

Illumina Microbial Amplicon Prep for viral surveillance

Flexible performance for
comprehensive viral
genome coverage



Introduction

Genomic surveillance for pathogenic microbes is a critical tool for public health. The need is justified by continued outbreaks, the impact of climate change, habitat encroachment, and continued zoonotic transmission events.¹

Illumina Microbial Amplicon Prep provides a customizable genomic surveillance tool with a streamlined, established workflow using the same chemistry as the Illumina COVIDSeq™ Assay (Figure 1). The workflow integrates sample and library prep (including cDNA conversion for RNA targets, PCR amplification, tagmentation, library amplification, and bead cleanup reagents), proven Illumina sequencing, and award-winning DRAGEN™ secondary analysis. To demonstrate the broad capability of virus whole-genome sequencing (WGS) with Illumina Microbial Amplicon Prep, several arboviruses (chikungunya, dengue 1 and zika), Mpox virus, and human Respiratory Syncytial Virus (hRSV) A/B were assayed using primer sets from open-source primer design tools or established scientific literature with minor protocol modifications. Results show comprehensive coverage across viral genomes of varying size for effective surveillance.

Methods

Sample preparation

Arboviruses

To characterize Illumina Microbial Amplicon Prep assay performance for arboviruses, commercially available genomic RNA controls of chikungunya, dengue 1, and zika

viruses were evaluated (Table 1). Defined copy numbers of genomic RNA were spiked into 10 ng of Universal Human Reference RNA (Agilent, Catalog no. 740000) to function as a contrived sample of RNA viruses.

Table 1: Viral samples used for evaluation

Virus	Vendor	Product no.
Chikungunya	Vircell	MBC099-R
Dengue 1	Vircell	MBC055-R
Zika	ATCC	VR-1843DQ

Mpox

To characterize DNA virus performance, DNA samples from Mpox positive skin lesions were extracted using the QIAamp DSP DNA Blood Mini Kit (QIAGEN, Catalog no. 61104) and used as input to the Illumina Microbial Amplicon Prep assay at the "Amplify cDNA" step of the protocol. Viral load of the samples was determined using qPCR performed by Aegis Sciences Corporation on the AB 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems, Catalog no. 4406985/4406984) to obtain cycle threshold (Ct) values.

hRSV

To characterize assay performance for hRSV, nasal swab samples (280 µl) stored in viral transport media were extracted using the QIAamp Viral RNA kit (QIAGEN, Catalog no. 52904). Extracted nucleic acid was then DNase-treated using the Quick-RNA MicroPrep Kit (Zymo Research, Catalog no. R1050). Samples were then diluted 1:10 to obtain additional volume for repeated testing and/or assay optimization. qPCR was performed using the

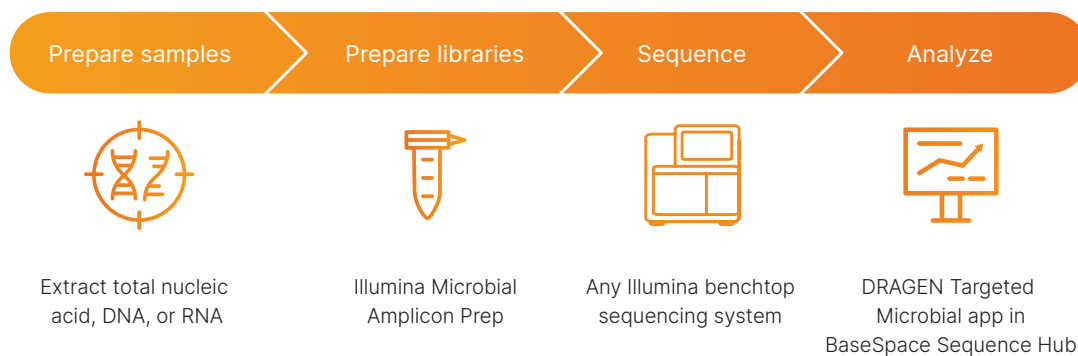


Figure 1: Illumina Microbial Amplicon Prep workflow—Illumina Microbial Amplicon Prep is part of a streamlined workflow for viral WGS that integrates sample and library preparation with sequencing on any Illumina benchtop sequencing system, and DRAGEN secondary analysis.

protocol and probes designed by the Centers for Disease Control (CDC) and the iTaq Universal Probes One-Step Kit 500 × 20 µl kit (Bio-Rad Laboratories, Catalog no. 1725141). Including an undiluted extracted sample in the Illumina Microbial Amplicon Prep assay is suggested when working with samples of unknown target copy numbers to maximize target amplification.

