APPLICATION NOTE

Rapid detection of respiratory pathogens using the MiniSeq™ System

- Comprehensive coverage of critical respiratory viruses, bacteria, and fungi, and antimicrobial resistance genes
- A 24-hour, sample-to-results sequencing workflow using Illumina RNA Prep with Enrichment and the Respiratory Pathogen ID/AMR Panel

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Introduction

Respiratory tract infections are a public health concern, often caused by viral, bacterial, and fungal pathogens. Upper respiratory tract infections are typically less severe and include the common cold (viral rhinitis), sinusitis, pharyngitis, and others. Lower respiratory tract infections comprise bronchitis, bronchiolitis, and pneumonia. These illnesses, particularly pneumonia, can be severe and require hospitalization, or even be fatal.[1,2]

Accurate identification of respiratory pathogens can be challenging, particularly for mixed or co-infections. Conventional methods for detection include *in vitro* culture, antibody- or antigen-based assays, and PCR.[3] PCR has become a preferred method for pathogen identification due to its speed, high sensitivity, and specificity.[4] However, due to the limited number of targets per assay, PCR can require multiple, sequential assays to test for even common pathogens. Compounding the COVID-19 pandemic with a flu season increases the potential for co-infections and complications with SARS-CoV-2 and other respiratory pathogens,[5] highlighting the need for rapid, accurate, and broad-spectrum pathogen detection.

Next-generation sequencing (NGS) provides an effective way to analyze samples and detect known and emerging respiratory pathogens from a variety of sample types, including those with multiple infectious agents, in a single assay. To that end, Illumina offers the Respiratory Pathogen ID/AMR Panel, which targets ~280 respiratory pathogens, including viruses, bacteria, and fungi, and associated antimicrobial resistance (AMR) markers. Combining the Illumina Respiratory Pathogen ID/AMR Panel with Illumina RNA Prep with Enrichment and sequencing with the MiniSeq Rapid Reagent Kit offers several advantages, including:

- Sensitive detection of both DNA- and RNA-based respiratory pathogens in a single assay in under 24 hours
- Comprehensive genome coverage of critical viral pathogens, including SARS-CoV-2 and Influenza A virus
- Concurrent profiling of AMR marker expression for pathogen characterization in the same assay

This application note highlights a streamlined workflow for detecting and analyzing DNA- and RNA-based respiratory pathogens using Illumina RNA Prep with Enrichment combined with the Illumina Respiratory Pathogens ID/AMR Panel, rapid sequencing on the MiniSeq System, and simplified data analysis with Explify RPIP Data Analysis (Figure 1).

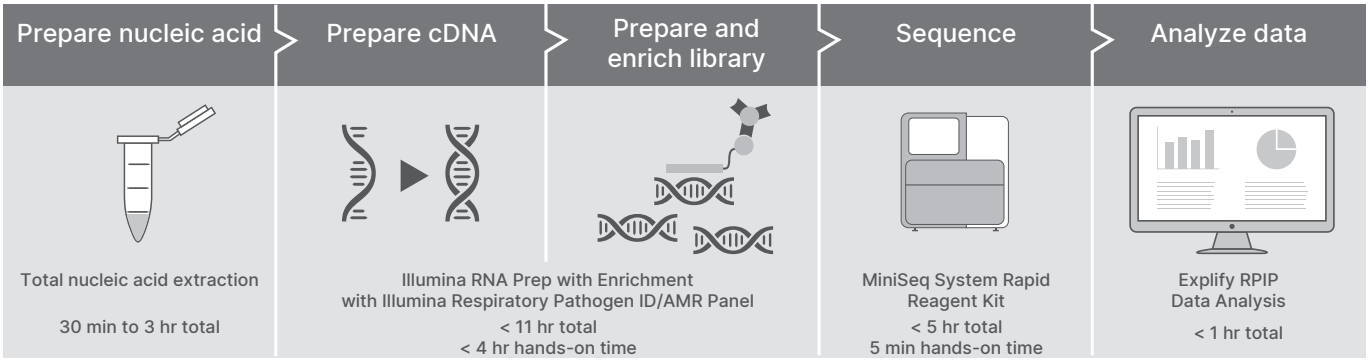

Figure 1: Enrichment workflow for respiratory pathogen detection—The streamlined NGS workflow for respiratory pathogen detection integrates sample preparation, library preparation, target enrichment, sequencing, and data analysis.

