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APPLICATION NOTE

Sequencing respiratory viruses with the MiSeq™ i100 Series

Accurate detection of respiratory syncytial viruses A and B and SARS-CoV-2 using targeted next-generation sequencing

Accurate detection and genomic characterization of respiratory viruses

Flexible amplicon- and enrichment-based library preparation solutions to meet user needs

Efficient, end-to-end workflow that provides same-day results for respiratory virus surveillance

Introduction

Respiratory syncytial virus (RSV) and SARS-CoV-2 have overlapping disease presentation and routinely co-circulate.[1-3] Continuous monitoring in the form of robust and timely viral surveillance is important to guide public health response and resource allocation.[4,5]

Historically, surveillance efforts have heavily depended on virus isolation and amplification using cell culture methods before genetic characterization. While effective, this method produces significant delays in time to results and could bias viral genome sequences due to adaptation during culture.[6,7] While PCR offers a rapid and simple method for detecting viral nucleic acids and antigens, it provides limited information and is vulnerable to false negative results if mutations occur in targeted regions.[8,9]

Targeted next-generation sequencing (NGS) methods, including amplicon- and enrichment-based sequencing, enable rapid, unbiased, and comprehensive genomic profiling, improving the speed and resolution of respiratory virus detection and characterization to provide highly accurate variant information directly from samples.[10] This is important for RNA viruses that accrue mutations at a higher rate than other viral pathogens[11] and may impact tracking viral transmission and evolution, and the efficacy of medical countermeasures.[4,5,8]

This application note demonstrates detection and characterization of respiratory viruses, including RSV-A, RSV-B, and SARS-CoV-2, in contrived samples and real-world nasal swab specimens using available Illumina NGS workflows that include Illumina amplicon- and enrichment-based library preparation solutions, the MiSeq i100 Series, and onboard DRAGEN™ secondary analysis to deliver same-day results (Figure 1).

Methods

Samples

A total of 54 samples were tested, including 12 contrived samples and 42 de-identified, viral-positive remnant nasopharyngeal swab specimens (Table 1). Deidentified remnant clinical samples and associated RT-PCR results were provided as part of a research collaboration with the Arizona State University Biodesign Clinical Testing Laboratory. Contrived samples were prepared by spiking 10–10,000 total copies of pathogen RNA into a matrix of molecular biology grade water + 10 ng of Human Genomic DNA (gDNA) (Promega, Catalog no. G1521) with a total volume of 8.5 µl (Table 2). The nasal swab samples were obtained from two commercial laboratories, where they previously underwent diagnostic molecular testing for RSV-A, RSV-B, and SARS-CoV-2. DNA and RNA were