Primer design

All primers were ordered from Integrated DNA Technologies (normalized to 100 µM) and pooled with equimolar concentrations. Primers were assembled into two pools to generate two overlapping sets of amplicons.

Arboviruses

While 400-bp amplicon designs are recommended as the default amplicon size for primer design, longer amplicons are possible with both RNA and DNA targets. Longer amplicons reduce the number of primers needed, the risk of heterodimer interactions, off-target binding interactions, and may be necessary to cover regions with high variability. Shorter amplicons can be advantageous as their performance can be more robust with degraded samples and difficult-to-amplify regions (viral genome secondary structure).

For chikungunya virus, genomic sequence NC_004162.2 was processed by the [PrimalScheme software tool](#), targeting a 400-bp amplicon size, to generate the primer pool design CHIK-PP. This primer pool (CHIK-PP) was used to assay the Amplirun Chikungunya Virus RNA Control (Vircell, Catalog no. MBC099-R). For dengue 1 virus, genomic sequence KM204119.1 was processed by the PrimalScheme software tool, targeting a 400-bp amplicon size. This primer pool (DENV1-PP) was used to assay the Amplirun Dengue 1 Virus RNA Control (Vircell, Catalog no. MBC055-R). For the zika virus, an existing primer design targeting a 400 bp amplicon size generated through PrimalScheme was used.² Primers were pooled in an equimolar manner and tested in the assay using Zika Virus RNA Control (ATCC, Catalog no. VR-1843DQ).



Learn more about PrimalScheme tool at www.primalscheme.com

Mpox

For Mpox virus, gDNA was assayed using an optimized primer pool designed using PrimalScheme targeting a ~2000-bp amplicon size.³ The initial Mpox primer pool contains 326 primers optimized through the addition of five additional primers to address dropouts seen in amplicons 11, 75, and 118 to create primer pool MPX-GL-Yv2.

hRSV

A primer design from the CDC⁴ and a primer design from the WHO Collaborating Centre for Reference and Research on Influenza (WCCRR1)⁵ were assayed with hRSV-positive nasopharyngeal swab samples acquired from Discovery Life Sciences. The CDC primer design (hRSV-A/B-CDC20) consisted of one primer design for hRSVA and another primer design for hRSVB. The hRSV-A/B-CDC20 primer designs generated 19 (hRSVA) or 20 (hRSVB) amplicons total targeting an amplicon size of ~925 bp. If using the CDC primer design, users should determine hRSV subtype (A/B) using qPCR with established probe sets to decide which primer design to use.⁶ The WCCRR1 primer design (hRSV-WCCRR16) is designed to generate six amplicons for both hRSVA or hRSVB, targeting variable amplicon sizes from ~2000–4400 bp. With the WCCRR1 primer design, determination of hRSV subtype is not required.

Library preparation

The [Illumina Microbial Amplicon Prep protocol](#) was followed to prepare libraries for chikungunya, dengue 1, and zika virus without modifications. For Mpox virus, DNA extracted from Mpox-positive samples was processed starting at the "Amplify cDNA" step of the protocol, as the reverse transcription step is not required for DNA virus samples. For hRSV, extracted RNA was processed following the Illumina Microbial Amplicon Prep protocol, with the following modifications:

- The "Anneal RNA" step was modified to add 2.5 µl of EPH3 and 6 µl of molecular grade water instead of the default input of 8.5 µl of EPH3. Previous testing has shown that reducing EPH3 input improves performance with amplicons larger than 400 bp (data not shown).
- The "Amplify cDNA" PCR program was changed to lower the annealing temperature to facilitate proper primer annealing ([Table 2](#)). This temperature was determined using the primer sequences from the CDC20 and WCCRR1 primer designs and an online [Tm Calculator](#).

Table 2: Changes to "Amplify cDNA" PCR program

Step	Temperature (°C)	Time (s)	No. of cycles
1	98	180	1
2	98	15	
3	56/59 ^a	30	35
4	72	180	
5	4	hold	—

a. Annealing temperature was lowered from 63°C to 56°C and 59°C for CDC20-RSV-A/B and WCCRRRI primer sets, respectively. PCR program was run for 35 cycles.

