



Targeted Sequencing of Respiratory Viruses in Clinical Specimens for Pathogen Identification and Genome-Wide Analysis

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Abstract

A large number of viruses can individually and concurrently cause various respiratory illnesses. Metagenomic sequencing using next-generation sequencing (NGS) technology is capable of identifying a variety of pathogens. Here, we describe a method using a large panel of oligo probes to enrich sequence targets of 34 respiratory DNA and RNA viruses that reduces non-viral reads in NGS data and achieves high performance of sequencing-based pathogen identification. The approach can be applied to total nucleic acids purified from respiratory swabs stored in viral transport medium. Illumina TruSeq RNA Access Library procedure is used in targeted sequencing of respiratory viruses. The samples are subjected to RNA fragmentation, random reverse transcription, random PCR amplification, and ligation with barcoded library adaptors. The libraries are pooled and subjected to two rounds of enrichments by using a large panel of oligos designed to capture whole genomes of 34 respiratory viruses. The enriched libraries are amplified and sequenced using Illumina MiSeq sequencing system and reagents. This method can achieve viral detection sensitivity comparable with molecular assay and obtain partial to complete genome sequences for each virus to allow accurate genotyping and variant analysis.

Key words Targeted sequencing, Next-generation sequencing, Genotyping, Respiratory disease, Respiratory virus, Viral enrichment, Pathogen discovery, Virome

1 Introduction

In targeted DNA or RNA sequencing, a panel of oligonucleotide probes are used to capture nucleotide contents containing interested sequences, the enriched targets are sequenced using next-generation sequencing (NGS) to achieve sensitive detection and sequence-based analysis [1]. Several methods with comparable performance had been developed and successfully applied to

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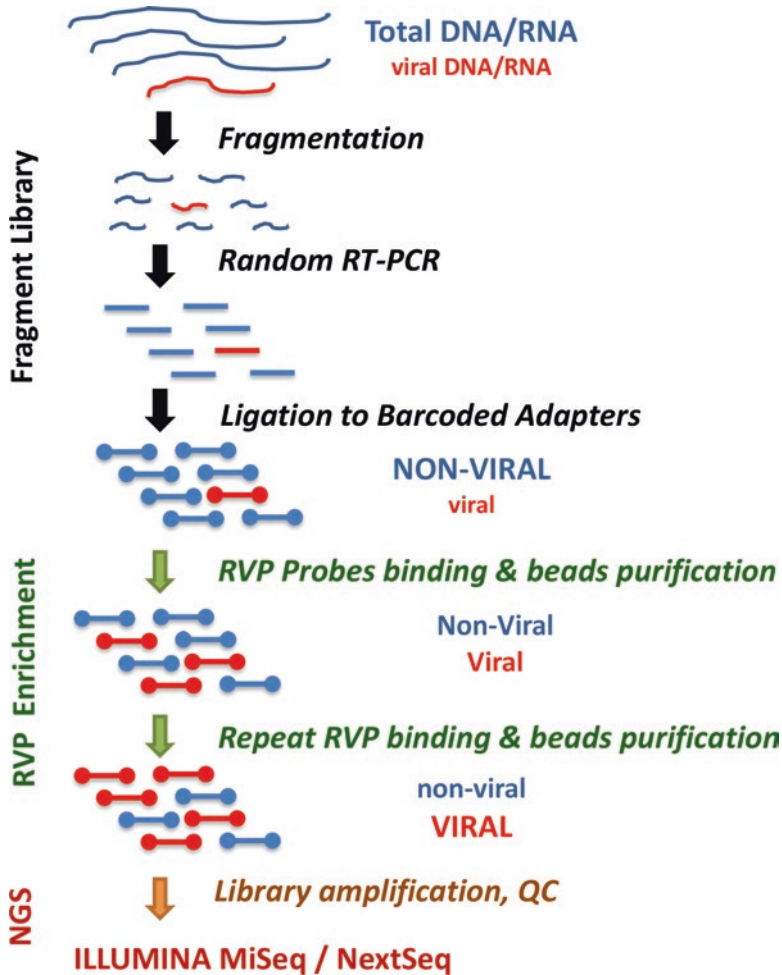


Fig. 1 Outline of the targeted sequencing of respiratory viruses in total nucleic acids from clinical specimens. The method uses TruSeq RNA Access Library Prep protocol and custom RVP oligos for genome-wide capture of 34 human respiratory viruses for next-generation sequencing (NGS)-based detections

sequencing of exomes, transcriptomes, cancer genes, significant pathogens, etc. [2–8]. Utilizing TruSeq RNA Access protocol and a large panel of oligos for whole-genome capture of 34 human respiratory viral pathogens (TruSeq RVP) enables enriching sequences of respiratory DNA and RNA viruses out of complex clinical specimens and production of genome sequences for genotyping and genetic variant analysis. The process includes collection, transportation, and storage of respiratory specimens, extraction of DNA and RNA, preparation of TruSeq fragment library, RVP enrichment, NGS and sequence data analysis (Fig. 1). The laboratory procedures require approximately 5 days from specimens to NGS run excluding specimen acquisition, NGS instrument run time and post-run data processing and analyses.