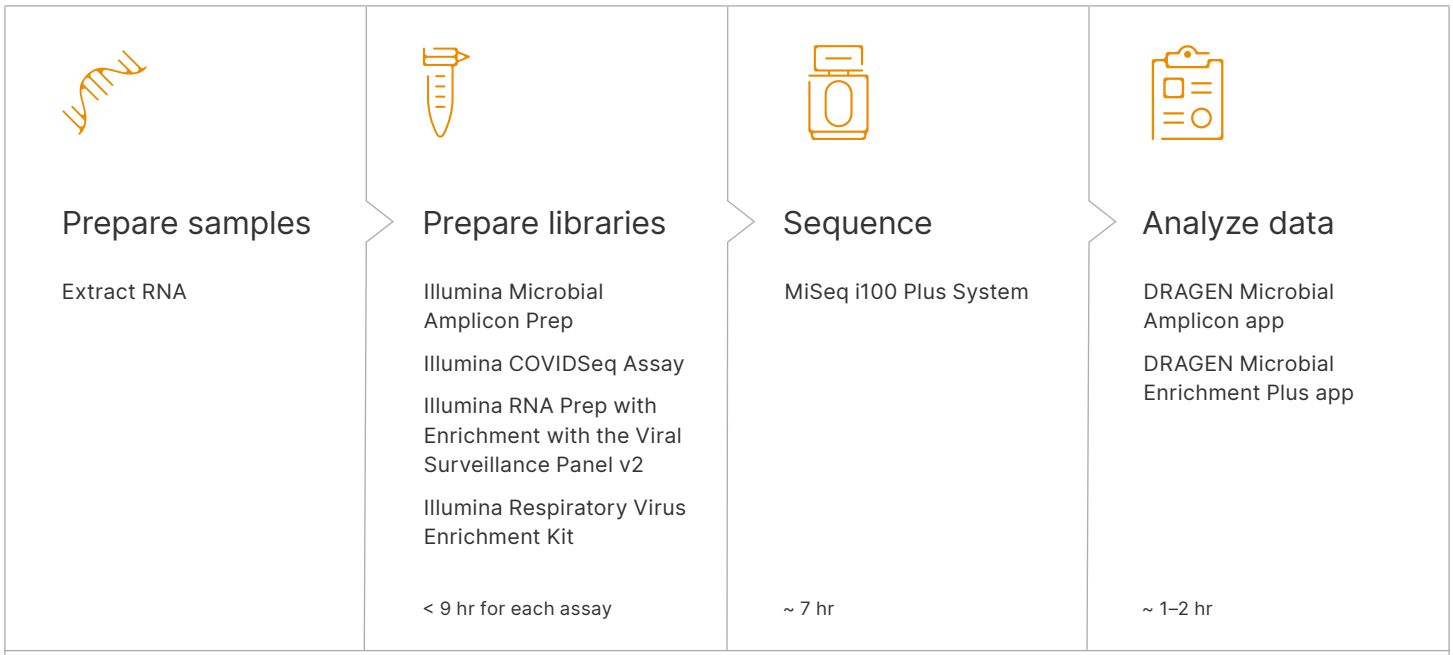

Figure 1: Comprehensive NGS workflow for respiratory virus sequencing
Combine Illumina library prep (including cDNA synthesis) using either an amplicon-based or targeted-enrichment approach with sequencing on the MiSeq i100 Series and DRAGEN secondary analysis for accurate detection and characterization of RSV-A/B and SARS-CoV-2.

Table 1: Samples assayed for performance evaluation

Sample type	Expected detection	No. of samples
Contrived samples (genomic RNA in gDNA)	RSV-A	4
	RSV-B	4
	SARS-CoV-2 (Omicron)	4
Total no. of contrived samples		**12**
Viral-positive nasopharyngeal swab specimens	RSV-A	16
	RSV-B	6
	SARS-CoV-2 (Omicron)	20
Total no. of swab specimens		**42**
Total no. of samples		**54**

extracted separately using the QIAsymphony DSP Virus/Pathogen Mini Kit (QIAGEN, Catalog no. 937036) (200 µl input volume) or the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, Catalog no. R2002) (400 µl input volume), according to the manufacturer's instructions, and 8.5 µl of RNA was input for library preparation. qPCR was performed on RSV- A and RSV-B nasal swab samples (with no Ct information provided) following a published protocol.[12] Five microliters of sample was used with the iTaq Universal Probes One-Step Kit, 500 × 20 µl reactions (Bio-Rad Laboratories, Inc., Catalog no. 1725141) and the AriaMx Real-Time PCR System (Agilent Technologies, Inc., Catalog no. G8830A).

Library preparation

Sequencing-ready libraries were prepared from all samples using amplicon and target enrichment methods. Amplicon libraries were prepared using Illumina Microbial Amplicon Prep (Illumina, Catalog no. 20097857) or the COVIDSeq™ Assay (96 Samples) (Illumina, Catalog no. 20049393) using published primers for RSV-A and RSV-B.[13] Enrichment libraries were prepared using Illumina RNA Prep with Enrichment (L) Tagmentation with the Viral Surveillance Panel v2 Kit (Illumina, Catalog no. 20108081) or with the Respiratory Virus Enrichment Kit (Illumina, Catalog no. 20100469) in 3-plex reactions. Unenriched Illumina RNA Prep with Enrichment libraries were used for comparative purposes.