Sequencing

Prepared libraries were denatured and diluted from a pooled library according to the NextSeq™ 500 and 550 Sequencing Systems Denature and Dilute Libraries Guide. Libraries were sequenced on the NextSeq 550 System at 2 × 149 bp read length and normalized to 1M paired-end read depth based on current sequencing recommendations for the COVIDSeq assay.

Data analysis

Sequencing data was analyzed using the DRAGEN Targeted Microbial App, available in BaseSpace™ Sequence Hub. The easy-to-use app aligns reads to reference genomes, calls variants, and generates a consensus genome sequence representing the population of nucleic acid species in the sample. When available, external curated databases were accessed for additional lineage analysis.

Results

Arbovirus sequencing

Sequencing of arbovirus libraries resulted in a technical replicate median of 80% and 96% of ≥ 10× genome coverage at 500 and 5000 viral copy inputs, respectively for chikungunya virus (Figure 2A, 2B); a median of 94% and 98.5% of the genome covered at ≥ 10× genome coverage at 500 and 5000 viral copy inputs, respectively for dengue 1 virus (Figure 2C, 2D); and a median of 97.2% and 98.5% of ≥ 10× genome coverage at 500 and 5,000 viral copy inputs, respectively for zika virus (Figure 2E, 2F).

For all arboviruses sequenced, coverage increased with greater viral copy input, demonstrating performance variability by viral titer. Sequencing read alignment yielded detection of multiple substitutions across the viral reference genome used for analysis and primer design. These substitutions could cause reduced coverage or amplicon dropout if they occur at primer binding sites, especially towards the 3' end of the primers. Substitution discrepancy was observed between libraries assaying the same viral sample or control (Figure 2E, 2F). Technical replicates can be helpful for amplicons that amplify inconsistently and are recommended to confirm the variant calling in the DRAGEN Targeted Microbial App.

Mpox sequencing

Sequencing Mpox virus resulted in robust genome coverage at 1M paired-end reads, despite a genome ~20× larger than the arboviruses assayed (Figure 3A). While coverage of inverted terminal repeats (ITR) is not represented, (due to the analysis pipeline omitting reads with multiple alignments), subsequent analysis of BAM file supplementary alignments showed that these regions were successfully amplified (data not shown). Evaluating the effect of viral input on performance demonstrated that mapped reads may not translate to full genome coverage for low-titer samples (Figure 3B).

hRSV sequencing

Sequencing results showed that both primer pools evaluated were able to amplify hRSV-A/B genomes (Figure 4). Further analysis showed that the reduced coverage depth seen in hRSV-A/B amplified with the WCCRRRI primer pool corresponded to the longest amplicon (~4300 bp, Figure 4A, 4C).⁵ While PrimalScheme is recommended for amplicon design, these results show that other primer designs can provide comprehensive genome coverage with protocol modifications. Further optimization could incorporate the latest hRSV genomes submitted to GISAID into primer design to target the most prevalent and current hRSV strains.