The method is expected to detect all commonly known respiratory viral pathogens that are diagnosed with molecular tests such as Luminex xTAG RVP assay, FilmArray Respiratory Panel (RP) tests, multiplex real-time PCR tests for respiratory viral infection with comparable sensitivity and accuracy [9–11]. The large capture panel and the scheme of genome-wide capture allow detection of most known respiratory viruses and viruses with high sequence divergences. The method is robust in handling specimens with viruses of a wide range of concentrations as well as specimens of compromised quality, e.g., degraded nucleic acids or high non-viral contents. The high capture efficiency and the superior sensitivity from this method make it a powerful tool for discovery of respiratory virome. However, there is an elevated risk of false positivity which demands more stringent contamination control procedure for the investigation of clinical specimens (*see Note 1*).

2 Materials

Perform the procedures in a Biosafety Level 2 (BSL-2) laboratory with a certified biosafety cabinet (BSC). Use clinical specimens and agents that can cause infection within the biosafety cabinet.

2.1 Nucleic Acids from Respiratory Specimens

Extract and purify DNA and RNA contents from respiratory swabs stored in a viral transport medium. Divide purified DNA/RNA into small aliquots, e.g., 10 μL per vial and store at $-80\text{ }^{\circ}\text{C}$ (*see Note 2*).

2.2 Library Preparation

1. Illumina TruSeq RNA Access Library Prep Kit (*see Note 3*). In this protocol, Coding Exome Oligos provided with the TruSeq RNA Access Library Prep kit are replaced with custom RVP oligos (*see* Supplementary file RVP capture probes for oligo sequences). The RVP oligos were designed to target whole genomes of 34 human respiratory viral pathogens (Table 1).
2. Illumina PhiX control V3 kit.
3. SuperScript III Reverse Transcriptase (200 U/ μL) (*see Note 4*).
4. Agencourt AMPure XP beads. Store at $4\text{ }^{\circ}\text{C}$. Before use, equilibrate the beads at room temperature for 30 min, vortex vigorously for 1 min to thoroughly suspend the beads.
5. Nuclease-free water.
6. 80% ethanol (EtOH): Prepare fresh 80% EtOH and use within a day.
7. 0.2 M Tris-HCl, pH 7.0.
8. 10 nM Tris-HCl, pH 8.5 with 0.1% Tween 20.

Table 1
Respiratory viruses targeted by TruSeq RVP RNA access sequencing

Respiratory virus (34)	Genome size	Type	Target genome sequences
Respiratory syncytial virus B (S2)	15,190	(-)ssRNA	NC_001803.1
Respiratory syncytial virus A	15,225	(-)ssRNA	AY353550
Influenza A (H9N2)	13,500	(-)ssRNA	NC_004910.1 NC_004911.1 NC_004912.1 NC_004908.1 NC_004905.2 NC_004909.1 NC_004907.1 NC_004906.1
Influenza A (H2N2)	13,460	(-)ssRNA	NC_007378.1 NC_007375.1 NC_007376.1 NC_007374.1 NC_007381.1 NC_007382.1 NC_007377.1 NC_007380.1
Influenza A (H3N2)	13,630	(-)ssRNA	NC_007373.1 NC_007372.1 NC_007371.1 NC_007366.1 NC_007369.1 NC_007368.1 NC_007367.1 NC_007370.1
Influenza A (H1N1)	13,590	(-)ssRNA	NC_002023.1 NC_002021.1 NC_002022.1 NC_002017.1 NC_002019.1 NC_002018.1 NC_002016.1 NC_002020.1
Influenza A (H5N1)	13,590	(-)ssRNA	NC_007357.1 NC_007358.1 NC_007359.1 NC_007362.1 NC_007360.1 NC_007361.1 NC_007363.1 NC_007364.1
Influenza A (H7N9)	13,590	(-)ssRNA	KC885955 KC885956 KC885957 KC885958 KC885959 KC885960 KC885961 KC885962
Influenza B	14,450	(-)ssRNA	NC_002204.1 NC_002205.1 NC_002206.1 NC_002207.1 NC_002208.1 NC_002209.1 NC_002210.1 NC_002211.1
Parainfluenza 1	15,600	(-)ssRNA	NC_003461.1
Parainfluenza 2	15,650	(-)ssRNA	NC_003443.1
Parainfluenza 3	15,460	(-)ssRNA	NC_001796.2
Parainfluenza 4	17,050	(-)ssRNA	NC_021928.1
Human metapneumovirus	13,340	(-)ssRNA	NC_004148.2
Adenovirus C	35,937	dsDNA	NC_001405.1
Adenovirus B	35,343	dsDNA	NC_011203.1

