

Primer ID Next-Generation Sequencing for the Analysis of a Broad Spectrum Antiviral Induced Transition Mutations and Errors Rates in a Coronavirus Genome

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[Abstract] Next generations sequencing (NGS) has become an important tool in biomedical research. The Primer ID approach combined with the MiSeq platform overcomes the limitation of PCR errors and reveals the true sampling depth of population sequencing, making it an ideal tool to study mutagenic effects of potential broad-spectrum antivirals on RNA viruses. In this report we describe a protocol using Primer ID sequencing to study the mutations induced by antivirals in a coronavirus genome from an *in vitro* cell culture model and an *in vivo* mouse model. Viral RNA or total lung tissue RNA is tagged with Primer ID-containing cDNA primers during the initial reverse transcription step, followed by two rounds of PCR to amplify viral sequences and incorporate sequencing adaptors. Purified and pooled libraries are sequenced using the MiSeq platform. Sequencing data are processed using the template consensus sequence (TCS) web-app. The Primer ID approach provides an accurate sequencing protocol to measure mutation error rates in viral RNA genomes and host mRNA. Sequencing results suggested that β -D-N₄-hydroxycytidine (NHC) greatly increased the transition substitution rate but not the transversion substitution rate in the viral RNA genomes, and cytosine (C) to uridine (U) was found as the most frequently seen mutation.

Keywords: Coronavirus, Antivirals, Next generation sequencing, Primer ID, Mutation

[Background] Next generation sequencing (NGS) has been extensively used in biomedical research for the last decade. When applying NGS to study intra-host viral populations for RNA viruses, modifications in library prep and sequencing protocols need to be considered. The virus titers (or viral loads) vary greatly from specimen to specimen. While conventional NGS platforms require 1-500 ng of DNA (or RNA) in a sequencing run, in most cases a clinical sample will have less than 100 fg of viral RNA, requiring that the viral RNA first be converted to cDNA using reverse transcriptase, followed by one or two rounds of PCR amplification to generate enough material for sequencing. However, the extensive cycles of PCR amplification will cause nucleotide mis-incorporation, recombination and amplification bias. Furthermore, the NGS platforms have relatively high error rates adding additional uncertainty to the resulting sequences (Liu *et al.*, 2012).

We have developed the Primer ID NGS approach to overcome the errors and bias from the conventional NGS approaches (Jabara *et al.*, 2011; Zhou *et al.*, 2015). During the initial cDNA synthesis step, we use cDNA primers with an embedded 11-base degenerate block of nucleotides (the Primer ID) to tag each viral RNA template with a unique ID, a version of the approach of adding a unique molecular identifier (UMI). The Primer ID is carried on throughout the downstream amplifications and sequencing. After sequencing, all raw sequences with the same ID are collapsed to make a template consensus sequence (TCS). Each TCS is linked to an original template that was queried in the initial cDNA synthesis step, and the number of TCS shows the sequencing sampling depth of the viral population. By the construction of consensus sequence from multiple raw sequencing reads for each viral template, we can greatly reduce the PCR and sequencing errors. We have shown that the Primer ID approach can greatly reduce the sequencing error to 1 in 10,000 nucleotides (100-fold reduction from the raw sequencing reads) and also reveal the true sampling depth of the viral population, making it possible to accurately measure the substitution rates and diversity in the viral genomes in a population (Figure 1) (Zhou *et al.*, 2015). We have further developed a multiplexing Primer ID approach, allowing the sequencing of multiple regions of the viral genome in a single cDNA synthesis/PCR (Figure 2) (Dennis *et al.*, 2018).