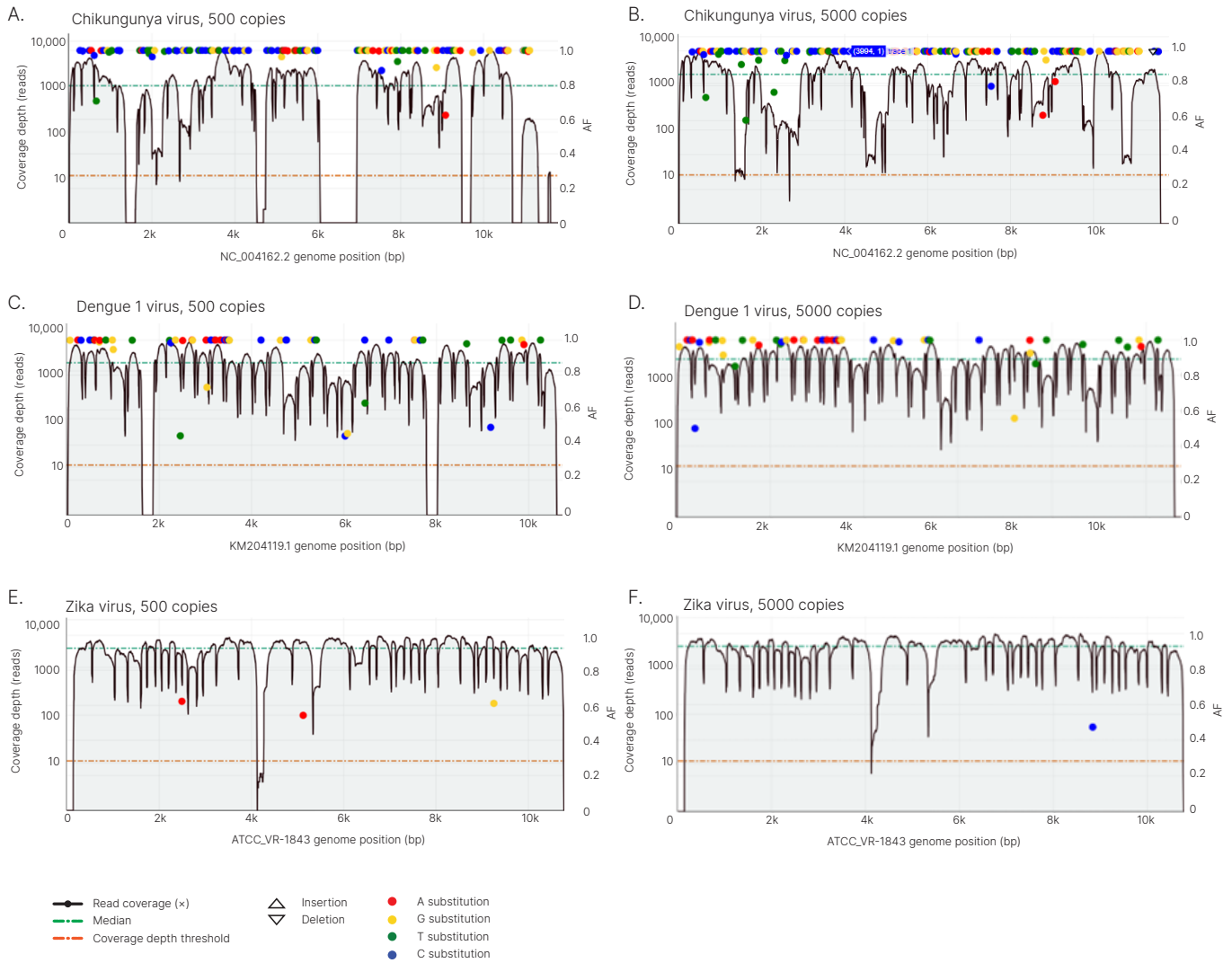


Figure 2: Arbovirus genome coverage from DRAGEN Targeted Microbial App—Sequencing results of arboviruses, including (A, B) Chikungunya virus, (C, D) Dengue 1 virus, and (E, F) Zika virus showed high median coverage at 500 copies of input, and increased coverage with 5000 copies.