(continued)

Table 1
(continued)

Respiratory virus (34)	Genome size	Type	Target genome sequences
Adenovirus E	35,994	dsDNA	NC_003266.2
Human coronavirus HKU1	29,930	(+)ssRNA	NC_006577.2
Human coronavirus NL63	27,550	(+)ssRNA	NC_005831.2
Human coronavirus 229E	27,320	(+)ssRNA	NC_002645.1
Human coronavirus OC43	30,738	(+)ssRNA	AY391777.1
Rhinovirus A	7150	(+)ssRNA	NC_001617.1
Rhinovirus C	7100	(+)ssRNA	NC_009996.1
Rhinovirus B14	7210	(+)ssRNA	NC_001490.1
Human Bocavirus 1	5299	ssDNA	NC_007455.1
Human Bocavirus 2	5196	ssDNA	NC_012042.1
Human Bocavirus 3	5242	ssDNA	NC_012564.1
Human Bocavirus 4	5104	ssDNA	NC_012729.2
KI polyomavirus	5040	dsDNA	NC_009238.1
WU polyomavirus	5229	dsDNA	NC_009539.1
HPeV type 1	7296	(+)ssRNA	FM242866.1
HPeV type 6	7347	(+)ssRNA	AB252582.1
Human enterovirus C104	7408	(+)ssRNA	AB686524.1
Human enterovirus C109	7354	(+)ssRNA	GQ865517.1

9. Microplate sealing film: Use one sheet to seal a 96-well microplate. Discard the seal after the removal from the plate. Use a new sheet to reseal the plate if needed.
10. Vortex mixer for small volumes in 96-well microplates and microcentrifuge tubes: Control speed and duration to obtain uniform mixing of solutions in microcentrifuge tubes or microplates.

11. Agilent 2100 Bioanalyzer, DNA 1000 (or 7500) chips and reagents. Store DNA chips at room temperature. Store the reagents at -20°C . Before use equilibrate the reagents at room temperature for 30 min.
12. Magnetic beads separation stand for a 96-well microplate, magnetic beads separation rack for tubes.
13. Centrifuges: benchtop centrifuge with microplate carriers, microcentrifuge, mini centrifuge.
14. PCR Thermocycler for 96-well microplate.
15. Two water baths.
16. A set of pipettors with pipetting volumes from 2 μL to 1000 μL . One 20 μL 8-channel pipettor.

2.3 NGS Run Using MiSeq or NextSeq System

1. 0.5% Tween 20.
2. Illumina MiSeq Reagent kit v3 or v2 (*see Note 5*).
3. Molecular biology grade water.
4. Illumina MiSeq or NextSeq sequencing system.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Brief centrifugation or quick spin is done at $280 \times g$ for 1 min in a benchtop centrifuge with microplate carriers for 96-well microplate or by a touch-spin using a microcentrifuge or a mini-centrifuge for microcentrifuge tubes. Thaw frozen reagents at room temperature completely, vortex and centrifuge briefly, then keep them at room temperature or on ice until use. Immediately return all reagents to their original storage condition after use except for Resuspension Buffer which is kept at 4°C after the initial use.

3.1 TruSeq RVP RNA Access Library Preparation

3.1.1 Fragment RNA

1. Label a clean 96-well microplate as DFP, add 8.5 μL of Elute/Prime/Fragment High Mix and 8.5 μL of RNA samples (*see Note 6*) into each well, seal and shake the DFP plate at 1,600 rpm for 20 s.
2. Put the DFP plate in a thermocycler for incubation, using parameters: lid temperature at 100°C , incubation at 94°C for 8 min and then at 4°C holding.
3. Remove the DFP plate from thermocycler when it reaches 4°C , centrifuge, then proceed to **step 3.1.2** immediately.

3.1.2 Synthesize First Strand cDNA

1. Remove the seal from the DFP plate, add 7.2 μL of First Strand Synthesis Act D Mix and 0.8 μL of SuperScript III Reverse Transcriptase into each well, seal and shake the plate at 1,600 rpm for 20 s.

2. Put the DFP plate in the thermocycler for incubation, using parameters: lid temperature at 100 °C, incubation at 25 °C for 10 min, 42 °C for 15 min, 70 °C for 15 min, and finally at 4 °C holding.
3. Remove the DFP plate from the thermocycler when it reaches 4 °C, centrifuge, then proceed to **step 3.1.3** immediately.