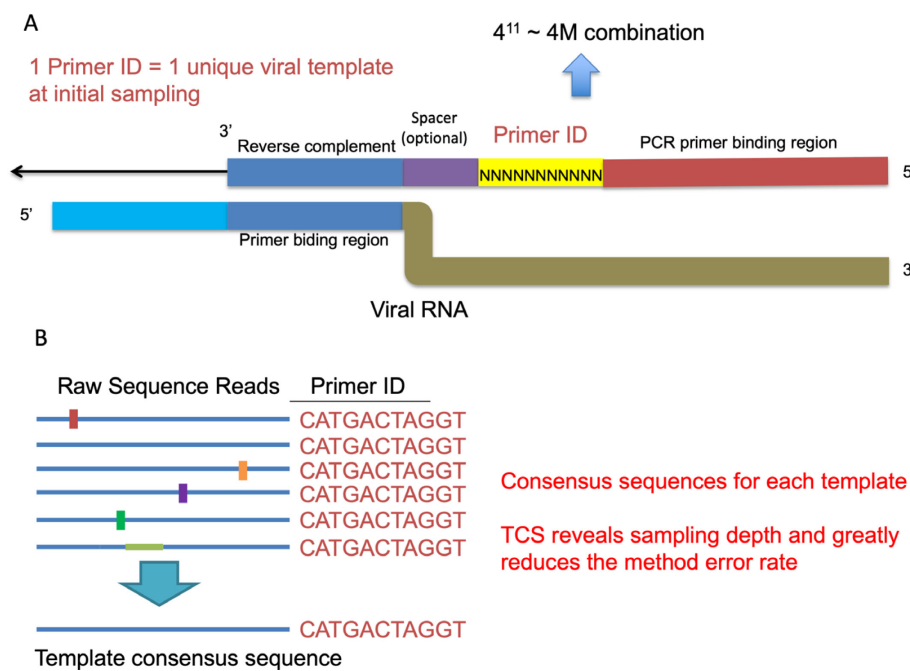


Figure 1. Primer ID Approach. A. The structure of the Primer ID primer and its binding to the viral RNA template. B. An example of creating the template consensus sequence from raw sequence reads.

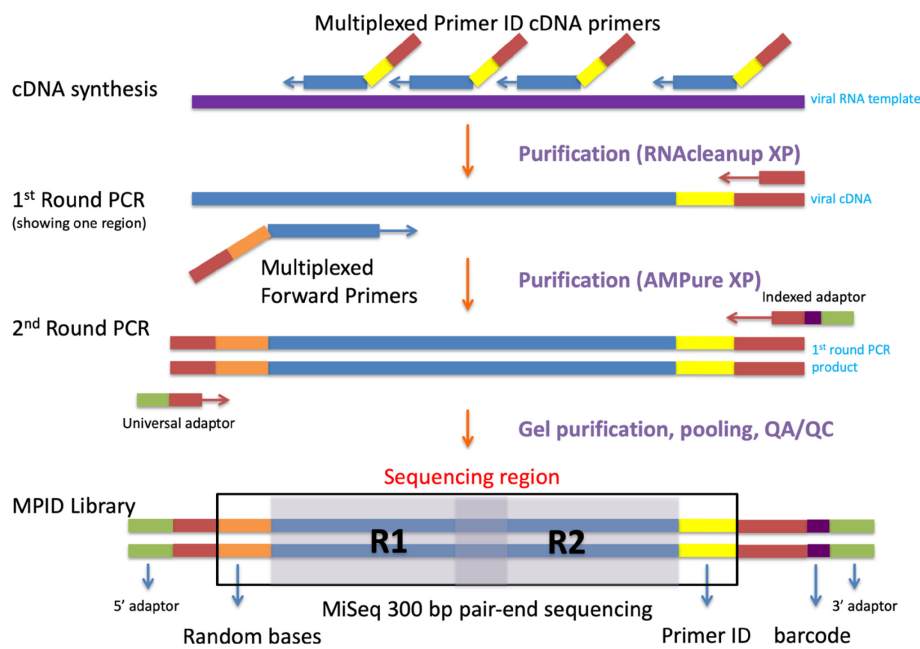


Figure 2. Library prep steps for the Multiplexed Primer ID sequencing. Biological sequence region in blue color, Primer ID region in yellow color, forward spacer region in orange, MiSeq barcode sequence region in purple.