Methods

Sample preparation

Residual clinical samples were used that were collected from the upper and lower respiratory tract and tested positive for viral, bacterial, and fungal pathogens, as determined by conventional assays (Table 1). DNA and RNA were extracted from each residual sample and used as input for library preparation and enrichment. To mimic total nucleic acid extracted samples for spike-in studies, 10 ng of DNA and 10 ng of RNA were pooled together. The nucleic acid pool was carried through cDNA conversion and subsequent library preparation and then used to evaluate genomic coverage and detection of AMR.

Table 1: Samples procured for analysis

Sample type	Number	Expected pathogens	Count
Bronchoalveolar lavage	6	Bacteria	7[a]
		Fungi	1
Nasopharyngeal swabs	13	Viruses	14[b]
Sputum	5	Bacteria	5
Tracheal aspirate	2	Bacteria	2
Total	26		29

a. One sample was positive for three bacteria.
b. One sample was positive for two viruses.

Library preparation

Sequencing-ready libraries were prepared with Illumina RNA Prep with Enrichment (Illumina, Catalog no. 20040536) and IDT for Illumina DNA/RNA UD Indexes (Illumina, Catalog no. 20027213). Illumina RNA Prep with Enrichment uses on-bead tagmentation followed by a single hybridization step to generate enriched DNA and RNA libraries. The combined cDNA and DNA samples can be carried straight through into tagmentation with no quantification required.

After amplification, libraries were enriched as 1-plex or 3-plex reactions using the Illumina Respiratory Pathogen ID/AMR Panel. This panel provides targeted detection of 282 pathogens selected to cover > 95% of common and rare causes of respiratory infections using cutting-edge implementations of a combination of alignment-based, alignment-free, assembly-based, and machine learning–enhanced approaches. In addition, 2097 AMR markers are targeted to characterize resistance of 75 common bacterial pathogens to 26 drug classes (Table 2).

Sequencing

Libraries were denatured and diluted to a final loading concentration of 2 pM, according to the MiniSeq System Denature and Dilute Libraries Guide (Document no. 1000000002697 v07) and sequenced on the MiniSeq System at 1 × 101 bp read length using either the MiniSeq High Output Reagent Kit (Illumina, Catalog no. FC-420-1002) or the MiniSeq Rapid Reagent Kit (Illumina, Catalog no. 20044338). Reads were trimmed to 1 × 75 bp, unless otherwise noted. The read recommendation for this workflow is 1M reads per sample, but these numbers can vary and this is only a recommended starting point.

Data analysis

FASTQ sequencing data files were input to Explify RPIP Data Analysis, which can be accessed in BaseSpace™ Sequence Hub, for analysis. The Explify platform offers a paired and custom tailored data analysis solution for the Illumina Respiratory Pathogen ID/AMR Panel. It provides information on sample composition, including host and microbial abundance, and proportion of targeted vs untargeted sequences. Users can select up to 35 individual samples or project folders containing any number of samples for analysis. Reports include a detailed text-based (JSON) format and PDF report.

Table 2: Targets on the Respiratory Pathogen ID/AMR Panel

	Viruses	42 targets	Bacteria	187 targets	Fungi	53 targets	AMR	2097 markers
Antimicrobial classes	Aminoglycosides		Glycopeptides		Sulfonamides		Ethionamides	
	Beta-lactam + beta-lactamase inhibitor		Lincosamides		Tetracyclines		Para-aminosalicylic acids	
	Carbapenems		Macrolides		Isoniazids		Aminoglycosides	
	Cephalosporins		Oxazolidinones		Polyamine antibiotics		Fluoroquinolones	
	Diaminopyrimidine		Penicillins		Pyrazinamides		Neuraminidase inhibitors	
	Fluoroquinolones		Polymyxins		Rifamycin antibiotics		Endonuclease inhibitors	
	Fosfomycin							
Curated bacterial pathogens	*Achromobacter xylosoxidans*		*Corynebacterium jeikeium*		*Klebsiella pneumoniae*		*Proteus vulgaris*	
	Acinetobacter baumannii		*Corynebacterium striatum*		*Klebsiella quasipneumoniae*		*Providencia stuartii*	
	Acinetobacter lwoffii		*Cronobacter sakazakii*		*Leclercia adecarboxylata*		*Pseudomonas aeruginosa*	
	Acinetobacter nosocomialis		*Delftia acidovorans*		*Legionella pneumophila*		*Pseudomonas fluorescens*	
	Acinetobacter pittii		*Elizabethkingia meningoseptica*		*Listeria monocytogenes*		*Pseudomonas stutzeri*	
	Aeromonas caviae		*Enterobacter cloacae complex*		*Moraxella catarrhalis*		*Ralstonia pickettii*	
	Aeromonas hydrophila		*Enterococcus faecalis*		*Moraxella osloensis*		*Raoultella planticola*	
	Aeromonas sobria		*Enterococcus faecium*		*Morganella morganii*		*Salmonella enterica*	
	Aeromonas veronii		*Escherichia coli*		*Mycobacterium fortuitum (Mycolicibacterium fortuitum)*		*Serratia marcescens*	
	Bacillus anthracis		*Eubacterium limosum*		*Mycobacterium tuberculosis complex*		*Shewanella putrefaciens*	
	Bacillus cereus		*Francisella tularensis*		*Mycobacteroides abscessus (Mycobacterium abscessus)*		*Staphylococcus aureus*	
	Bacteroides fragilis		*Fusobacterium nucleatum*		*Mycoplasma pneumoniae*		*Stenotrophomonas maltophilia*	
	Brucella abortus		*Gemella morbillorum*		*Neisseria meningitidis*		*Streptococcus agalactiae*	
	Burkholderia cepacia complex		*Haemophilus influenzae*		*Pandoraea pulmonicola*		*Streptococcus anginosus*	
	Burkholderia pseudomallei		*Haemophilus parainfluenzae*		*Pantoea agglomerans*		*Streptococcus constellatus*	
	Chlamydia trachomatis		*Hafnia alvei*		*Parvimonas micra*		*Streptococcus pneumoniae*	
	Citrobacter freundii complex		*Kingella kingae*		*Pasteurella multocida*		*Streptococcus pyogenes*	
	Citrobacter koseri		*Klebsiella aerogenes (Enterobacter aerogenes)*		*Prevotella intermedia*		*Yersinia enterocolitica*	
	Corynebacterium diphtheriae		*Klebsiella oxytoca*		*Proteus mirabilis*			

