcriptase (RT)-PCR, that allows the identification of the S gene mutations 69–70del, E484K and N501Y. The COVID-19 Variant Catcher kit must be used on extracted RNA from SARS-CoV-2 positive samples that have shown amplification within the 35th Cycle Threshold (Ct) in a previous Real Time RT-PCR-based method. The COVID-19 Variant Catcher kit was developed and validated to be used with the following instruments: Rotor Gene Q MDx from QIAGEN, CFX96 from Biorad and 7500 from LifeTechnologies. (Instructions For Use COVID-19 Variant Catcher, Clonit distribuited by Biomedica). An updated version called “COVID-19 Ultra Variant Catcher” discriminates the L452R, E484K, E484Q and N501Y mutations allowing the identification of a larger number of lineages (see **Table 1**).

Table 1. Characteristics of commercially methods for SARS-CoV-2 variants identification.

Company	SARS-CoV-2 Variants Assay's Name	Regulatory Approval Status	Including RNA Extraction	Turnaround Time	Limit of Detection or Ct Cutoff Value	Mutation Detected	Interpretation of SARS-CoV-2 Variants Based on Detection of Mutations
ABL Advanced Biological Laboratories	UltraGene Assay SARS-CoV-2 Multi Variants Deletions V1	CE - IVD	No	<1.30 h	1150 TCID50/mL (QuantStudio 5) 115 TCID50/mL (UltraGene qPCR)	69-70del, Y144del, 242-244del, 3675-3677del	69-70del + Y144del + 3675-3677del --> B.1.1.7 242-244del + 3675-3677del --> B.1.351 3675-3677del --> P1 Lineage
Clonit S.r.l.	COVID-19 Variant Catcher COVID-19 Ultra Variant Catcher	CE - IVD	No	≈2 h <1.30 h	SARS-CoV-2 Positive RNA Ct < 35 SARS-CoV-2 Positive RNA Ct < 30	69-70del, E484K, N501Y L452R, E484K, E484Q, N501Y	69-70del + N501Y --> B.1.1.7 E484K + N501Y --> B.1.351/P1 Lineage N501Y --> B.1.1.7 N501Y + E484K --> B.1.351/P.1 L452R --> B.1.617.2/B.1.427/B.1.429/B.1.526/B.1.526.1
Diasorin Molecular	Simplexa™ SARS-CoV-2 Variants Direct assay	RUO	Yes	<2 h	500 copies/mL	N501Y, E484K, E484Q, L452	N501Y + E484K --> B.1.351/P.1 L452R --> B.1.617.2/B.1.427/B.1.429/B.1.526/B.1.526.1 N501Y --> B.1.1.7 E484K -->B.1.525/P2 L452R + E484Q --> B.1.617.1/B.1.617.3 N501Y + E484Q --> B.1.621
ELITech Group	SARS-CoV-2 Variants ELITe MGB® Kit SARS-CoV-2 Extended ELITe MGB® Kit	RUO	No	INA	INA	E484K, N501Y L452R, E484K, E484Q, N501Y	N501Y --> B.1.1.7 N501Y + E484K --> B.1.351/P.1 E484K -->B.1.525/P2 N501Y --> B.1.1.7 N501Y + E484K --> B.1.351/P.1 L452R --> B.1.617.2/B.1.427/B.1.429/B.1.526/B.1.526.1
Life Technologies	TaqPath™ COVID 19 CE IVD RT PCR Kit	CE - IVD	No	<1.30 h	10 GCE/reaction	69-70del	69-70del --> B.1.1.7/B.1.525/BA.1
Seegene	Allplex™ SARS-CoV-2 Variants I Assay	CE - IVD	No (possibility of automated extraction	INA	INA	69-70del, E484K, N501Y	69-70del + N501Y --> B.1.1.7/B.1.1.529 E484K + N501Y --> B.1.351/P1/P.3 69-70del + E484K --> B.1.525