This approach is ideal for studying intra-host RNA virus populations due to its ability to accurately characterize individual viral genomes. We have used this approach to determine the genetic structures of intra-host viral populations in HIV (Zhou *et al.*, 2016; Lee *et al.*, 2017; Dennis *et al.*, 2018; Abrahams *et al.*, 2019; Adewumi *et al.*, 2020; Council *et al.*, 2020), to study drug resistance mutations in HIV and HCV (Clutter *et al.*, 2017; Zhou *et al.*, 2018; Keys *et al.*, 2015), and to detect nucleotide mutation rates in single-stranded DNA molecule after heating (Lewis *et al.*, 2016).

In this report we describe a protocol using the Primer ID MiSeq sequencing to detect the mutagenic effect of EIDD-1931(β -D-N4-hydroxycytidine, NHC) and EIDD-2801 (an orally bioavailable prodrug of EIDD-1931 and is also recognized as MK-4482 or Molnupiravir) in the Middle East respiratory syndrome coronavirus (MERS-CoV) genome from *in vitro* cell culture and *in vivo* mouse models. In the cell culture sequencing experiment, we used a multiplexed Primer ID protocol to sequence multiple regions in the MERS-CoV ORF1b in a single library prep and sequencing reaction, while in the sequencing of total lung RNA from MERS-CoV infected mice, we used a multiplexed Primer ID protocol targeting both MERS-CoV genomes and two selected mouse mRNAs in a single library prep and sequencing reaction, in which we can directly compared the mutagenic effect induced by EIDD-2801 on MERS-CoV genomes and host mRNA. This protocol is not restricted to MERS-CoV and EIDD-1931 study only, but it can be used to detect viral RNA mutation rate in general.

Materials and Reagents

1. Pipette tips
2. Collection tubes (QIAGEN, catalog number: 19201, stored at room temperature)
3. Qubit Assay Tubes (ThermoFisher Scientific, Invitrogen, catalog number: Q32856, stored at room temperature)
4. SuperScript III One-Step RT-PCR System (ThermoFisher Scientific, Invitrogen, catalog number: 12574026, stored at -20 °C)
5. Primer ID primers (Integrated DNA Technologies) at 100 µM in Tris-HCl buffer stored at -20 °C
6. SuperScript III Reverse Transcriptase (ThermoFisher Scientific, Invitrogen, catalog number: 18080085, stored at -20 °C)
7. RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher Scientific, Invitrogen, catalog number: 10777019, stored at -20 °C)
8. Ribonuclease H (ThermoFisher Scientific, Invitrogen, catalog number: 18021071, stored at -20 °C)
9. KAPA2G Robust PCR kits (Hotstart PCR kits with dNTPs) (Roche, catalog number: KK5516, stored at -20 °C)
10. KAPA2G HiFi HotStart (with dNTPs) (Roche, catalog number: KK2502, stored at -20 °C)
11. RNAClean XP (Beckman Coulter, catalog number: A63987, stored at 4 °C)
12. AMPure XP (Beckman Coulter, catalog number: A63881, stored at 4 °C)
13. Ethanol 200 Proof (Decon Laboratories, catalog number: 2716)
14. DNase/RNase-free water
15. MinElute Gel Extraction kit (QIAGEN, catalog number: 28606, stored at 4 °C for MinElute columns, room temperature for other reagents)
16. Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Invitrogen, catalog number: Q32853, stored at 4 °C for standard 1 and standard 2, room temperature for other reagents)
17. Experion DNA 12K analysis kit (Bio-Rad, catalog number: 7007108, stored at room temperature for the chips, 4 °C for other reagents)
18. MiSeq Reagent Kit v3 (600-cycle) (Illumina, catalog number: MS-102-3003, stored at -20 °C for box 1 and 4 °C for box 2)
19. Sodium hydroxide (Sigma-Aldrich, catalog number: 221465, stored at room temperature)
20. Tris hydrochloride (Sigma-Aldrich, catalog number: 10812846001, stored at room temperature)
21. PhiX Control V3 (Illumina, catalog number: FC-110-3001, stored at -20 °C)
22. 10 mM Tris-HCl with 0.1% TWEEN 20 (Teknova, catalog number: T7724, stored at room temperature)