Results

Improved respiratory pathogen detection with enrichment

Detection of various respiratory pathogens in clinical sample libraries was significantly improved with enrichment using the Respiratory Pathogen ID/AMR Panel, as compared to aliquots of the same libraries pre-enrichment (Table 3). Indeed, normalized pathogen reads were increased by a median of 330-fold in enriched samples (Figure 2). Enrichment was most pronounced in samples with lower pathogen load, ie, fewer identified pathogens in the pre-enriched libraries.

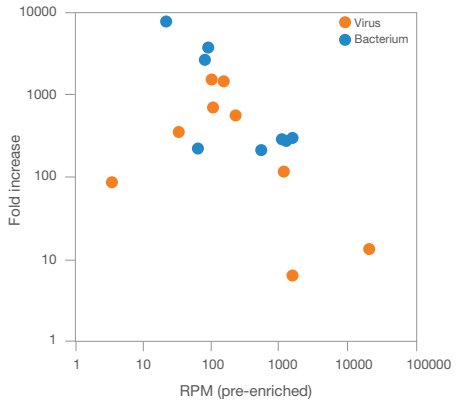

Figure 2: Enrichment increases normalized pathogen reads—Normalized pathogen reads were increased by a median of 330-fold in enriched viral (orange) and bacterial (blue) samples.

Table 3: Improved detection of pathogens with enrichment using the Respiratory Pathogen ID/AMR Panel

Pathogen		Detected without enrichment	Detected only with enrichment
Viruses	Human adenovirus B[a]	1	1
	Human adenovirus C	2	2
	Influenza A virus (H1N1)	0	1
	Human metapneumovirus	1	2
	Influenza B virus	0	1
	Human parainfluenza virus 1	1	2
	Human parainfluenza virus 3	2	2
	Respiratory syncytial virus B	1	1
	SARS-CoV-2	1	2
Bacteria	*Enterobacter cloacae complex*	0	1
	Escherichia coli	1	2
	Haemophilus influenzae	2	2
	Klebsiella pneumoniae	1	2
	Legionella pneumophila	0	1
	Proteus mirabilis	0	1
	Pseudomonas aeruginosa	1	1
	Serratia marcescens	0	1
	Staphylococcus aureus	2	2
	Streptococcus pneumoniae	1	1
Fungi	*Pneumocystis jirovecii*	0	1
	Total	17/29	29/29

a. Reported adenovirus B/E by conventional test.

Comprehensive genomic coverage of critical viral pathogens

Full genome coverage of SARS-CoV-2 and Influenza A virus was achieved at ≥ 100,000 viral copies (Figure 3). These results are significant, as they demonstrate that the Respiratory Pathogen ID/AMR Panel provides sufficient coverage of critical viral pathogens for analysis of viral evolution or viral surveillance.