Table 2: Viral strains used for contrived samples

Virus	Strain	Vendor	Catalog no.
RSV-A	A2	ATCC	VR-1540DQ
RSV-B	B2-18537	ATCC	VR-1580DQ
SARS-CoV-2 (Omicron)	B.1.1.529/ BA.2	Twist Bioscience	105346
Copies/ reaction	**No. of copies[a]**	**RSV-A Ct values**	**RSV-B Ct values**
10	1.2	38.56	36.28
100	12	34.22	32.47
1000	118	31.26	29.14
10,000	1175	27.40	26.18

a. Number of copies input to library preparation.

Sequencing

Amplicon and enrichment libraries were sequenced on the MiSeq i100 Plus System (Illumina, Catalog no. 20115695) using a 25M flow cell with a run configuration of 2 × 151 bp. Shotgun (unenriched) libraries were sequenced on the NextSeq™ 550 System (Illumina, Catalog no. SY-415-1002) with a run configuration of 2 × 151 bp for comparison.

Data analysis

Resulting FASTQ data sets were downsampled to 500,000 clusters or 1M paired-end (PE) reads for amplicon libraries, and 1M clusters or 2M PE reads for enriched libraries. FASTQ files were analyzed using the DRAGEN Microbial Enrichment Plus app either onboard the MiSeq i100 Plus System or in BaseSpace™ Sequence Hub with Viral Surveillance Panel v2 or Illumina Respiratory Virus Enrichment Kit references. Amplicon libraries were processed using the DRAGEN Microbial Amplicon app. Shotgun libraries were analyzed with the DRAGEN Microbial Enrichment Plus app and Viral Surveillance Panel v2 references. Statistical analyses and data visualization were performed using GraphPad Prism 10 and JMP 18.

Results

Sequencing metrics

Amplicon and enrichment libraries were sequenced across four runs on the MiSeq i100 Plus System. All four runs generated high-quality data with > 93% of reads passing filter (% PF) and average Q30 scores above 84%, supporting high-confidence downstream analysis. For all four runs, the combined instrument run time and analysis times were under 10 hours (Table 3). This demonstrates that the MiSeq i100 Plus System offers the speed and efficiency to provide timely results important for public health monitoring and response.

Detection across varying viral genome concentrations

A titration experiment was carried out to evaluate the sensitivity of respiratory virus detection with contrived samples of varying viral copy number. Libraries prepared with Illumina Microbial Amplicon Prep and the COVIDSeq Assay demonstrated reliable detection of RSV-A/B and SARS-CoV-2, respectively, down to 100 viral copies (Figure 2).* RSV-B showed higher coverage (> 10×) and more aligned reads at all conditions, perhaps due to greater sequence conservation and fewer SNPs, compared to RSV-A.[14]

* Genome coverage was measured at a threshold of > 10× depth, and aligned read counts were derived from 1M downsampled PE reads. Limit of detection data were generated using limited replicates and are intended for qualitative comparison.

The reduced sensitivity of SARS-CoV-2 detection, relative to RSV detection, was due to using synthetic RNA as the viral genome material in contrived samples, which are more prone to degradation, lack protective biological matrices, and can be inaccurately quantified, making them less reflective of real-world sample conditions compared to genomic RNA.[15]

Evaluation of genome coverage and reads per kilobase per million mapped reads (RPKM) values showed the strong performance of both amplicon- and enrichment-based library prep solutions, with > 85% genome coverage reliably achieved for RSV-A and RSV-B viruses in contrived samples with greater than or equal to 1000 total genome copies (Ct < 30 in the qPCR assay used for the present study) (Figure 3). In contrived samples with 100 copies or less (Ct < 35 in the qPCR assay used for the present study), sequencing with enrichment panels provided greater coverage of the RSV viral genomes than amplicon-sequencing in this experiment (Figure 3). This result is consistent with the need for a nested amplification protocol with this primer design for samples with Ct values generally considered to be low (Ct < 24).[12]