Equipment

1. Pipettes

2. Magnetic rack
3. Thermal cycler (ThermoFisher Scientific, Applied Biosystems, catalog number: 4384638)
4. Centrifuge (Eppendorf, model: 5424)
5. DynaMag-2 Magnet (ThermoFisher Scientific, Invitrogen, catalog number: 12321D)
6. Qubit 2.0 Fluorometer (ThermoFisher Scientific, Invitrogen, catalog number: Q32866)
7. Experion Electrophoresis Station (Bio-Rad, catalog number: 7007010)
8. MiSeq System (Illumina, catalog number: SY-410-1003)

Software

1. Primer-BLAST (NCBI, <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Ye *et al.*, 2012)
2. bcl2fastq pipeline (v.2.20.0) (Illumina, https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html)
3. TCS web app (UNC, <https://tcs-dr-dept-tcs.cloudapps.unc.edu/>)
4. TCS pipeline (v.1.3.8) (UNC, <https://github.com/SwanstromLab/PID>)
5. RUBY package viral_seq (v.1.0.8) (UNC, https://github.com/ViralSeq/viral_seq)

Procedure

We first design the primers for the multiplexed Primer ID library prep. After extraction of viral RNA from the tissue culture supernatants or total RNA from lung tissues, we construct MiSeq sequencing libraries using the multiplexed Primer ID library prep protocol from each specimen. The multiplexed Primer ID protocol allows us to make a sequencing library of multiple regions of the viral RNA in one reaction. We pool up to 24 barcoded libraries (from 24 different specimens) for each sequencing run. We use Illumina MiSeq V3 kit (600 cycle) for the sequencing. We choose paired-end sequencing to have longer read length and better error correction from overlapping regions over single-end sequencing.

A. Primer ID cDNA primer and PCR primer design

1. Design primer sequences using NCBI Primer-BLAST.
 - a. Use ORF1b region of the reference genome MERS-CoV NC_019843 as “PCR template” in the Primer-BLAST interface to design primers for MERS-CoV. Use mouse interferon-induced protein with tetratricopeptide repeats 2 (Ifit2, reference: NM_008332), interferon-induced protein with tetratricopeptide repeats 3 (Ifit3, reference: NM_010501), ISG15 ubiquitin-like modifier (Isg15, reference: NM_015783), interferon-induced protein with tetratricopeptide repeats 1 (Ifit1, reference: NM_008331) and chemokine (C-X-C motif) ligand 10 (Cxcl10, reference: NM_021274) genomes as templates to design primers for mouse mRNA.
 - b. Set PCR product size as Min: 400 bp and Max: 600 bp.

Primer set 2 had the best amplification efficiency thus it was selected for downstream Primer ID primer design. NC, no-reaction control.

3. Design the Primer ID cDNA primers and other primers for the MiSeq library prep.
 - a. Design cDNA primers by attaching the Primer ID (a degenerate block of 11-base long sequence) and adaptor sequence to the reverse primers.
 - b. Forward primer of the 1st round PCR is designed by joining a common sequence “GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNN” on the 5' end with the forward primer sequencing designed at step a) on the 3'. The Ns in the joined primer sequences are random sequences to increase the sequencing quality.
 - c. Reverse primer of the 1st round PCR is designed against the adaptor sequence section of the Primer ID cDNA primer, and is universal regardless of regions (Table 1).
 - d. The 2nd round PCR primers are designed to incorporate the MiSeq sequencing adaptors with barcodes.
 - e. Order primers designed above in Steps A1a-A1d from Integrated DNA Technologies (IDT). Request hand-mixing of the precursors for the degenerate block of sequence of the cDNA primers to have an even distribution of bases at each position.
 - f. Table 1 shows the complete list of primers used in this protocol including primers for Primer ID MiSeq library prep and customized MiSeq sequencing primer. Table 2 shows the sequences of the indexed primers.