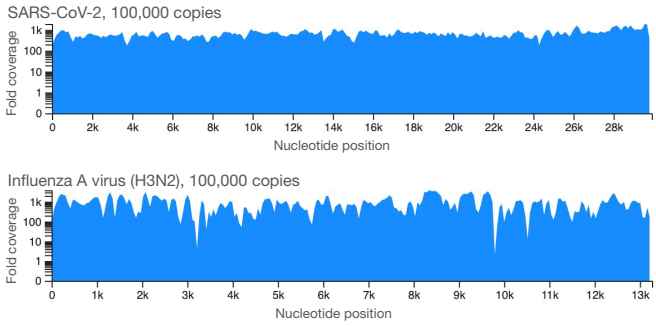

Figure 3: Comprehensive genomic coverage of critical viral pathogens—The Respiratory Pathogen ID/AMR Panel enables comprehensive coverage of viral genomes, including SARS-CoV-2 and Influenza A virus (H3N2), at 100,000 viral copies. Sequencing was run on the MiniSeq System at 1 × 75 bp.

Pathogen detection using the MiniSeq Rapid Kit

To assess performance with the MiniSeq Rapid Kit, one pool of enriched libraries (n=19, including 17 libraries from clinical samples) was sequenced separately with the MiniSeq High Output Reagent Kit (150 cycle) and the MiniSeq Rapid Reagent Kit (100 cycle), both run with 1 × 101 bp. Sequencing metrics were equivalent with the MiniSeq High Output Kit and MiniSeq Rapid Kit (Table 4).

In this experiment, clinical samples were positive for four viruses, eight bacteria, and one fungus. All respiratory pathogens detected using the MiniSeq High Output Kit were also detected using the MiniSeq Rapid Kit (Figure 4). These results demonstrate the highly consistent data and equivalent performance of the two reagent kits.

Table 4: Comparison of sequencing metrics by reagent kit

Metric	MiniSeq High Output Kit (1 × 101 bp)	MiniSeq Rapid Kit (1 × 101 bp)
Total run yield	4.3 Gb	3.2 Gb
% bases ≥ Q30	94%	94%
Cluster density	241 K/mm^2	210 K/mm^2
% clusters passing filter	87%	87%
PhiX error rate	0.50%	0.30%
% tile pass	100%	100%

Detection of AMR markers

A titration experiment was conducted in which diminishing amounts of *Klebsiella oxytoca* was spiked into human background RNA and taken though library preparation and enrichment with the Respiratory Pathogen ID/AMR Panel. Analysis of sequencing data showed that all AMR genes expressed by *Klebsiella oxytoca* were detected as expected for this pathogen (Table 5).

Enrichment plexity and pathogen detection

To evaluate the effect of plexity, the number of pre-enriched libraries that are pooled together in a single enrichment reaction, on pathogen detection, libraries from 21 clinical samples were prepared and enriched with the Respiratory Pathogen ID/AMR Panel either at 1-plex or 3-plex. Clinical samples were positive for 11 viruses, 13 bacteria, and one fungus. While libraries enriched in the 3-plex format resulted in more variable read counts than 1-plex enrichment (data not shown), pathogen detection was highly consistent, with all respiratory pathogens detected in all libraries at both plexities (Figure 5).

