TECHNICAL NOTE

Guidelines for detecting the SARS-CoV-2 Omicron variant using the Illumina COVIDSeq™ Test (RUO Version)

Spike-in of 11 additional oligos to the ARTIC v4 primer pool provides near-complete genomic coverage for the Omicron variant

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Introduction

The novel SARS-CoV-2 virus is responsible for the COVID-19 pandemic that has affected billions of lives worldwide in one of the largest public health emergencies of the last century.[1] This highly infectious virus has acquired mutations and evolved into various strains, some of which have been identified by the public health community as variants of concern, highlighting the need for rapid, accurate viral detection and characterization.[2-5] Mutations in these variants can be problematic for amplicon sequencing methods because they can have a detrimental effect on primer binding. In response, primer pool updates are necessary to continue to sequence evolving viral genomes accurately.

ARTIC Network developed and released primer sequences that can effectively sequence the SARS-CoV-2 genome in late 2020 (ARTIC v3). The rise of the Delta variant (B.1.617.2) created amplification issues across the viral genome with the ARTIC v3 primers due to Delta's many mutations.[6,7] In response, the ARTIC Network redesigned primer sequences for low-coverage amplicons, leading to the formation of the ARTIC v4 primer pool. In early November 2021, the Omicron variant (B.1.1.529) was first identified in South Africa and quickly became the dominant variant across multiple regions due to its high transmission rates.[8,9] Additional mutations in the Omicron variant in primer binding sites were speculated to adversely affect amplification using the ARTIC v4 primer pool. The pool was updated with 11 primers to resolve this issue, resulting in the ARTIC v4.1 pool.

Alternatively, hybrid capture–based enrichment is a targeted sequencing approach that remains effective even within highly mutagenic regions. The Respiratory Virus Oligo Panel v2 with Illumina RNA Prep with Enrichment enables detection and characterization of common respiratory viruses, including SARS-CoV-2 variants. This enrichment-based panel offers potential use as a "future-proof" method for surveillance of additional SARS-CoV-2 variants.

This technical note presents a pooling scheme for users to spike-in the 11 additional oligos to formulate the ARTIC v4.1 pool. It highlights results from functional testing of the ARTIC v4 and v4.1 pools with nasopharyngeal (NP) swab samples of known Omicron lineage. Finally, the Respiratory Virus Oligo Panel v2 with Illumina RNA Prep with Enrichment is evaluated using Omicron-positive samples to determine its capacity to overcome mutations in SARS-CoV-2 variant genomes.

Experimental methods

Sample preparation

Thirty upper respiratory tract specimens were collected from individuals experiencing COVID-19-related symptoms using a flocked nasal swab and PrimeStore Molecular Transport Media (MTM) (Longhorn Vaccines & Diagnostics, LLC). The KingFisher Flex Magnetic Particle Processor with 96 Deep Well head (Thermo Fisher Scientific) and the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Catalog no. A48383) were used for high-throughput nucleic acid extraction. Synthetic RNA Control 48 (B.1.1.529/BA.1) (Twist Bioscience, Catalog no. 105204) was assayed with 200 synthetic viral copies as a positive control for detection of the Omicron variant. The TaqPath COVID-19 Combo Kit (Thermo Fisher Scientific, Catalog no. A47814) was used to detect the SARS-CoV-2 ORF1ab and N protein genes by real-time qPCR with the Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Catalog no. 4485701) and COVID-19 Interpretive Software (Thermo Fisher Scientific).

For the Respiratory Virus Oligo Panel, one NP swab sample was collected and the RNA extracted and amplified following the same methods described above. This sample had a qPCR Ct value of 14.4 for the N-gene and 13.2 for Orf_1ab. Synthetic RNA Control 48 (B.1.1.529/BA.1) was assayed with 1700 synthetic viral copies as a positive control for detection of the Omicron variant.

Library preparation

Samples were processed in duplicate using the Illumina COVIDSeq Test (RUO Version)* (Illumina, Catalog no. 20043675) with either the ARTIC v4 or v4.1 pool. Primers to formulate the v4.1 pool were ordered from Integrated DNA Technologies (IDT) as custom 25 nmole DNA Oligos and resuspended at 100 µM in IDTE buffer† (Table 1). Primers were pooled with equal volume from each 100-µM tube and then diluted to 10 µM to create two spike-in pools to add to C4P1 and C4P2 primer pools tubes, respectively.

* While not evaluated as part of this study, similar results may be expected with using the Illumina COVIDSeq Assay (96 samples).