Notes:

1. *Primer-BLAST is not necessary to design primers. Primers can be manually designed based on known reference genomes.*
2. *Hand-mixing of nucleotide precursors prior to Primer ID cDNA primer synthesis is important to ensure the degenerate sequences have an even distribution of bases (A, C, G, T) at each position of the degenerate block.*

Table 1. Primers used for MiSeq library prep and sequencing. Primer sequences with gene-specific regions are highlighted in blue. They can be replaced with other gene-specific primer sequences when designing primers for other viruses.

Primer	5'-3' sequence	Comment
41R_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTATGACCTT CCTGTTGCTTCT	cDNA primer. Targeting 20331-20350 on the reference genome.
nsp10_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTCCTAAAGA CGACATCAGTGG	cDNA primer. Targeting 13488-13507 on the reference genome.
nsp12_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTATAGCCAA AGACACAAACCG	cDNA primer. Targeting 15983-16002 on the reference genome.
nsp14_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTGAACATC GACAAAGAAAGGG	cDNA primer. Targeting 18715-18734 on the reference genome.
ifit3_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTTTCAGCC ACTCCTTTATCCC	cDNA primer. Targeting mice IFIT3 mRNA.
isg15_PID11*	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNCAGTGGGGCTT TAGGCCATACTC	cDNA primer. Targeting mice ISG15 mRNA.
41F_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNGCTACAAGTTCGTCC TTTGG	1 st round PCR forward primer. Targeting 19812-19831 on the reference genome
nsp10_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTGCTCAGGTGCTAAG CGAAT	1 st round PCR forward primer. Targeting 12983-13002 on the reference genome
nsp12_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNATAGGCTTCGATGTT GAGGG	1 st round PCR forward primer. Targeting 15388-15407 on the reference genome
nsp14_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNATTGCAAGCTGGTTC TAACA	1 st round PCR forward primer. Targeting 18260-18279 on the reference genome
ifit3_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNCGATCCACAGTGAAC AACAG	1 st round PCR forward primer. Targeting mice IFIT3 mRNA.
isg15_AD	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTGGGACCTAAAGGTG AAGATG	1 st round PCR forward primer. Targeting mice ISG15 mRNA.

Table 1. Continued

Adapter R	GTGACTGGAGTTCAGACGTGTGCTC	1 st round PCR reverse primer
Universal Adapter	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG	2 nd round PCR forward primer with Illumina adapter sequence
Indexed Adapter**	CAAGCAGAAGACGGCATACGAGAT NNNNNN GTGACTGGAGTTCAGACGTGTGCTC	2 nd round PCR reverse primer with Illumina adapter sequence and indices (NNNNNNN)
Old Nextera	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAG	Customized sequencing primer

* cDNA primer has a block of degenerate nucleotides (Ns) as the Primer ID.

** Ns in the Indexed Adapter primers are a set of 24 pre-designed barcodes for multiplexing samples for one MiSeq run. See the index sequences and adapter sequences in Table 2.