Company	SARS-CoV-2 Variants Assay's Name	Regulatory Approval Status	Including RNA Extraction	Turnaround Time	Limit of Detection or Ct Cutoff Value	Mutation Detected	Interpretation of SARS-CoV-2 Variants Based on Detection of Mutations
	Allplex™ SARS-CoV-2 Variants II Assay	CE - IVD in progress	and PCR setup)	INA		L452R, W152C, K417T, K417N	K417N --> B.1.351/B.1.1.529/AY.1/BA.2
							K417T --> P.1
							L452R + W152C --> B.1.429/ B.1.427
							L452R --> B.1.617.1/ B.1.617.2
	Novaplex™ SARS-CoV-2 Variants Assays I	RUO		4 h (starting from extraction)		69-70del, E484K, N501Y	69-70del + N501Y --> B.1.1.7/B.1.529
							E484K + N501Y --> B.1.351/P1/P.3
							69-70del + E484K --> B.1.525
	Novaplex™ SARS-CoV-2 Variants II Assay					L452R, W152C, K417T, K417N	K417N --> B.1.351/B.1.1.529/AY.1/BA.2
							K417T --> P.1
							L452R + W152C --> B.1.429/ B.1.427
							L452R --> B.1.617.1/ B.1.617.2
	Novaplex™ SARS-CoV-2 Variants IV Assay					L452R, P681R, K417N	L452R + P681R --> B.1.617.1/ B.1.617.2
							K417N --> B.1.351/B.1.1.529/BA.2
							L452R + P681R + K417N --> AY.1
							L425R --> B.1.427/B.1.429
	Novaplex™ SARS-CoV-2 Variants V Assay					L452Q, F490S,P681R, L452R	P681R + L452R --> B.1.617.1/B.1.617.2/AY.1
							L425R --> B.1.427/B.1.429
							L452Q+F490S --> C.37
	Novaplex™ SARS-CoV-2 Variants VI Assay					L452Q, F490S, R346K, D950N	D950N --> B.1.617.1/ B.1.617.2/AY.1
							L452Q + F490S --> C.37
							R346K + D950N --> B.1.621
	Novaplex™ SARS-CoV-2 Variants VII Assay					69-70del, E484A, N501Y, RdRp	69-70del + N501Y + RdRp --> B.1.1.7
							N501Y + RdRp --> B.1.351/P.1/P.3
							69-70del + E484A + N501Y + RdRp--> B.1.1.529

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ning 7 samples were not amplified, due to the low viral load. For the 7 NPS not amplified by the assay, a Real Time RT-PCR on thawed samples showed positive results although with high Ct values (median Ct were > 30.for S and ORF1ab gene) [8].

During the last year and a half, with the aim to identify the circulating variants, the Seegene Inc developed a series of multiplex Real Time RT-PCR based typing tests called "Novaplex SARS-CoV-2 Variants Assay" detecting different panels of spike protein mutations such as: 69–70del, E484K, N501Y (Variants I); L452R, W152C, K417T and K417N (Variants II), L452R, P681R, AND K417N (Variants IV), L452Q, F490S,P681R, and L452R (Variants V), L452Q, F490S, R346K, and D950N (Variants VI), and 69–70del, E484A, N501Y and RdRp (Variants VII). The system provides an automated extraction and Real Time RT-PCR setup followed by an automated interpretation reporting data for each specific mutation probe as a Ct value. These assays must be used with Seegene instruments: STARIet (for the extraction and PCR setup), CFX96Dx (for the Real Time RT PCR) and a specific Seegene Viewer (for the automated interpretation). A recent paper compared the results obtained from the Novaplex Variants I, II, and IV assays with S gene Sanger sequencing resulting in a 100% overall agreement in variants identification when using extracted RNA, while a RNA-extraction free protocol was less sensitive in detecting some mutations especially with Ct values > 30 [9].

Company	SARS-CoV-2 Variants Assay's Name	Regulatory Approval Status	Including RNA Extraction	Turnaround Time	Limit of Detection or Ct Cutoff Value	Mutation Detected	Interpretation of SARS-CoV-2 Variants Based on Detection of Mutations
							E484A + N501Y + RdRp --> BA.2
							RdRp --> B.1.617.2/AY1/B.1.427/429/P.2/B.1.526/B.1.617.1/C.37/B.1.621
							69-70del + RdRp --> B.1.525

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amplification stage, is usable with any qPCR instrument compatible with the FAM, HEX, ROX, Cy5 channels. The LoD is 1150 TCID50/mL for the QuantStudio 5 Real Time RT-PCR instrument and 115 TCID50/mL with the UltraGene qPCR 48 instrument.

The "SARS-CoV-2 Extended ELITe MGB (ELITechGroup)" molecular kit is a RUO multitarget designed assay intended for use as a test for the detection and discrimination of the mutations L452R, E484K, E484Q and N501Y of the S gene of SARS-CoV-2. This assay foresees a Real Time RT-PCR performed using the ELITe InGenius instrument (ELITechGroup) and the identification of mutations by analysis of the melting curve.