† IDTE (10 mM Tris, 0.2 mM EDTA) is recommended by IDT for resuspending and storing oligos.

Table 1: Spike-in pool primers

C4P1.1 spike-in pool primers	
Primer name	Primer sequence
SARS-CoV-2_23_RIGHT_alt1	*AGAATCTAAACCACTAAGACAAACACTAC*
SARS-CoV-2_27_RIGHT_alt1	*AATGTTGTGACTTTTTGCTACCTGC*
SARS-CoV-2_79_RIGHT_alt1	*AATTGGTGGTGTTTTGTAAATTTGTTTGAC*
SARS-CoV-2_89_LEFT_alt1	*TAGGTTTCCTATTCCTTACATGGATTTGT*
SARS-CoV-2_89_RIGHT_alt1	*CTAGATGGTGTCCAGCAATACGAAG*
C4P2.1 spike-in pool primers	
Primer name	Primer sequence
SARS-CoV-2_10_LEFT_alt1	*TGAATATCACTTTTGAACTTGATGAAAGGATTG*
SARS-CoV-2_10_RIGHT_alt1	*GGTTGAAGAGCAGCAGAAGTG*
SARS-CoV-2_76_LEFT_alt1	*ATGTCTATGCAGATTCATTTGTAATTAGAGGT*
SARS-CoV-2_76_RIGHT_alt1	*GTCCACAAACAGTTGCTGGTG*
SARS-CoV-2_88_LEFT_alt1	*TTATGTACTCATTCGTTTCGGAAGAG*
SARS-CoV-2_90_RIGHT_alt1	*ATTAGTAATATCTCTGCTATAGTAACCTGAAAG*

Table 2: Primer pool formulation

C4P1.1 formulation			
No. of samples	Total volume of C4P1.1 pool	Volume of C4P1	Volume of C4P1.1 spike-in pool
1	4.30 µl	4.10 µl	0.20 µl
96	412.80 µl	393.14 µl	19.66 µl
384	1651.20 µl	1572.57 µl	78.63 µl
C4P2.1 formulation			
No. of samples	Total volume of C4P2.1 pool	Volume of C4P2	Volume of C4P2.1 spike-in pool
1	4.30 µl	4.06 µl	0.24 µl
96	412.80 µl	389.43 µl	23.37 µl
384	1651.20 µl	1557.74 µl	93.46 µl

Spike-in pool volumes were calculated to attain equimolar input of each primer (Table 2).

COVIDSeq v4 and v4.1 libraries were assayed using the Bioanalyzer High Sensitivity DNA Kit (Agilent, Catalog no. 5067-4626) to confirm expected library yield and size. Both v4 and v4.1 library pools had sufficient yield for sequencing (60.12 nM and 76.96 nM, respectively) and the expected median library size (310 bp and 315 bp, respectively).

Respiratory Virus Oligo Panel

Libraries were prepared using RNA from the NP swab sample with Illumina RNA Prep with Enrichment (Illumina, Catalog no. 20040536) and IDT for Illumina DNA/RNA UD Indexes (Illumina, Catalog no. 20027213). Synthetic RNA Control 48 was also assayed as a positive control with 1700 synthetic viral copies input at the "Denature RNA" step of the protocol. After amplification, samples were enriched as single-plex reactions using the Respiratory Virus Oligos Panel v2 (Illumina, Catalog no. 20044311). Both the NP swab sample and control 48 were assayed in duplicate. Enriched libraries were assayed as described above to confirm expected library yield (11.65 nM) and size (421 bp).

Sequencing

Prepared libraries were denatured and diluted following the NextSeq™ System Denature and Dilute Libraries Guide and sequenced on the NextSeq 550 System using the NextSeq High Output Kit v2.5 (150 cycles) (Illumina, Catalog no. 20024907) with a 2 × 74 bp paired-end read configuration.

Data analysis

FASTQ files were generated from run data using the BaseSpace™ FASTQ Generation v1.0.0 app and randomly downsampled to the same depth at 1 million clusters/2 million paired-end reads. Further analysis was performed using the DRAGEN™ COVID Lineage app v3.5.5 in BaseSpace Sequence Hub. Default parameters with either the v4 or v4.1 browser extensible data (BED) file selected for primer trimming were used for COVIDSeq libraries. Default parameters with "None" BED file selected for primer trimming were used for enriched libraries. Amplicon coverage was reported by the DRAGEN COVID Lineage app as “counts”.