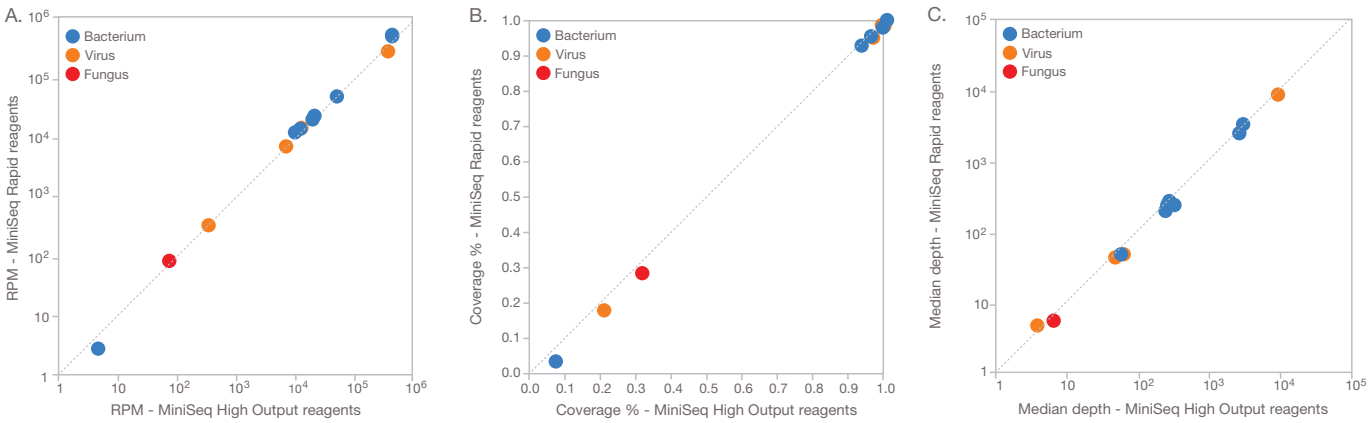

Figure 4: Consistent detection of respiratory pathogens using MiniSeq High Output and Rapid reagents—All respiratory pathogens detected using MiniSeq High Output reagents were also detected using MiniSeq Rapid reagents. Performance metrics, including (A) reads per million (RPM), (B) coverage %, and (C) median depth were highly consistent between reagent kits.

Table 5: Detection of *Klebsiella oxytoca* and expressed AMR genes with the Respiratory Pathogen ID/AMR Panel

Genome copy number	*K. oxytoca*	AAC(6')-Ib-cr[a]	aadA	ANT(3'')-IIa	dfrA1	SHV-5	sul1
100,000	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10,000	3/3	3/3	3/3	3/3	3/3	3/3	3/3
1000	3/3	2/3	1/3	3/3	2/3	3/3	3/3
100	1/3	0/3	0/3	0/3	0/3	0/3	0/3
10	0/3	0/3	0/3	0/3	0/3	0/3	0/3

a. The best hit for each resistance gene was used.

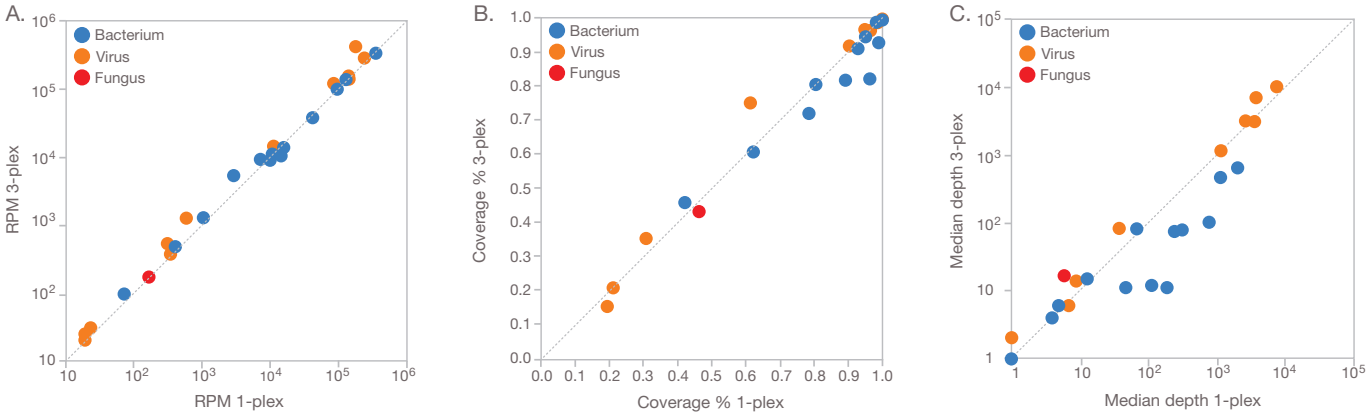

Figure 5: Consistent detection of respiratory pathogens at different plexities—All respiratory pathogens detected at 1-plex were also detected at 3-plex enrichment. Performance metrics, including (A) RPM, (B) coverage %, and (C) median depth were highly consistent between plexities.

Summary

The identification and characterization of respiratory pathogens is central to improving public health. NGS is a powerful method for simultaneous, broad-range detection of multiple infectious agents. Combining Illumina RNA Prep with Enrichment and the Respiratory Pathogen ID/AMR Panel with MiniSeq Rapid sequencing and Explify RPIP Data Analysis enables detection of hundreds of DNA and RNA respiratory pathogens in a single assay. By including AMR markers, the panel analyzes the expression of resistance genes to widely used antibiotics by common bacterial pathogens. Together, this sample-to-results workflow provides rapid, accurate, and broad-spectrum respiratory pathogen detection and characterization.

Learn more

Respiratory Pathogen ID/AMR Panel

Explify RPIP Data Analysis on BaseSpace Sequence Hub

Using NGS for infectious disease detection

Target enrichment detection of respiratory viruses with the MiniSeq Rapid Reagent Kit

MiniSeq Sequencing System

Illumina RNA Prep with Enrichment

References

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