Respiratory virus detection in nasopharyngeal swab specimens

To evaluate performance in real-world samples, viral-positive nasopharyngeal swab specimens were processed using amplicon, enrichment, and shotgun sequencing approaches. Shotgun libraries (unenriched Illumina RNA Prep with Enrichment libraries) sequenced on the NextSeq 550 System demonstrated lower genome

Table 3: Sequencing metrics for the MiSeq i100 Series[a]

Run	Library type	No. of samples	Sequencing run time	Analysis time with DRAGEN Microbial Enrichment Plus[b]	Analysis time with DRAGEN Microbial Amplicon[c]	% PF	% Q30
1	Viral Surveillance Panel v2	32	7 hr 13 min	41 min	N/A	94.17%	85.99%
2	Illumina Respiratory Virus Enrichment Kit	32	7 hr 11 min	41 min	N/A	93.03%	84.25%
3	Illumina Microbial Amplicon Prep	36	7 hr 13 min	N/A	1 hr 34 min	96.85%	85.96%
4	COVIDSeq Assay	24	6 hr 47 min	N/A	1 hr 07 min	94.85%	87.12%

a. Metrics for shotgun libraries are not included in this table.
b. Analysis was performed onboard the MiSeq i100 Plus System.
c. Analysis was performed in BaseSpace Sequence Hub.

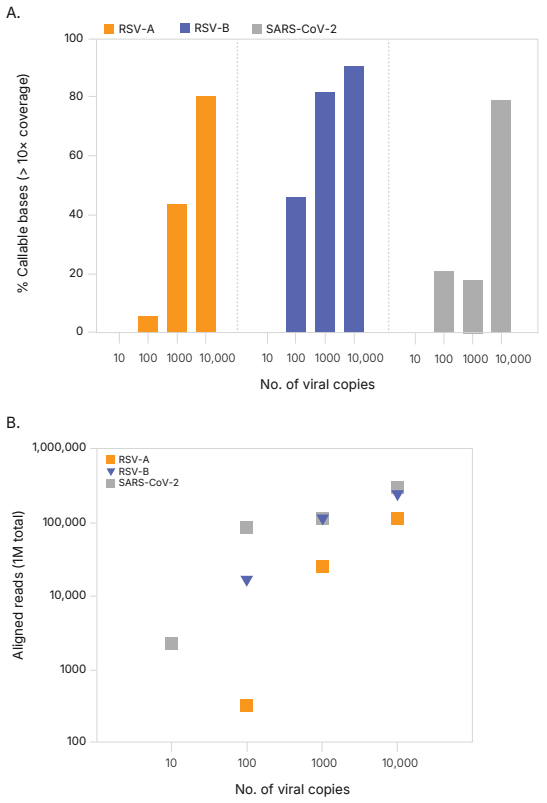

Figure 2: Detection of RSV-A/B with Illumina Microbial Amplicon Prep and SARS-CoV-2 with the COVIDSeq Assay

Results from a limit of detection assay with contrived samples showed sensitive detection of RSV-A, RSV-B, and SARS-CoV-2 down to 100 copies, as shown by (A) percent genome coverage and (B) total number of reads aligning to detected viral genomes from 1M total PE reads. Data were analyzed using the DRAGEN Microbial Amplicon app with pathogen-specific primers.