3.1.3 Synthesize Second Strand cDNA

1. Remove the seal from the DFP plate, add 5 µL of Resuspension Buffer and 20 µL of Second Strand Marking Master Mix into each well, seal and shake the plate at 1,600 rpm for 20 s.
2. Put the DFP plate in the thermocycler for incubation, using parameters: lid temperature at 30 °C, incubation at 16 °C for 60 min and then at 24 °C for 2 min.
3. Immediately following the incubation, remove the DFP plate from thermocycler, remove the seal, add 90 µL of well-mixed AMPure XP beads to each well, seal and shake the plate at 1,800 rpm for 2 min.
Follow **steps 4–10** in Subheading **3** for AMPure XP beads binding and washing.
4. Incubate the plate at room temperature for 5 min.
5. Centrifuge the plate briefly, place the plate on a magnet stand for 5 min.
6. Remove the seal from the plate, discard all supernatants from each well.
7. With the plate on the magnet stand, add 200 µL of 80% EtOH to each well.
8. Incubate the plate at room temperature for 30 s, then remove all supernatants.
9. Repeat **steps 7** and **8** once. Using a 20 µL multi-channel pipettor, carefully remove the remaining 80% EtOH from the wells.
10. Keep the plate stand at room temperature for 5 min to allow the EtOH to evaporate, then remove the plate from the magnet stand.
11. Add 17.5 µL of Resuspension Buffer into each well of the DFP plate, seal and shake the plate at 1,900 rpm for 2 min.
12. Incubate the DFP plate at room temperature for 2 min.
13. Centrifuge the DFP plate, and then place the plate on the magnet stand for 5 min.
14. With the DFP plate on the magnet stand, transfer 15 µL of clear supernatant from each well of the DFP plate to a new 96-well microplate labeled as ALP, without touching the magnetic beads. Seal the ALP plate. Discard the DFP plate.
15. Store the ALP plate at –20 °C and continue to **step 3.1.4** within 7 days.

3.1.4 *Adenylate 3'End*

If the ALP plate has been stored at $-20\text{ }^{\circ}\text{C}$, thaw it at room temperature. Set up two water baths, one at $37\text{ }^{\circ}\text{C}$, the other at $70\text{ }^{\circ}\text{C}$.

1. Centrifuge the ALP plate. Remove the seal from the plate, add $2.5\text{ }\mu\text{L}$ of Resuspension Buffer and $12.5\text{ }\mu\text{L}$ of A-Tailing Mix to each well, seal the plate and shake it at $1,800\text{ rpm}$ for 2 min.
2. Incubate the ALP plate at $37\text{ }^{\circ}\text{C}$ for 30 min, and then immediately transfer the plate to $70\text{ }^{\circ}\text{C}$ and incubate for 5 min. After incubation, immediately place the plate on ice for at least 1 min.

3.1.5 *Ligate Adapters*

1. Remove the seal from the ALP plate, add $2.5\text{ }\mu\text{L}$ of Resuspension Buffer, $2.5\text{ }\mu\text{L}$ of Ligation Mix, and $2.5\text{ }\mu\text{L}$ of RNA Adapter Index to each well, seal and shake the plate at $1,800\text{ rpm}$ for 2 min, and then centrifuge the ALP plate.
2. Incubate the ALP plate at $30\text{ }^{\circ}\text{C}$ for 10 min in a water bath, then move the plate to room temperature, and keep for 5 min to allow the ALP plate temperature drop to room temperature before proceeding to the next step.
3. Remove the seal from the ALP plate, add $5\text{ }\mu\text{L}$ of Stop Ligation Buffer to each well, seal and shake the plate at $1,800\text{ rpm}$ for 2 min, then centrifuge the plate.
4. Remove the seal from the ALP plate, add $42\text{ }\mu\text{L}$ of well-mixed AMPure XP beads to each well of the ALP plate, seal and shake the ALP plate at $1,800\text{ rpm}$ for 2 min.
5. Follow 3.1.3. **steps 4 to 10** for AMPure XP beads binding and washing.
6. Add $52.5\text{ }\mu\text{L}$ of Resuspension Buffer into each well of the ALP plate, seal and shake the ALP plate at $1,800\text{ rpm}$ for 2 min.
7. Incubate the ALP plate at room temperature for 2 min.
8. Centrifuge the ALP plate, remove the seal of the ALP plate, then place the plate on the magnet stand for 5 min.
9. Carefully transfer $50\text{ }\mu\text{L}$ of clear supernatant from each well of the ALP plate to a new 96-well microplate labeled as CAP. Discard the ALP plate.
10. Add $50\text{ }\mu\text{L}$ of well-mixed AMPure XP beads to each well of the CAP plate, seal and shake at $1,800\text{ rpm}$ for 2 min.
11. Follow 3.1.3. **steps 4 to 10** for AMPure XP beads binding and washing.
12. Add $22.5\text{ }\mu\text{L}$ of Resuspension Buffer into each well of the CAP plate, seal and shake the plate at $1,900\text{ rpm}$ for 2 min.
13. Incubate the CAP plate at room temperature for 2 min.
14. Centrifuge the CAP plate, remove the seal from the CAP plate, then place the plate on the magnet stand for 5 min.
15. Transfer $20\text{ }\mu\text{L}$ of clear supernatant from each well of the CAP plate to a new 96-well microplate labeled as PCR.