Results

Evaluation of ARTIC v4 and v4.1 pools

Presence of SARS-CoV-2 RNA was confirmed in all NP samples and controls by qPCR (Figure 1). Analysis of sequencing data with the DRAGEN COVID Lineage app resulted in classification as the Omicron variant (BA.1) (data not shown).

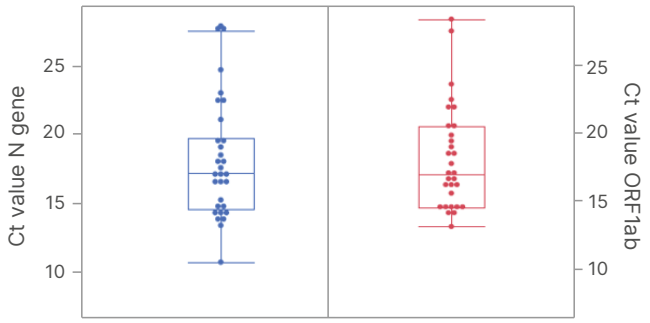

Figure 1: Detection of SARS-CoV-2 RNA by qPCR—
Ct values plotted for N protein (blue) and ORF1ab (red) gene regions, confirming the presence of SARS-CoV-2 RNA in all samples assayed.

The performance of the ARTIC v4 and v4.1 primer pools was evaluated using the Illumina COVIDSeq Test (RUO). The mean percent of genome captured, reported as percent of non-N bases (coverage ≥ 10×), was 98.50% and 99.66% for v4 and v4.1, respectively. The mean median coverage was 1788 and 1748 for v4 and v4.1, respectively (Figure 2).

To investigate the difference between using ARTIC v4 and v4.1 primer pools for amplification, coverage was plotted for each amplicon. *In silico* analysis performed by the ARTIC Network predicted that amplicons 10, 23, 27, 76, 79, 88, 89, and 90 may display reduced performance in Omicron-classified samples. Synthetic RNA Control 48 may be used as a quality control (QC) for amplicon coverage. Results from sequencing three technical replicates showed that amplicons 10, 23, 27, and 88 were unaffected with either primer pool (data not shown). Amplicons 76, 89, and 90 had near complete drop out in coverage, while amplicon 79 showed a wider range in counts between replicates with the v4 pool. With the exception of amplicon 90, all amplicons were rescued with the v4.1 pool (Table 3).