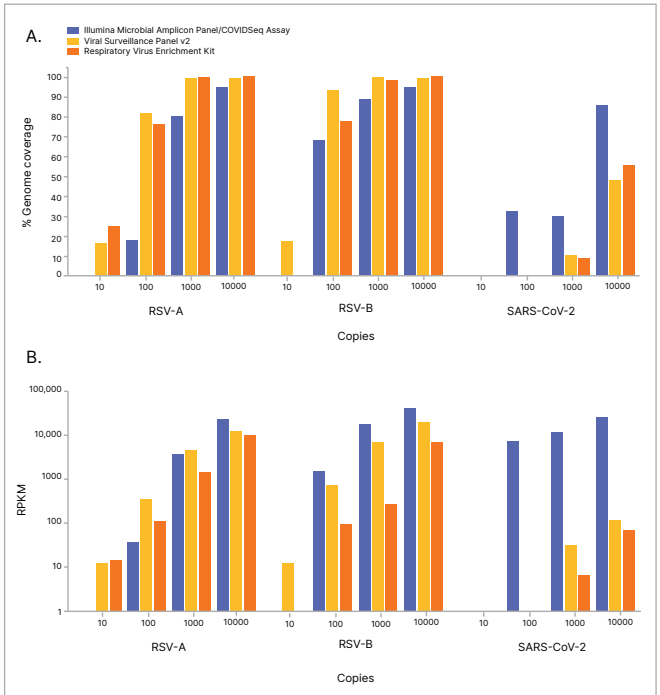

Figure 3: Comparison of viral detection with amplicon and enrichment panels

(A) Genome coverage and (B) RPKM were evaluated for 10, 100, 1000, and 10,000 viral copies numbers in contrived samples. Illumina Microbial Amplicon Prep and enrichment panels (Respiratory Virus Enrichment Kit and Viral Surveillance Panel v2) showed comparable performance in detecting RSV-A and RSV-B down to 100 copies, with the Viral Surveillance Panel v2 able to detect both RSV subtypes down to 10 copies. The COVIDSeq Assay showed superior detection of SARS-CoV-2 as compared to enrichment panels. Data were analyzed using the DRAGEN Microbial Enrichment Plus app with with Viral Surveillance Panel v2 or Respiratory Virus Enrichment Kit pipelines.

coverage for SARS-CoV-2, RSV-A, and RSV-B particularly in samples with lower viral abundance (as indicated by higher qPCR Ct values), relative to amplicon and enrichment workflows on the MiSeq i100 Plus System (Figure 4–Figure 6).

The COVIDSeq Assay, the Respiratory Virus Enrichment Kit, and Illumina RNA Prep with Enrichment with the Viral Surveillance Panel v2 all successfully detected SARS-CoV-2 across a range of nasal swab samples with varying viral abundance, achieving ~100% callable bases in high abundance samples and maintaining strong coverage in samples with lower viral loads (Figure 4A). Representative nasal swab samples with high viral abundance (indicated by low qPCR Ct values) demonstrated comprehensive genome coverage with both amplicon- (Figure 4B) and enrichment-based library preparation kits (Figure 4C).

Illumina Microbial Amplicon Prep, the Respiratory Virus Enrichment Kit, and Illumina RNA Prep with Enrichment with the Viral Surveillance Panel v2 all successfully detected RSV-A (Figure 5A) and RSV-B (Figure 6A) across a range of nasal swab samples with varying viral abundance, achieving ~100% callable bases in high abundance samples and maintaining strong coverage in samples with lower viral loads. Representative nasal swab samples with high viral abundance (indicated by low qPCR Ct values) demonstrated comprehensive genome coverage with both amplicon- (Figure 5B and Figure 6B) and enrichment-based library preparation kits (Figure 5C and Figure 6C).