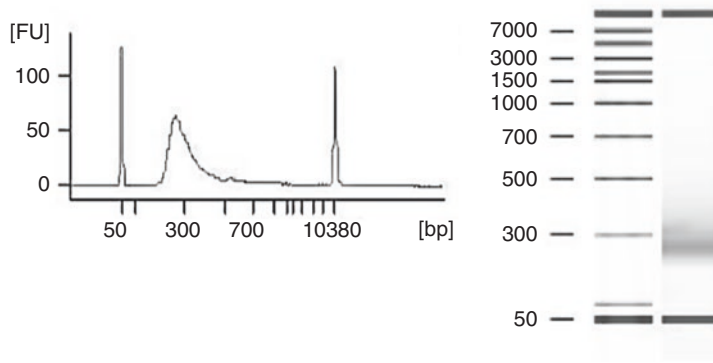


Fig. 2 Analysis of fragment library using Agilent 2100 Bioanalyzer and DNA 7500 kit

3.1.6 First PCR Amplification

1. Add 5 μL of PCR Primer Cocktail and 25 μL of PCR Master Mix to each well of the PCR plate.
2. Seal the PCR plate and shake at 1,600 rpm for 20 s. Centrifuge the PCR plate.
3. Put the PCR plate in a thermocycler for PCR amplification, using parameters: lid temperature at 100 $^{\circ}\text{C}$, 98 $^{\circ}\text{C}$ for 30 s, then 15 cycles of 98 $^{\circ}\text{C}$ for 10 s, 60 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 30 s, followed by 72 $^{\circ}\text{C}$ for 5 min and finally at 4 $^{\circ}\text{C}$ holding.
4. Remove the PCR plate from the thermocycler, centrifuge the plate, remove the seal, add 50 μL of well-mixed AMPure XP beads to each well, seal and shake the plate at 1,800 rpm for 2 min.
5. Follow 3.1.3. **steps 4 to 10** for AMPure XP beads binding and washing.
6. Add 17.5 μL of Resuspension Buffer into each well of the PCR plate, seal and shake the plate at 1,900 rpm for 2 min.
7. Incubate the PCR plate at room temperature for 2 min.
8. Centrifuge the PCR plate, remove the seal, then place the plate on the magnet stand for 5 min.
9. Transfer 15 μL of clear supernatant from each well of the PCR plate to a new 96-well microplate labeled as TSP1. Discard the PCR plate.

3.1.7 Quality Examination of the Library

Use 1 μL of each library from the TSP1 plate to measure DNA concentration and size distribution on Agilent 2100 Bioanalyzer with DNA 1000 (or 7500) kit (*see Note 7*). Determine DNA concentration in $\text{ng}/\mu\text{L}$ for each library. The expected size distribution for the library is 200–500 bp with apparent peak at approximately 260 bp (Fig. 2).

3.1.8 First Hybridization

1. Combine up to four libraries (200 ng of DNA for each library) to one pool. If the total volume of a pool is greater than 45 μL , use a centrifugal ultrafiltration unit with a molecular weight cutoff of 30 kDa to concentrate the mixture. Bring the total volume to 45 μL with Resuspension Buffer if a pool volume is less than 45 μL . Save the library pools in a new 96-well microplate labeled as RAH1.
2. Add 50 μL of Capture Target Buffer 3 and 5 μL of the RVP oligos to each pool of the RAH1 plate.
3. Seal and shake the plate at 1,200 rpm for 1 min, then centrifuge the plate.
4. Put the plate in a thermocycler for incubation, using parameters: lid temperature at 100 $^{\circ}\text{C}$, incubation at 95 $^{\circ}\text{C}$ for 10 min, 18 steps of decreasing temperatures starting at 94 $^{\circ}\text{C}$, decreased by 2 $^{\circ}\text{C}$ at each step and kept for 1 min at each temperature, then final incubation at 58 $^{\circ}\text{C}$ for 90 min (*see Note 8*).
5. During the incubation, prepare Pre-Mixed Elution in a microcentrifuge tube (to be used in 3.1.9. **step 7**): mix 28.5 μL of Enrichment Elution Buffer 1 and 1.5 μL of 2 N NaOH for each pool, and keep at room temperature until use.
6. Once the incubation is over, immediately remove the plate from the thermocycler and proceed to **step 3.1.9**.