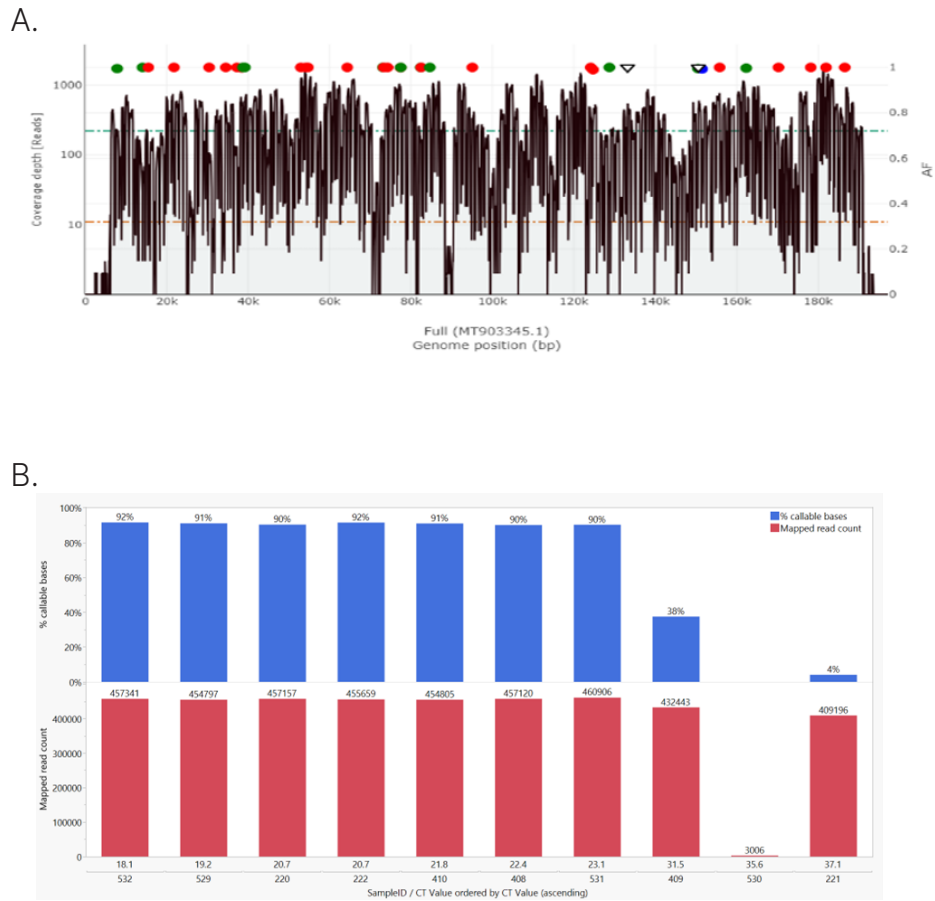


Figure 3: Mpox genome coverage—Sequencing results with (A) a representative sample of Mpox virus showed coverage across the genome; (B) low viral-titer samples showed reduced coverage.

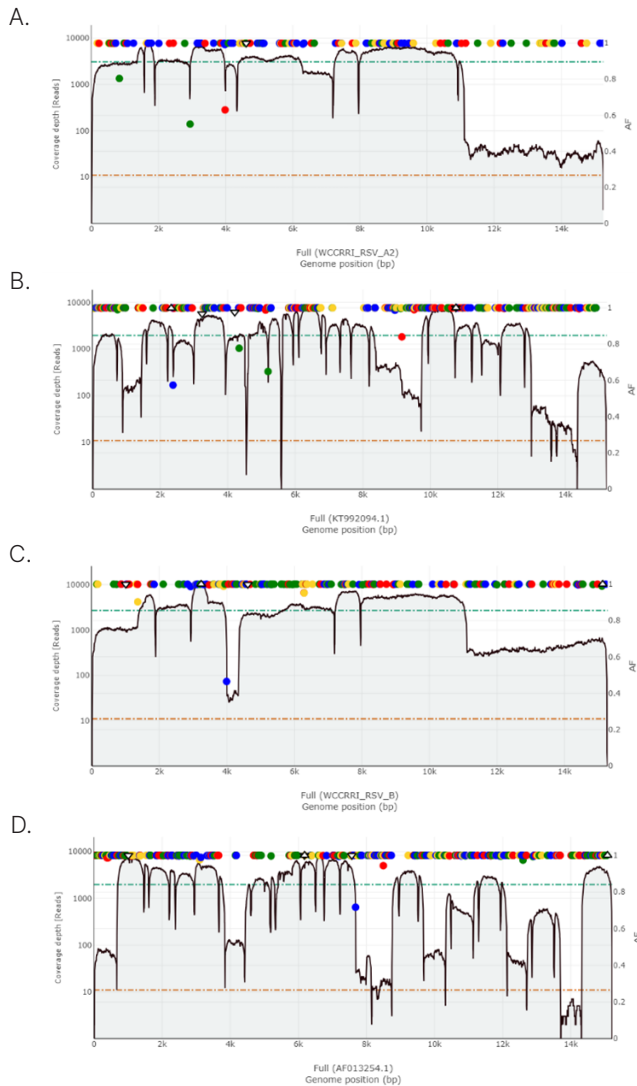


Figure 4: hRSV A/B genome coverage—Sequencing results with representative samples of (A, B) hRSV-A and (C, D) hRSV-B showed that while both primer designs amplified viral genomes, reduced coverage seen with the (A, C) WCCRR1 primer pool corresponded to the longest amplicon (~4300 bp).

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Summary

Illumina Microbial Amplicon Prep provides a genomic surveillance solution with demonstrated performance across a range of RNA and DNA viral families and genome sizes. As shown in this application note, Illumina Microbial Amplicon Prep provides comprehensive genome coverage (defined here as > 90% of the viral genome covered at $\geq 10\times$ coverage) for all viruses evaluated. This kit delivers a universal workflow with the flexibility to customize to virtually any microbial target of interest.

Learn more

[Illumina Microbial Amplicon Prep](#)

References

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