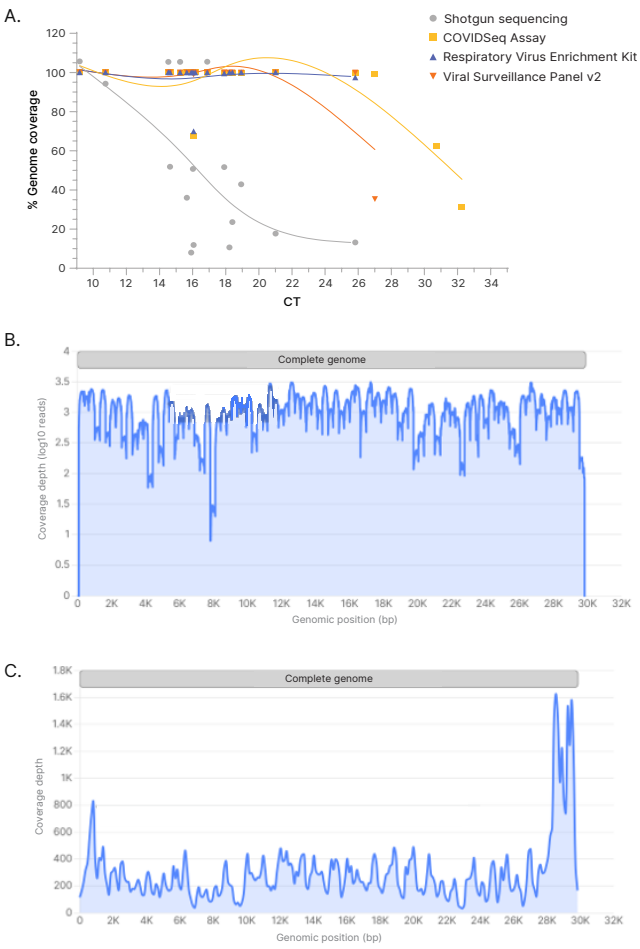

Figure 4: Detection of SARS-CoV-2 in nasopharyngeal swab specimens

As indicated by (A) percent genome coverage, the COVIDSeq Assay, the Respiratory Virus Enrichment Kit, and the Viral Surveillance Panel v2 all successfully detected SARS-CoV-2 in clinical specimens across a range of viral abundance (indicated by qPCR Ct value). Genome coverage was maintained across most viral abundance levels with reduced coverage for specimens with low viral levels. Representative coverage plots for SARS-CoV-2 analyzed with (B) the DRAGEN Microbial Amplicon (log scale) and (C) the DRAGEN Microbial Enrichment Plus app (linear scale) demonstrate that full genome assembly was achieved.

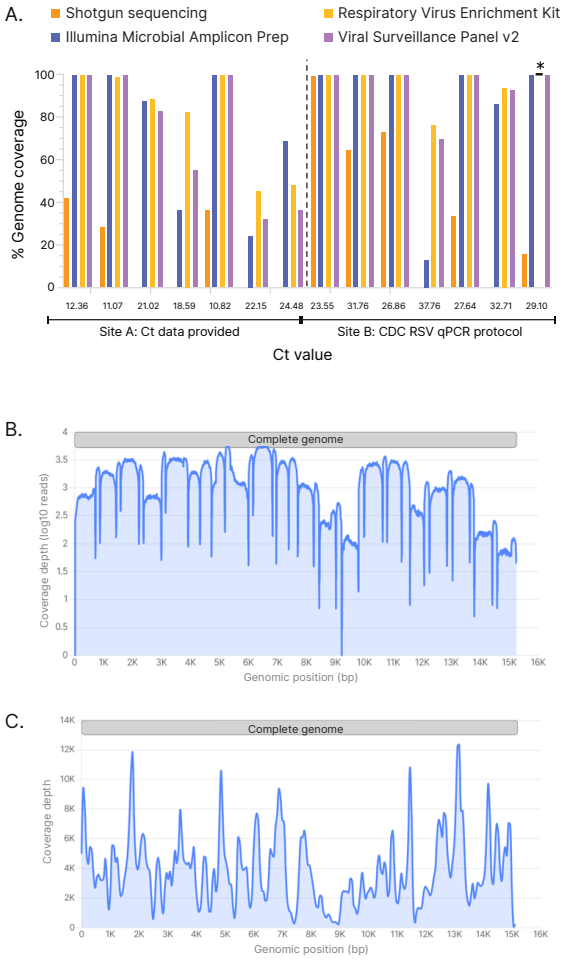

Figure 5: Detection of RSV-A in nasopharyngeal swab specimens

As indicated by (A) percent genome coverage, Illumina Microbial Amplicon Prep, the Respiratory Virus Enrichment Kit, and the Viral Surveillance Panel v2 all successfully detected RSV-A in clinical specimens across a range of viral abundance (indicated by qPCR Ct value). Genome coverage was maintained across most viral abundance levels with reduced coverage for specimens with low viral levels. Representative coverage plots for RSV-A analyzed with (B) the DRAGEN Microbial Amplicon (log scale) and (C) the DRAGEN Microbial Enrichment Plus app (linear scale) demonstrate that full genome assembly was achieved.