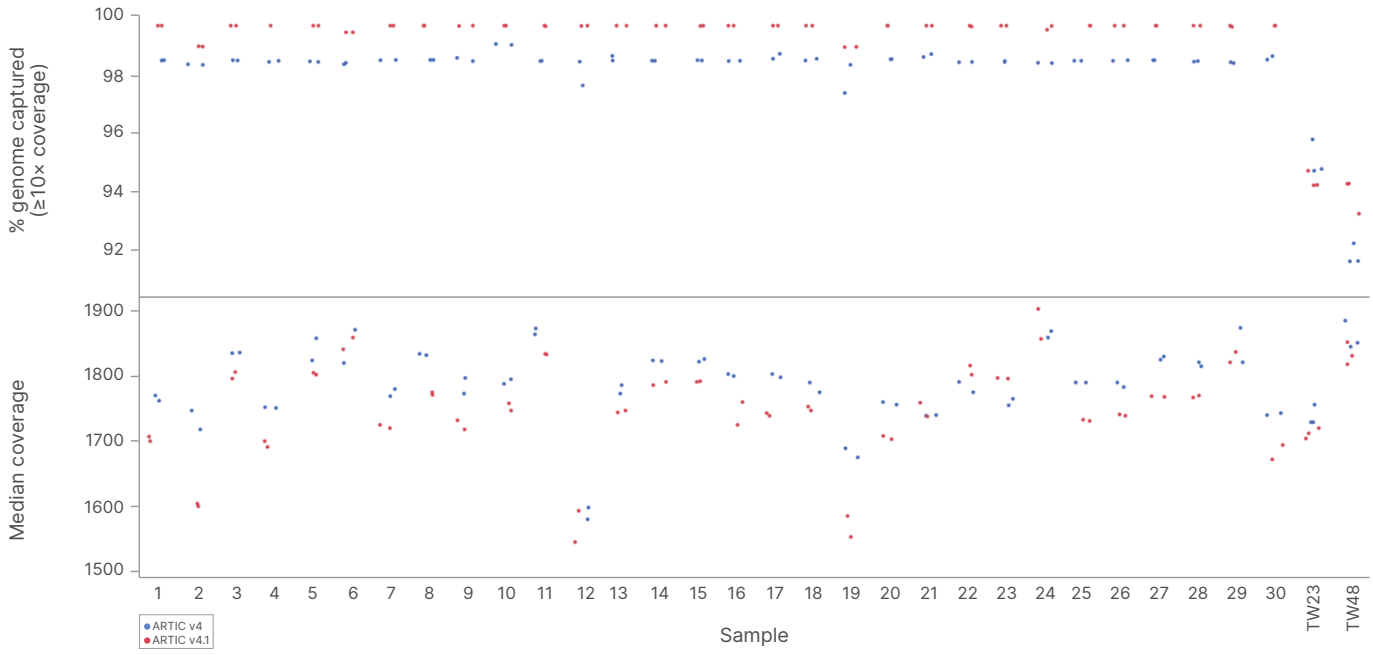

Figure 2: Viral genome coverage for NP samples assayed with ARTIC v4 and v4.1 primer pools—Viral genome coverage was assayed for v4 (blue) and v4.1 (red) primer pools. The mean percent of genome captured, reported as percent of non-N bases (coverage ≥ 10×), was 98.50% and 99.66% for v4 and v4.1, respectively. The mean median coverage was 1788 and 1748 for v4 and v4.1, respectively.

Table 3: Amplicon coverage with RNA Control 48

Amplicon	ARTIC v4	ARTIC v4.1
76	Drop out	Rescued
79	Wide count range	Rescued
89	Drop out	Rescued
90	Drop out	Drop out

For sample libraries amplified with the v4 pool, amplicons 76 and 90 had near complete dropout in coverage, and amplicons 79, 88, and 89 showed a greater range in counts between samples (Figure 3A, Figure 3C). Coverage of amplicons 10, 23, and 27 was not affected in any v4-amplified libraries, similar to results using Synthetic RNA Control 48 (Figure 3A, Figure 3C).

Comparing these results to the same sample libraries amplified with the v4.1 pool showed rescue of coverage for amplicons 76 and 90 and a reduced range in counts of amplicons 79, 88, and 89 (Figure 3B, Figure 3D). The improved performance across these amplicons can be attributed to the primers added to the v4.1 pool. These results also validate the use of Synthetic Control 48 for QC of amplicon coverage with the v4 and v4.1 primer pools.

To determine if the 11 primers formulating the v4.1 pool might affect analytical sensitivity in variant detection or introduce new PCR artifacts, variant calls were compared between samples amplified with the v4 and v4.1 pools. Results showed that variants G22813T, T22882G, and G22898A were consistently detected in amplicon 76 in 55/58 of libraries amplified with v4.1 but not with v4.

Further investigation using Integrated Genomics Viewer (IGV) to view sequencing depth showed coverage gaps in amplicon 76 causing a lack of variant calls at G22813T, T22882G, and G22898A in v4-amplified libraries (Figure 4).