One of the first commercially available methods intended for the qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory specimens was "TaqPath™ COVID-19 CE-IVD RT-PCR Kit" (by ThermoFisher Scientific, Waltham, Massachusetts, Stati Uniti). TaqPath™ COVID-19 is a multiplexed assay that contains three primer/probe sets specific to ORF1ab, N gene and S gene of SARS-CoV-2. This system needs extracted RNA (the minimum recommended elution volume is 50 µL). The TaqPath COVID-19 was developed and validated to be used with the following instruments: QuantStudio 5, QuantStudio 7 Flex, 7500 Fast Dx, 7500 Fast, and 7500 from LifeTechnologies. The LoD study established that the lowest SARS-CoV-2 viral concentration (Genomic Copy Equivalents or GCE) that can be detected by the TaqPath™ was 10 Genomic copy equivalent/reaction (GCE/reaction) for both nasopharyngeal swab and bronchoalveolar lavage specimens. The Ct cut-off value for clinical target was ≤37 (TaqPath™ COVID-19 CE-IVD RT-PCR Kit Instructions For Use). Interestingly, although this test was developed with the diagnostic intent of quickly diagnose COVID-19 caused by SARS-CoV-2 infection, if a sample with the 69–70del S-gene mutation is tested using this kit, it will result in an S-gene dropout in presence of ORF1ab and N gene amplifications (also indicated as a S gene Target Failure or SGTF). Considering the importance of this mutation in identifying some SARS-CoV-2 variants, namely the Alpha and Omicron BA.1, this assay, has been used as a fast screening method. On 26 November 2021, the European Center for Disease Control and Prevention (ECDC) released a document ("Implications of the emergence and spread of the SARS-CoV-2 B.1.1.529 variant of concern (Omicron) for the EU/EEA") [10] in which the SGTF from the Thermo Fisher TaqPath assay was indicated as a good proxy for Omicron identification in the scenario present at the time with the Delta variant dominating and the Omicron (BA.1) rising.

De Pace at al. evaluated the diagnostic performance of five qualitative Real Time RT-PCR based tests (SARS-CoV-2 Variants II Assay—Allplex-Seegene Inc.; UltraGene Assay SARS-CoV-2 452R & 484K & 484Q Mutations V1.x—Advanced Biological Laboratories; COVID-19 Ultra Variant Catcher—Clonit S.r.l; SARS-CoV-2 Extended ELITe MGB—ELITechGroup; Simplexa SARS-CoV-2 Variants Direct - Diasorin Molecular) as compared with Next Generation Sequencing (NGS) finding that the overall accuracy of these assays ranged from 96.9% to 100% and specificity and sensitivity were 100% and 96–100%, respectively. The authors recommend the use of these assays

as second-level tests in the routine workflow of SARS-CoV-2 laboratory diagnostics, as they are accurate, user friendly, low cost, may identify specific mutations in about 2–3 h and, therefore, optimize the surveillance of SARS-CoV-2 variants [11].

Vice versa Alejo-Cancho et al. describe three cases in which a Mu strain containing the mutation K417N was initially misclassified as the Beta variant using a multiplex Real Time RT-PCR (Allplex SARS-CoV-2 Variants Assay -Seegene), in this case the authors recommend to use NGS or other methods for the detection of P681H to distinguish between these two variants [12].

3. In House Rapid Methods for SARS-CoV-2 Variants Identification

Viral genome sequencing procedures are expensive and time-consuming, so with a view to reducing labor intensive and to quickly screen for the different SARS-CoV-2 circulating variants, many laboratories have developed an in house rapid methods for SARS-CoV-2 variants identification.

Several studies reported delectable results by using in-house molecular tests for identifying a specific single SARS-CoV-2 variant screening, in the contest of knowledge of variants circulating in a given period. Hamill et al. developed a Real Time RT-PCR for deletions of Δ156–157 in the spike gene that is characteristic of the Delta variant with the purpose, primarily to monitor and identify the Delta variant strains, but data analysis indicated that to increase the identification of both Delta and Omicron variants this newly designed assay need to be used in combination with CDC N1 target [13].

Barua et al. developed a reverse transcription fluorescence resonance energy transfer-polymerase chain reaction (RT-FRET-PCR) designed to identify the T478K mutation (present in 99.73% of the Delta variant) that can be used both to diagnose COVID-19 patients and simultaneously identify if they are infected with the Delta variant; but the Delta variant is just one of several SARS-CoV-2 variants, so a continuous monitoring of strains will still be necessary [14].