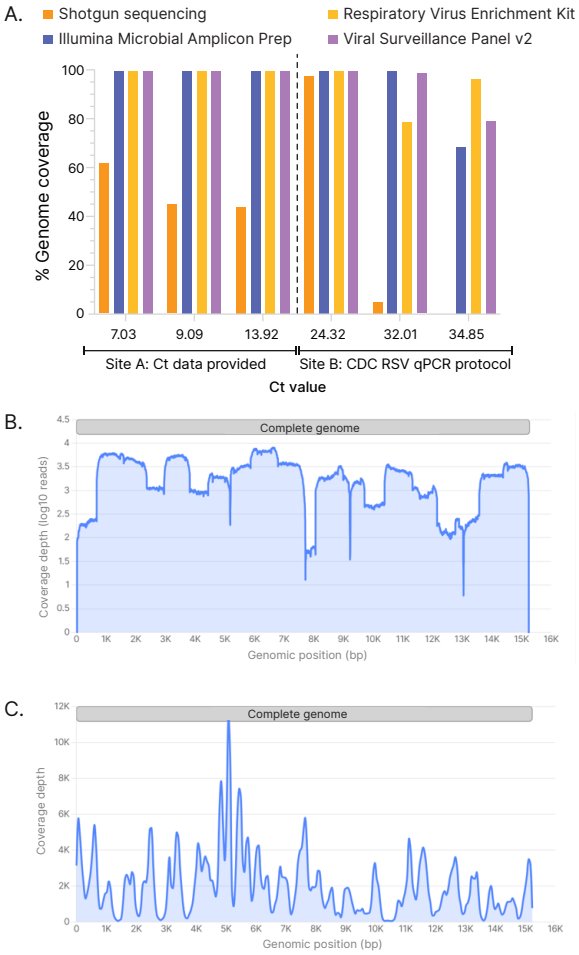

Figure 6: Detection of RSV-B in nasopharyngeal swab specimens

As indicated by (A) plotting % genome coverage, Illumina Microbial Amplicon Prep, the Respiratory Virus Enrichment Kit, and the Viral Surveillance Panel v2 all successfully detected RSV-B in clinical specimens across a range of viral abundance (indicated by qPCR Ct value). Genome coverage was maintained across most viral abundance levels with reduced coverage for specimens with low viral levels. Representative coverage plots for RSV-B analyzed with (B) the DRAGEN Microbial Amplicon (log scale) and (C) the DRAGEN Microbial Enrichment Plus app (linear scale) demonstrate that full genome assembly was achieved.

Summary

Recovery of high-quality partial or full viral genomes rapidly and directly from primary samples offers unique information beyond the qualitative detection of respiratory viruses, which can then inform viral surveillance and outbreak investigation efforts. The MiSeq i100 workflows presented here all share this capability and the potential for more cost- and data-effective sequencing compared with shotgun sequencing, with some unique advantages. The Illumina Microbial Amplicon Prep and COVIDSeq Assay kits are well suited to single-pathogen surveillance and offer a simple building block to a standardized sequencing workflow. These kits can be easily adapted to new targets as public health needs demand, as illustrated in this application note by the substitution of RSV primers for SARS-CoV-2 primers and the selection of RSV reference genomes within the DRAGEN Microbial Amplicon app. Custom references can be easily uploaded into the app as needed.

Sequencing with enrichment unlocks multipathogen surveillance capabilities without sacrificing the ability to generate at least partial genome sequences, particularly in regions of interest such as vaccine epitopes and antiviral targets, as demonstrated in this application note with RSV-positive samples. The Respiratory Virus Enrichment Kit may be more appropriate and offer workflow consolidation opportunities for dedicated characterization of viruses from respiratory samples, whereas the Viral Surveillance Panel v2 can be used with diverse sample types and the detection and characterization of many other viral pathogens of public health interest.

Learn more →

MiSeq i100 Series

Illumina Microbial Amplicon Prep

Illumina Respiratory Virus Enrichment Kit

Viral Surveillance Panel v2

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