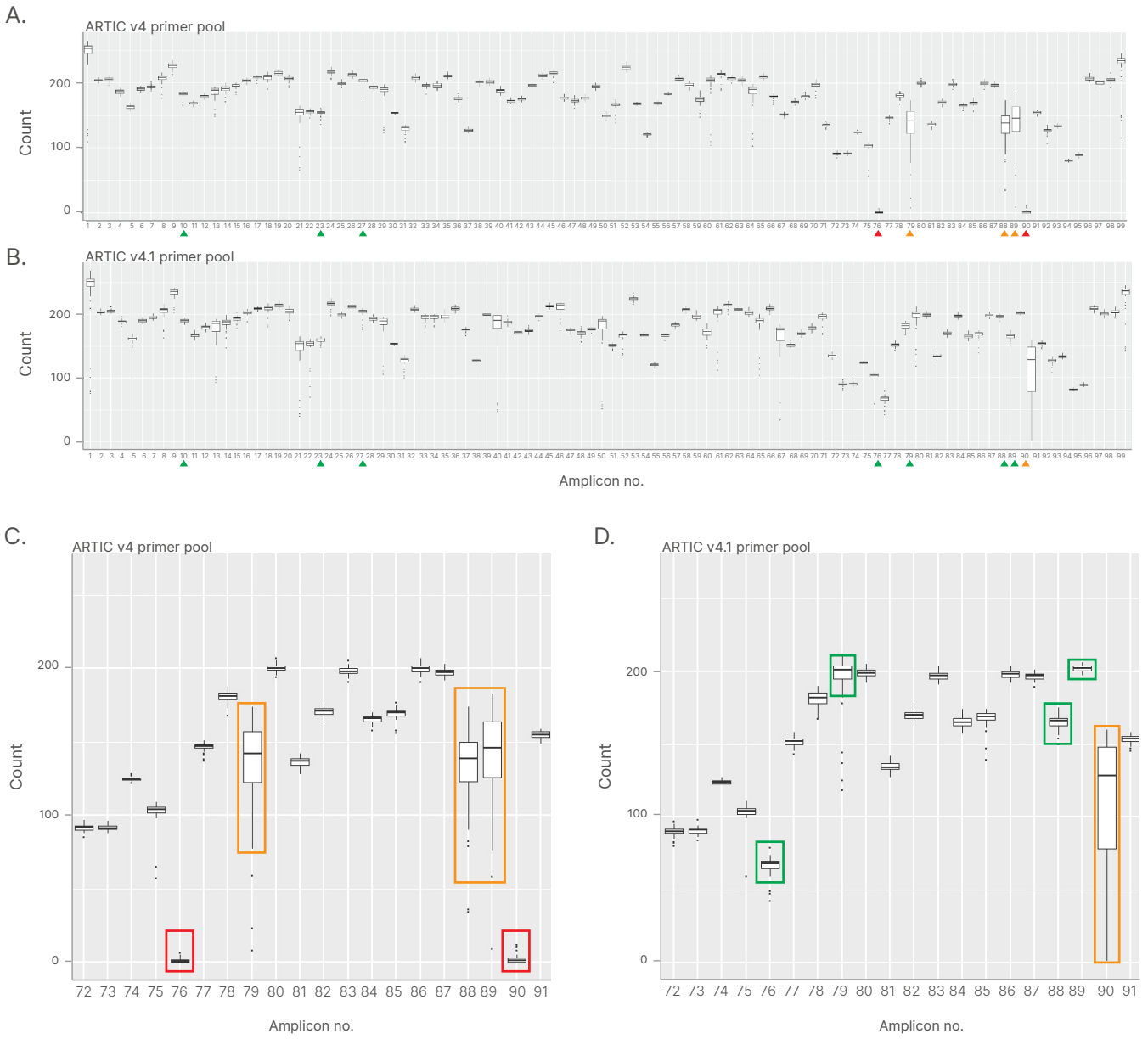

Figure 3: Amplicon coverage for NP samples assayed with ARTIC v4 and v4.1 primer pools—Coverage is plotted for (A, B) all amplicons and (C, D) amplicons 72-84, which span the spike protein region, generated with ARTIC v4 and ARTIC v4.1 primer pools. Amplicons 10, 23, and 27 showed no adverse effects on coverage with either pool (green arrowheads in A and B). Amplicons 76 and 90 showed near complete dropout with v4 (red arrowheads in A and red boxes in C), and amplicons 79, 88, and 89 showed a wide range in counts between samples (orange arrowheads in A and orange boxes in C). The ARTIC v4.1 pool rescued amplicons 76, 79, 88, and 89 (green arrowheads in B and green boxes in D).