3.1.9 First Capture

1. Centrifuge the RAH1 plate, remove the seal, and transfer the first pool content to a clean 1.5 mL microcentrifuge tube labeled as S1, the second pool content to tube S2, and so on (*see Note 9*). Discard the RAH1 plate.
2. Add 250 μL of well-mixed Streptavidin magnetic beads to each S tube. Shake S tubes at 1,200 rpm for 5 min. Incubate at room temperature for 25 min. Centrifuge S tubes briefly, then put S tubes in a magnet rack for 2 min. Discard all supernatants with all S tubes on the magnet rack.
3. Remove all S tubes from the magnet rack, add 200 μL of Enrichment Wash Solution, shake at 1,800 rpm for 4 min.
4. Heat S tubes in a 50 $^{\circ}\text{C}$ water bath for 20 min. Then put S tubes on the magnet rack for 2 min. Remove supernatants with S tubes in the magnet rack.
5. Remove all S tubes from the magnet rack, add 200 μL of Enrichment Wash Solution, shake at 1,800 rpm for 4 min. Quick spin, then transfer the mixture from each S tube into a new 96-well microplate labeled as RAW1. Seal the RAW1 plate. Discard S tubes.
6. Incubate the RAW1 plate at the 50 $^{\circ}\text{C}$ water bath for 20 min. Immediately put the plate on the magnet stand for 2 min, then remove the supernatants with the plate on the magnet stand.
7. Add 23 μL of Pre-Mixed Elution to each well on the RAW1 plate, seal the plate and shake at 1,800 rpm for 2 min, incubate

the plate at room temperature for 2 min, centrifuge and place the plate on the magnet stand for 2 min.

8. Transfer 21 μL of the clear supernatant from each well of the RAW1 plate to a new 96-well microplate labeled as RAH2. Discard the RAW1 plate.
9. Add 4 μL of Elute Target Buffer 2 to each well of the RAH2 plate to neutralize the elution.
10. Seal the RAH2 plate, shake at 1,800 rpm for 1 min, and then centrifuge the plate.

3.1.10 *Second Hybridization*

1. Remove seal from the RAH2 plate, add 20 μL of Resuspension Buffer, 50 μL of Capture Target Buffer 3 and 5 μL of RVP oligos into each well.
2. Follow 3.1.8. **steps 3 to 6** for hybridization, then proceed immediately to **step 3.1.11**.

3.1.11 *Second Capture*

1. Centrifuge the RAH2 plate, remove the seal and transfer the contents from each well to clean 1.5 mL tubes labeled as S1, S2, and so on. Discard the RAH2 plate.
2. Follow 3.1.9. **steps of 2 to 10** for capture, except that the new microplates are labeled as RAW2 and RAC1 respectively.
3. Remove the seal from the RAC1 plate, add 45 μL of well-mixed AMPure XP beads to each well, seal and shake the plate at 1,800 rpm for 1 min.
4. Follow 3.1.3. **steps 4 to 10** for AMPure XP beads binding and washing.
5. Add 27.5 μL of Resuspension Buffer into each well, seal and shake the RAC1 plate at 1,900 rpm for 1 min.
6. Keep the RAC1 plate at room temperature for 2 min.
7. Centrifuge the RAC1 plate, remove the seal and place the plate on the magnet stand for 2 min.
8. Transfer 25 μL of the clear supernatant from each well of the RAC1 plate to a new 96-well microplate labeled as RAA. Seal the RAA plate. Discard the RAC1 plate.
9. Store the RAA plate at $-20\text{ }^{\circ}\text{C}$ and continue within a week.

3.1.12 *Second PCR Amplification and Cleanup*

1. Thaw the RAA plate, centrifuge the plate, remove the seal, add 5 μL of PCR Primer Cocktail and 20 μL of Enhanced PCR Mix. Seal the plate, shake at 1,200 rpm for 1 min, and then centrifuge the plate.
2. Put the RAA plate in thermocycler for PCR amplification, using parameters: lid temperature at $100\text{ }^{\circ}\text{C}$, $98\text{ }^{\circ}\text{C}$ for 30 s, 17 cycles of $98\text{ }^{\circ}\text{C}$ for 10 s, $60\text{ }^{\circ}\text{C}$ for 30 s and $72\text{ }^{\circ}\text{C}$ for 30 s, followed by $72\text{ }^{\circ}\text{C}$ for 5 min and finally at $4\text{ }^{\circ}\text{C}$ holding.

