A sample-to-results workflow for Respiratory Syncytial Virus whole-genome sequencing

Kaylinnette Pinet[1], Anagha Kadam[1], Patrick Acer[2], Patrick Anderson[2], Matthew Angel[1], Ganit Pricer[2], V K Chaithanya Ponnaluri[1], Bradley W. Langhorst[1], Lauren Saunders[2], Eric Cantor[1], Keerthana Krishnan[1]

1. New England Biolabs, Ipswich, MA, USA | 2. Ceres Nanosciences Inc., Manassas, VA, USA

Introduction

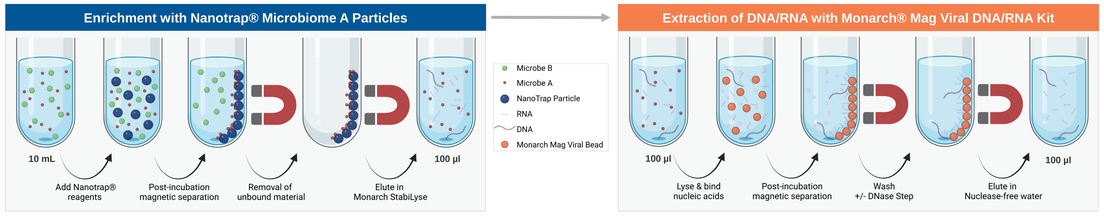
Human respiratory syncytial virus (RSV) is a single-stranded RNA virus that has persisted for over seven decades as a leading cause of respiratory illness in infants and young children. Applying multiplex targeted amplification sequencing techniques to RSV can support public health as well as research efforts to monitor vaccine or therapeutic evasion mutations and emergent strains. Here we describe a sample-to-result workflow for RSV concentration, extraction, and sequencing applied to commercial controls and contrived wastewater samples.

We employed Nanotrap® Microbiome A Particles and Enhancement Reagent 2 to concentrate RSV viral particles from wastewater samples in a semi-automated, high-throughput method. Nucleic acids were then extracted from samples that underwent enrichment, as well as unenriched samples, using the magnetic bead-based Monarch® Mag Viral DNA/RNA Extraction Kit. After DNase treatment to remove any contaminating DNA, the subsequent RNA served as a template for targeted cDNA synthesis and amplification with NEBNext® RSV Primers and the LunaScript® Multiplex One-Step RT-PCR Kit. This targeted amplicon RT-PCR method uses two multiplexed primer pools that generate overlapping amplicons and has been optimized to generate genome coverage for both major RSV subtypes, A and B. Finally, amplicon-library preparation was performed in under two hours with the NEBNext UltaExpress® FS DNA Library Prep Kit for Illumina sequencing. With this workflow end-users can take samples from raw wastewater and generate complete libraries in less than 8 hours. Additionally, this RSV sequencing method reduces plastic consumption, hands-on time, and the probability of sample-cross contamination.

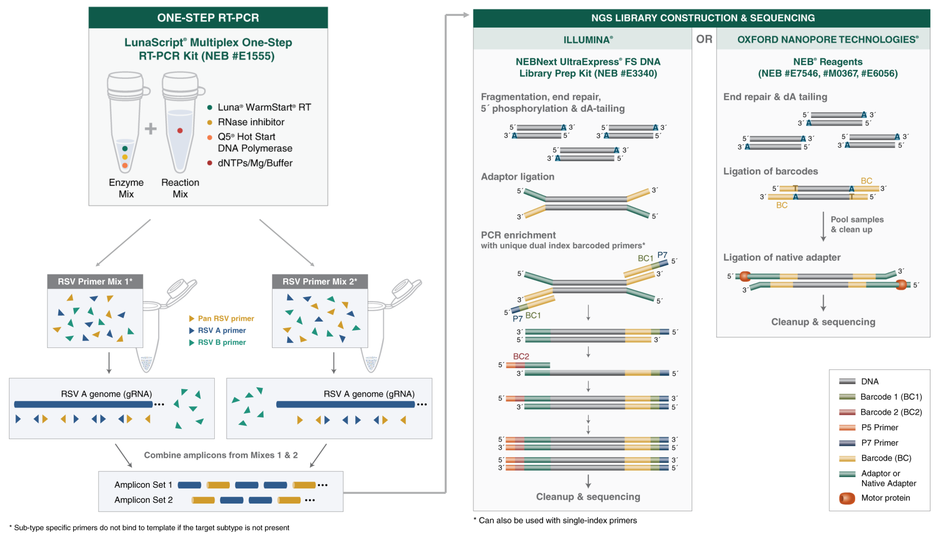
We found high genome coverage across all samples tested, with viral enrichment improving data observed. This workflow provides scientists and public health experts with a cost-effective solution for monitoring RSV viral genomes from population-level wastewater sources, to ensure and improve vaccine efficacy.

