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Editorial

PCR-Based Screening Tests for SARS-CoV-2 Mutations: What Is the Best Way to Identify Variants?

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Since late 2020, the World Health Organization (WHO) has classified the emergence of SARS-CoV-2 variants that have the potential to increase the risk to global public health as variants of concern (VOCs) and variants of interest (VOIs). Some VOCs, such as Alpha, Delta, and Omicron variants, rapidly spread across the entire globe and contributed to the COVID-19 pandemic (1). SARS-CoV-2 genetic surveillance is essential to track SARS-CoV-2 variants and protect against the spread of high-risk variants, including VOCs and VOIs.

Specimens from COVID-19 patients were used to determine the viral whole-genome sequences (30 kilobases) using a next-generation sequencer (NGS) (2). Therefore, high-income countries should continue to track the evolution of SARS-CoV-2 using NGS data. However, NGS analysis requires expensive equipment and bioinformatics skills. Additionally, this process takes a few days to identify viral whole-genome sequences. Some SARS-CoV-2 mutations lead to higher mortality rates and increased disease severity of COVID-19 (3). Screening tests can detect SARS-CoV-2 mutations within a day, thereby helping us decide the appropriate treatment for individual COVID-19 patients.

Several screening assays for detecting SARS-CoV-2 mutations have been reported (4, 5), among which not every assay can identify the variants in specimens containing low-copy viruses. The reverse transcription (RT)-polymerase chain reaction (PCR) method is widely adopted to identify SARS-CoV-2 variants because of its high sensitivity and detection specificity. The WHO declared that PCR-based approaches, combined with whole-genome sequencing, provide several advantages: PCR is readily available and less resource-intensive; PCR provides information rapidly; and a single PCR assay is performed on many samples (6). The TaqMan probe assay is the gold-standard PCR method for detecting single-nucleotide polymorphisms and many TaqMan probe kits for SARS-CoV-2 mutations are commercially available (7). The TaqPath COVID-19 assay kit (Thermo Fisher Scientific), one of the most popular TaqMan probe kits, supplies various probes and primer sets for SARS-CoV-2 mutations. These kits determine SARS-CoV-2 variants in clinical samples based on mutational spectra. Thus, multiplex PCR amplifies and detects various SARS-CoV-2 mutations, allowing the development of a high-throughput screening for each variant. Additionally, multicolor fluorometric probes lead to the development of multiplex PCR and improve assays' throughput. Realistically, up to 4-color multiplex PCR based on the TaqMan probe assay can be performed using a real-time PCR instrument. It is, hence, difficult to detect over 5 SARS-CoV-2 mutations simultaneously.

In this issue of *Clinical Chemistry*, Clark et al. (8) describe a study to validate an 8-plex fragment analysis assay, namely CoVarScan. This assay analyzes fluorescent-labeled RT-PCR amplicons by capillary electrophoresis and detects 8 mutational regions: 3 recurrently deleted regions (RDR: RDR1, RDR2, and RDR3-4), 3 receptor-binding domain mutations (N501Y, E484K, and L452R), and 2 open reading frames (ORF: ORF1A and ORF8). This assay also can identify at least 8 variants, such as Alpha, Beta, Gamma, Delta, Lambda, Mu, Iota, and Omicron, based on these mutational spectra. This 8-plex assay exhibited 95% sensitivity and 98% specificity compared to whole-genome sequencing. Variants were identified in 95% of samples with threshold cycle (C_T) values <30, 75% of them with C_T values from 34 to 35. These results suggest that this assay provides rapid, cost-effective, high-throughput screening with enough sensitivity and specificity to identify SARS-CoV-2 variants in clinical samples, indicating that CoVarScan is a powerful tool for identifying various VOCs and VOIs.

Multiplex PCR assays are affected by many factors such as PCR conditions, primer pair specificity, and PCR buffer composition. Optimization of these factors and method validation is essential for developing multiplex PCR assays. Clark et al. optimized various multiplex PCR factors and validated them for clinical use on 3238 respiratory specimens. Thus, the development of multiplex PCR requires substantial time and effort. Our group and others developed assays to detect SARS-CoV-2 mutations based on high-resolution

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melting (HRM) analysis, a post-PCR technique for genotyping based on the melting behavior of amplicons (9–12). Our HRM-based assay can detect 2 SARS-CoV-2 mutations using single-plex PCR. The development of the HRM-based assay requires little time since we designed only primer sets without specific probes. This simple assay distinguished not only Delta and Omicron variants (13) but also Omicron sublineages BA.1 and BA.2 (14). However, our HRM-based assay needs 2-step nested PCR to improve the detection limit. Additional work is required to make our assay more valuable as a one-step PCR assay.

What is the best way to adopt the screening tests to identify SARS-CoV-2 variants? Clark et al. developed one of the ultimate screening tests, CoVarScan, which can identify almost all VOCs and VOIs using a single assay. Interestingly, the assay uses a traditional capillary sequencer, ABI 3730XL (Thermo Fisher Scientific), for capillary electrophoresis, indicating that the conventional sequencer helps the next-generation sequencer rapidly diagnose SARS-CoV-2 variants. Additionally, CoVarScan can be a universal method since capillary sequencers, such as ABI 3730XL, have been widely used for Sanger sequencing in various clinical cases. We propose a 3-step model of screening tests combined with different assays to detect SARS-CoV-2 variants. First, quick-build and straightforward tests, such as the HRM assay, pick up the emergent variants. Second, the gold-standard TaqMan assay identifies the mainstream variants. Third, the multiplex assay, such as CoVarScan, classifies almost all variants simultaneously. It is hoped that combinations of these PCR-based methods will detect the newly emerging SARS-CoV-2 variants. Moreover, some new PCR instruments have been developed and these have helped to save human resources and reduce the time needed for PCR amplification. Based on these technologies, a quicker and more cost-effective screening test should be developed using CoVarScan and other PCR methods.

Nonstandard Abbreviations: VOC, variant of concern; VOI, variant of interest; HRM, high-resolution melting.

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