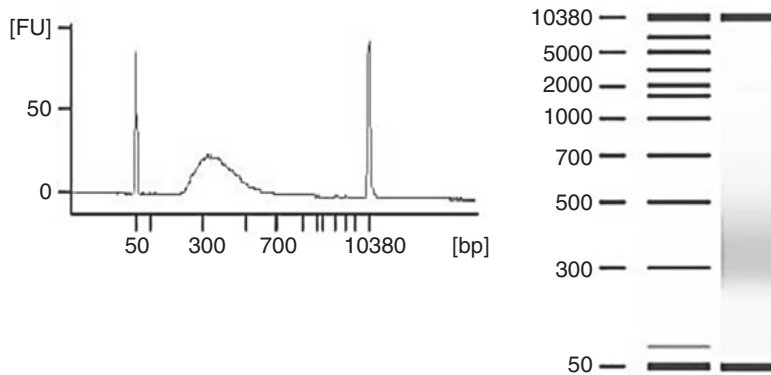


Fig. 3 Analysis of post-enrichment TruSeq RVP RNA Access library using Agilent 2100 Bioanalyzer and DNA 7500 kit

3. Remove the seal of the RAA plate, add 90 μL of well-mixed AMPure XP beads to each pool, seal and shake the plate at 1,800 rpm for 1 min.
4. Follow 3.1.3. **steps 4 to 10** for AMPure XP beads binding and washing.
5. Add 32 μL of Resuspension Buffer into each well of the RAA plate, seal and shake the plate at 1,900 rpm for 1 min.
6. Leave the RAA plate at room temperature for 2 min.
7. Centrifuge the RAA plate, remove the seal, and then place the plate on the magnet stand for 2 min.
8. Transfer 30 μL of clear supernatant from each well of the RAA plate to a new 96-well microplate labeled as RAL. Discard the RAA plate.

3.1.13 Quality Examination of the Final Library

Use 1 μL of the final library in the RAL plate to measure DNA concentration and size distribution on Agilent 2100 Bioanalyzer with DNA 1000 (or 7500) kit. Determine DNA concentration in nM for each pool. The expected size distribution for the library is 200–500 bp with apparent peak at approximately 300 bp (Fig. 3) (*see Note 10*).

3.1.14 Dilution and Denaturation of the Libraries for NGS

The enriched libraries can be sequenced on Illumina MiSeq or NextSeq systems. The loading concentration for libraries on MiSeq is 6–15 pM for reagents kit v2 or v3. We use loading concentration in the range of 8–14 pM for sequencing with MiSeq and reagents kit v3.

1. Dilute each library in the RAL plate from the concentration determined with Agilent 2100 Bioanalyzer to 1 nM with Resuspension Buffer. Prepare 22 μL of 1 nM of each library and pool all of them together into a new 1.5 mL tube labeled as 1 nM lib pool mix. Mix the tube at 1,200 rpm for 30 s and centrifuge. Keep the tube on ice until use. Store the RAL plate at $-20\text{ }^{\circ}\text{C}$.

2. Prepare fresh 0.2 N NaOH by adding 200 μL of 1 N NaOH into 800 μL of nuclease-free water in a clean 1.5 mL tube labeled as 0.2 N NaOH. Mix the tube at 1,200 rpm for 30 s and centrifuge.
3. In a new 1.5 mL tube labeled as 20 pM lib pool mix, add 20 μL of 1 nM lib pool mix and 20 μL of 0.2 N NaOH. Mix the tube at 1,200 rpm for 30 s and centrifuge. Keep it at room temperature for 5 min. Add 20 μL of 0.2 M Tris-HCl, pH 7.0 into the tube. Mix the tube at 1,200 rpm for 30 s and centrifuge. Add 940 μL of prechilled HT1. Mix the tube at 1,200 rpm for 30 s and centrifuge. Keep the 20 pM lib pool mix tube on ice. Store the 1 nM lib pool mix tube at -20°C .
4. In a new 1.5 mL tube labeled as loading conc. Pool, dilute the 20 pM denatured pool mix with prechilled HT1 using following chart as guide. Keep the loading conc. Pool tube on ice.

Loading concentration (pM)	6	8	10	12	14	20
20 pM lib pool mix (μL)	180	240	300	360	420	600
Pre-chilled HT1 (μL)	420	360	300	240	180	0

5. In a clean 1.5 mL tube labeled as 20 pM PhiX control (*see Note 11*), add 2 μL of 10 nM PhiX library, 3 μL of 10 nM Tris-Cl, pH 8.5 with 0.1% Tween 20, and 5 μL of 0.2 N NaOH. Mix the tube at 1,200 rpm for 30 s and centrifuge. Incubate at room temperature for 5 min. Add 990 μL of prechilled HT1. Mix the tube at 1,200 rpm for 30 s and centrifuge. Keep this tube on ice.
6. Add 6 μL of 20 pM PhiX control into 600 μL of the loading conc. pool tube. Mix the tube at 1,200 rpm for 30 s and centrifuge. Keep the tubes on ice until NGS run. (The mixture inside of the tube will be loaded into the reagent cartridge for sequencing run).

3.2 NGS and Sequence-Based Detection of Respiratory Pathogens

Sequence the lib pool with PhiX control using Illumina MiSeq or NextSeq system. The acquired sequence data can be applied to bioinformatics data analysis pipeline, which may include data quality processing, the removal of human genome and transcriptome sequences, de novo assembly, and comparison of sequence contigs and unassembled reads with sequence databases using BLAST programs [12] (*see Note 12*).