Methods

Viral Particle Concentration and Nucleic Acid Extraction[1]



Targeted Amplification & Sequencing of the RSV Genome



Results

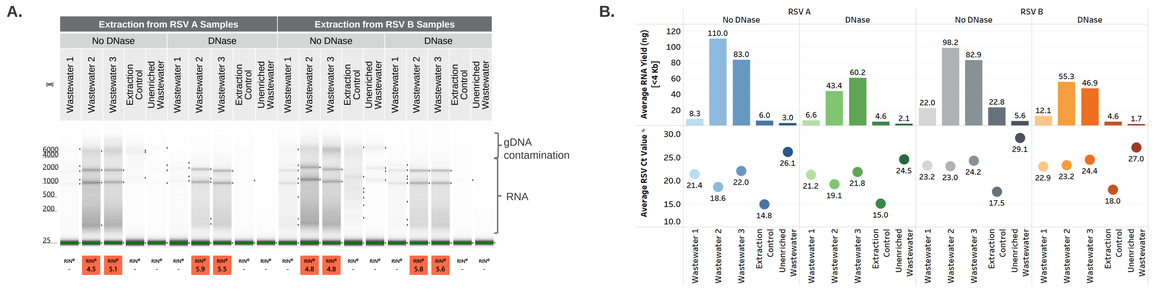


Figure 1. Nanotrap® concentration followed by Monarch Mag Viral Extraction with DNase treatment removes gDNA contaminants. Wastewater samples were acquired from 3 different locations and 10 mL aliquots from each were spiked with one million viral particles of either RSV A (Zeptomatrix; 0810040A) or RSV B (ATCC; VR-1580PQ). These contrived wastewater samples were enriched and then DNA/RNA was extracted, alongside an unenriched control and extraction control, as shown in the Methods section. Final extract volumes were 100 µl. A) Undiluted extracts from wastewater and lysis control samples were run on TapeStation® using RNA HS reagents. Traces show expected eukaryotic rRNA peaks within the 50 bp to 4 Kb size range and gDNA bands within the 4 to 6 Kb size range for samples extracted without the on-bead DNase treatment. B) Average Total RNA yields (n=1-2) and RSV target qPCR Ct value (n=3) for these extracts.