Many other groups have also developed homemade techniques for the identification of single position mutations in order to quickly identify a single variant circulating on the territory [15] but the major limitation of all this kind of assays is the inability to detect all the other major variants because of each variant is characterized by a multitude of mutations.

Erster et al. focused their attention on developing a test that was able to discriminate between two variants such as Alpha (B.1.1.7) and Beta (B.1.351) variants. This kind of approach can be useful in a territorial context in which two variants mainly circulate but does not allow the identification of new mutation or to the advent of new variants [16].

Because of tracking SARS-CoV-2 variants through Whole Genome Sequencing (WGS) can be time consuming and resource-heavy, some laboratories describe an in-house validation of an allele-specific polymerase chain

reaction (ASP) variant assay to detect SARS-CoV-2 VOC's. For example, Brito-Mutunayagam et al. described an ASP based three mutation targets: E484 K, L452R and P681R (based on the circulating variant epidemiology at the time) [17]. Despite the considerable advantage in terms of time, this type of methodology can help to identify a variant only in certain historical periods, when you already know what circulates in a specific area and when you want to discriminate between specific variants.

Other groups describe multiplex Real Time RT-PCR homemade methods capable to detect 9 mutations with specific primers and probes, these PCR typing strategy allowed the detection of the major variants and also provided an open-source PCR assay which could rapidly be deployed in laboratories around the world [18].

Given that genotyping approaches are rapid methods for monitoring SARS-CoV-2 variants but require continuous adaptation, fragment analysis may represent an approach for improved SARS-CoV-2 variant detection. Clark et al. described a multiplex fragment analysis approach using PCR targeting variants by size and fluorescent color. Eight SARS-CoV-2 mutational hot spots in VOCs were targeted. This kind of method could classify a variant with similar accuracy as sequencing without frequent target modification [19].

Moreover, given the possibility that Emerging variants pose the risk for target dropout and false-negative results secondary to primer/probe binding site (PBS) mismatches. Hernandez et al. described a method that combine RT-PCR and matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry to probe for five targets across N and ORF1ab genes, which provides a robust platform to accommodate PBS mismatches in divergent viruses [20].

Another important goal could be to develop a single platform with both diagnostic and surveillance capabilities for comprehensive SARS-CoV-2 Spike gene mutations. This is what was made by Welch et al. that selected up to 26 mutations to distinguish between or detect mutations shared among the Alpha, Beta, Gamma, Delta, Epsilon, and Omicron variant lineages in a cost-effective virus and variant detection platform, which combines CRISPR-based diagnostics and microfluidics with a streamlined workflow for clinical use [21] (**Table 2**).

Table 2. Characteristics of in house rapid methods for SARS-CoV-2 variants identification.

Method	Amino Acid Mutation Detected	Variants Identified	Ref
Real-Time RT-PCR	Δ156–157	B.1.617.2	[6]
RT-FRET-PCR	T478K	B.1.617.2	[7]
One-step RT-qPCR	N501Y	B.1.1.7 B.1.351 P.1 BA.1/2/4/5 (no discrimination)	[13]
Multiplex RT-qPCR	D3L and Δ242–244	B.1.1.7 or B.1.351	[14]
Allele-specific polymerase chain reaction (ASP)	E484 K, L452R and P681R	B.1.1.7, B.1.351, B.1.617.2	[15]

Method	Amino Acid Mutation Detected	Variants Identified	Ref
Multiplex RT-qPCR	Δ69–70, K417T, K417N, L452R, E484K, E484Q, N501Y, P681H, and P681R	B.1.1.7, B.1.351, P.1, B.1.617.2, BA.1	[16]
CoVarScan Multiplex fragment analysis (Fluorescently labeled RT-PCR amplicons analyzed by capillary electrophoresis)	8 defined hotspot regions: 5 recurrently deleted regions (RDRs; S:RDR1, S:RDR2, S: RDR3–4, ORF1A, and ORF8) and 3 SNPs (S:N501Y, S:L452R, and S:E484K)	B.1.1.7, B.1.351, P.1, B.1.617.2, BA.1	[17]
RT-PCR/MALDI-TOF	five targets: N1, N2, N3, ORF1A, ORF1AB.	B.1.1.7 (tested december 2020 to april 2021)	[18]
Multiplex CRISPR-based diagnostics and microfluidics	D614G, Δ69–70, N501Y, A570D, P681H/R, D80A, K417N, K417T, L18F, E484K/A, H655Y, P26S, Δ156–157, T478K, L452R/Q, S477N	B.1.1.7, B.1.351, P.1, B.1.617.2, BA.1	[19]

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