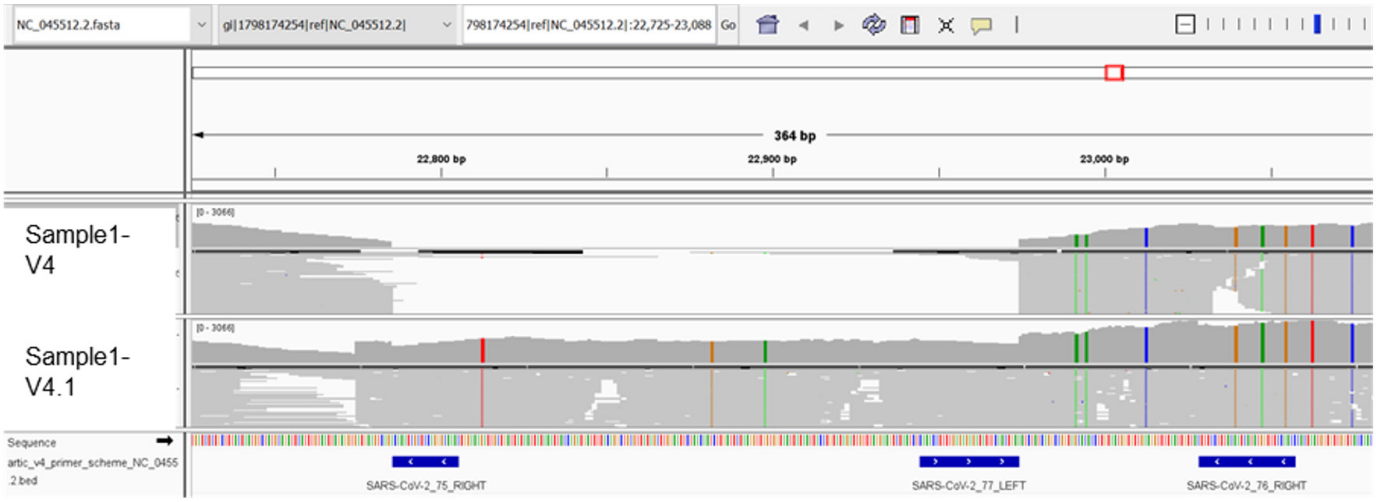

Figure 4: Amplicon 76 coverage with ARTIC v4 and v4.1 pools—Visualization of sequencing depth with IGV showed coverage gaps in amplicon 76 with the ARTIC v4 pool, causing a lack of variant calls at G22813T, T22882G, and G22898A, which are successfully called with the v4.1 pools.

Enrichment with the Respiratory Virus Oligo Panel for SARS-CoV-2 variant coverage

To evaluate using target-based enrichment with the Respiratory Virus Oligo Panel v2 to overcome mutations in SARS-CoV-2 variant genomes, one Omicron-positive NP swab and Synthetic RNA control 48 were taken through the Illumina RNA Prep with Enrichment workflow. Results showed that the percent of genome captured was 99.99% and 99.53% for the NP swab sample and control 48, respectively (Figure 5). The median coverages were 1963 and 82 for the NP swab sample and control 48, respectively (Figure 5).

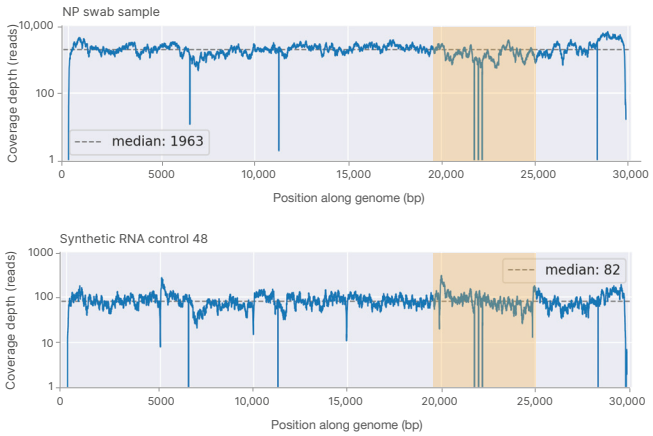

Figure 5: Genome coverage in SARS-CoV-2 Omicron (BA.1) variant using the Respiratory Virus Oligo Panel v2—The percent of genome captured was 99.99% and 99.53% for the NP swab sample (top) and control 48 (bottom), respectively, and the median coverage was 1963 and 82 for the NP swab sample and control 48, respectively. Note: Observed drops in coverage are due to deletions within the genome and not drops in enrichment or assay performance.

Summary

The emergence and spread of new SARS-CoV-2 variants of concern during the COVID-19 pandemic highlights the need for sequencing-based viral surveillance. This technical note demonstrates the Illumina COVIDSeq Test (RUO) used with the DRAGEN COVID Lineage app correctly classifies SARS-CoV-2 lineages using both the ARTIC v4 and v4.1 primer pools. Additionally, while the v4 pool delivers a near-complete consensus genome, some amplicons show diminished coverage in SARS-CoV-2 variants, including Omicron. For comprehensive coverage of the viral genome, guidelines are provided in this technical note to formulate and use the ARTIC v4.1 primer pool to overcome amplicon dropouts. Alternatively, for users who wish to avoid the burden of new probe design and panel maintenance, the Respiratory Virus Oligo Panel v2 with Illumina RNA Prep with Enrichment provides comprehensive genome coverage in emerging SARS-CoV-2 variants.

Learn more

Illumina COVIDSeq Test (RUO), illumina.com/covidseq

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1.800.809.4566 toll-free (US) | +1.858.202.4566 tel
techsupport@illumina.com | www.illumina.com

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