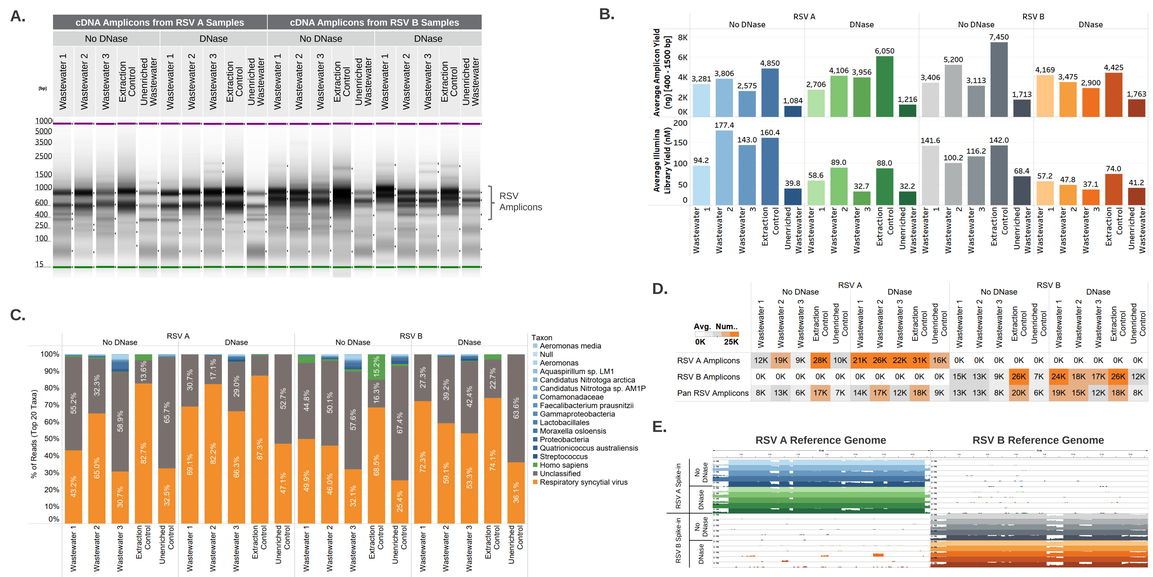


Figure 2. LunaScript® Multiplex One-Step cDNA synthesis and amplification of RSV genome targets of varying sizes provides robust amplicon yields, library yields, and high genome coverage. Overlapping amplicons were generated from 8 µl (~80,000 RSV genome copies) of DNA/RNA extracts described in Figure 1 with NEBNext RSV Primer Module and LunaScript® Multiplex One-Step RT-PCR Kit. Then Illumina libraries were generated with the NEBNext UltraExpress FS Kit and sequenced on a NextSeq® 500. Taxonomic labels were assigned to all reads via Kraken 2 and a custom database sourced from NCBI. Reads were down sampled to 2 million reads with Seqtk[2] for mapping to RSV reference genomes with Bowtie 2[3]. A) 1/5th diluted amplicons run on TapeStation® using DS 5000 HS reagents. TapeStation traces show expected amplicon peaks within the 400 to 1,400 bp range. B) Average amplicon yields (n=1-2) within 400 to 1400 bp window and average library yields (n=1-2) determined via TapeStation analysis. C) Percent of reads assigned to top 20 taxa. D) Average number of reads mapped to Pan RSV, RSV A, or RSV B amplicon regions. E) IGV visualization of coverage across RSV A or RSV B reference genomes for enriched wastewater samples, extraction controls, and unenriched wastewater samples (0-1000 log scale).

Conclusions

- Combining Nanotrap Microbiome A particle concentration, NEB Monarch Mag Viral extraction, and NEBNext RSV sequencing protocols provides a scalable, automation friendly workflow for wastewater surveillance through targeted sequencing
- Nanotrap technology enables better viral genome coverage by concentrating intact organisms prior to sequencing
- DNase treatment during nucleic acid extraction from wastewater reduces background DNA contamination and off target signal
- NEB is focused on developing variant-tolerant primer schemes and streamlined protocols for the targeted sequencing of viral pathogens
- Our amplicon-based targeted sequencing method for RSVA and RSVB using dual multiplexed primer pool provides high genome coverage

References

1. Created in https://BioRender.com
2. H. Li, Seqtk: a fast and lightweight tool for processing FASTA or FASTQ sequences, 2013. https://github.com/lh3/seqtk/
3. Langmead, B., Salzberg, S. Fast gapped-read alignment with Bowtie 2. Nat Methods 9, 357–359 (2012). https://doi.org/10.1038/nmeth.1923.

Poster #22W