4 Notes

1. The method we described in this chapter is for research use only. It is not validated for clinical diagnosis purpose. The potential contamination and cross-contamination can come

from specimen collection, shipping and handling, storage, laboratory surfaces and instruments, reagents, viral cultures, nucleic acids or PCR amplification products, etc. Conduct the experiments in a clean laboratory room where high titer cultures, high concentration nucleic acids and PCR amplifications are not present. Clean frequently used instruments and devices, e.g., centrifuge, thermocycler, mixer, pipettors, on a regular basis to prevent accumulative contaminations and before each experiment to minimize carry-over of nucleic acids from the previous run. Use newly opened consumable materials and freshly prepared reagents. We highly recommend dividing original specimens, DNA/RNA extracts, and stock solutions into small aliquots or fractions to avoid accumulative contamination and for independent repeating of the test when trouble-shooting or verification of results is needed.

2. Respiratory swabs can be stored in transport medium such as Copan Universal Transport Medium (UTM) (Murrieta, CA, USA) or PrimeStore Molecular Transport Medium (MTM) (Longhorn Vaccines and Diagnostics LLC, Bethesda, MD, USA). We use QIAGEN QIAamp Viral RNA Mini Kit in DNA/RNA preparation. Carrier RNA is used in DNA/RNA extraction with QIAamp Viral RNA kit to increase RNA yield and protect purified RNA from degradation. Store RNA extracts in small aliquots and minimize freeze thaw cycles. Use RNase-decontamination products, for example, RNase AWAY made by Molecular Bio-Products, Inc., to clean the surface of the hood, benches, instruments, etc. When handle RNA samples, always wear gloves and change them often. During the procedure, work quickly and keep tubes closed and plates covered.
3. Most reagents used in this method are parts in Illumina TruSeq RNA Access Library Prep Kit. The user's guide that we used with modification is RS-301-9001DOC Part# 15049525 Rev. B. The plate names in the protocol are DFP (Depleted RNA Fragmentation Plate), ALP (Adaptor Ligation Plate), CAP (Clean up ALP Plate), RAH1 (RNA Access Hybridization 1), RAW1 (RNA Access Wash 1), RAH2 (RNA Access Hybridization 2), RAW2 (RNA Access Wash 2), RAC1 (RNA Access Clean-up 1), RAA (RNA Access Amplification), TSP1 (Target Sample Plate 1) and RAL (RNA Access Library).
4. SuperScript II Reverse Transcriptase and the new SuperScript IV Reverse Transcriptase can also be used. SuperScript III Reverse Transcriptase is the most often used reverse transcriptase. It is advised that SuperScript IV can obtain good yield and length of cDNA synthesis with improvements on thermal stability, speed, and resistance to inhibitors.
5. The TruSeq RNA Access library has size range around 300 bp. Both Illumina MiSeq Reagent kits v2 and v3, which can read

150 bp and 300 bp from either end, can be used in sequencing the libraries.

6. The TruSeq RNA Access Library guide recommends using 10–100 ng of RNA as starting materials. Since total nucleic acids extracted from respiratory specimens contain both DNA and RNA and have highly variable concentrations from specimen to specimen, we use 8.5 μ L of DNA/RNA for each sample in RNA fragmentation.
7. Agilent 2100 Bioanalyzer can analyze up to 12 samples in one run. An alternative high-throughput system, the Agilent TapeStation 4200, can analyze up to 96 samples in one run.
8. The total hybridization time is about 2 h. Over hybridization may cause a high degree of nonspecific binding.
9. Instead of using microcentrifuge tubes and a magnetic beads separation rack, a 0.8 mL 96 deep well plate and a magnetic beads separation stand for 96-well plate can be used to process a large number of samples.
10. After two rounds of hybridization and capture and the second PCR amplification, the post-enrichment library appears having broadened size distribution with the peak shifted slightly to a higher molecular weight as compared with the fragment library shown in Fig. 2.
11. We always include Illumina PhiX Control v3 library in MiSeq runs. The PhiX control can be used for quick assessment of data quality and for technical trouble shooting. Besides, the addition of PhiX library can improve base calling quality for libraries with high G/C or A/T contents or low diversity sequences. The spiking ratio can be increased or reduced based on the needs and the nature of the specimens.
12. Using this method, partial to near-complete viral genome sequences can be obtained to allow genotyping and recombination and variant analysis. The method can detect both RNA and DNA viruses. During interpretation of the results, take cautious consideration of probable contamination and cross-contamination. If necessary, verify the results with additional tests such as molecular assays and/or by repeating the experiment with archived specimens or DNA/RNA aliquots.

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