Table 2. Indexed adapter sequences. Note the index sequences are the reverse complement sequences of the highlighted (red) region in the adapter sequences

Indexed Adapter	Index	Index Sequence	Sequence (5'-3')
PCR Primer, Index 1	1	ATCACGA	CAAGCAGAAGACGGCATACGAGAT TCGTGAT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 2	2	CGATGTA	CAAGCAGAAGACGGCATACGAGAT TACATCG GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 3	3	TTAGGCA	CAAGCAGAAGACGGCATACGAGAT TGCCTAA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 4	4	TGACCAA	CAAGCAGAAGACGGCATACGAGAT TTGGTCA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 5	5	ACAGTGA	CAAGCAGAAGACGGCATACGAGAT TCACTGT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 6	6	GCCAATA	CAAGCAGAAGACGGCATACGAGAT TATTGGC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 7	7	CAGATCA	CAAGCAGAAGACGGCATACGAGAT TGATCTG GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 8	8	ACTTGAA	CAAGCAGAAGACGGCATACGAGAT TTCAAGT GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 9	9	GATCAGA	CAAGCAGAAGACGGCATACGAGAT TCTGATC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 10	10	TAGCTTA	CAAGCAGAAGACGGCATACGAGAT TAAGCTA GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 11	11	GGCTACA	CAAGCAGAAGACGGCATACGAGAT TGTAGCC GTGACTGGAGTTCAGACGTGTGCTC
PCR Primer, Index 12	12	CTTGTA	CAAGCAGAAGACGGCATACGAGAT TTACAAG GTGACTGGAGTTCAGACGTGTGCTC

PCR Primer, Index 13	13	TCCATAA	CAAGCAGAAGACGGCATACGAGATTATGGAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 14	14	GTAATAA	CAAGCAGAAGACGGCATACGAGATTAGTACGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 15	15	ACAGTAA	CAAGCAGAAGACGGCATACGAGATTACTGTGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 16	16	CTCATGA	CAAGCAGAAGACGGCATACGAGATCATGAGGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 17	17	ACGATAA	CAAGCAGAAGACGGCATACGAGATTATCGTGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 18	18	TGCAGAA	CAAGCAGAAGACGGCATACGAGATTCTGCAAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 19	19	TTCATAA	CAAGCAGAAGACGGCATACGAGATTATGAAAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 20	20	TGCTGTA	CAAGCAGAAGACGGCATACGAGATACAGCAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 21	21	TATCACA	CAAGCAGAAGACGGCATACGAGATGTGATAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 22	22	TGGATAA	CAAGCAGAAGACGGCATACGAGATTATCCAAGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 23	23	CGCATAA	CAAGCAGAAGACGGCATACGAGATAATGCGGTGACTGGAGTTCAGACGTGTGCT C
PCR Primer, Index 24	24	GCCTTAA	CAAGCAGAAGACGGCATACGAGATTAAGGCGTGACTGGAGTTCAGACGTGTGCT C

B. Primer ID MiSeq library prep

1. cDNA synthesis

- a. Use viral RNA extracted from cell culture supernatants from infected cells or total lung tissue RNA from infected mice as amplification templates. cDNA synthesis step should be performed in BSL2+ facility until after the Ribonuclease H step.
- b. Completely thaw, vortex and spin down 5× buffer, dNTPs and DTT.
- c. Make cDNA primer working mix from the 100 μM primer stock. The final concentration for each primer is 10 μM in the primer mix.
 - i. Determine regions for sequencing.
 - ii. Add 10 μl of each cDNA primer (no more than 5 different primers to multiplex) to an Eppendorf microcentrifuge tube, and add DNase/RNase-free water to a total volume of 100 μl.
 - iii. Increase the amount of primers and DNase/RNase-free water proportionally if larger volume of primer mix is needed.
- d. Pipette the following components into a 0.5 ml RNase-free PCR tube.
 - 2 μl of dNTP mix (10 mM each)
 - 1 μl of Primer ID cDNA primer mix (10 μM each)
 - 23 μl of RNA template (vRNA or total lung RNA)
- e. Heat the PCR tube on a thermal cycler at 65°C for 5 min, then cool down to 4 °C for 2 min with lid closed (lid temperature at 105 °C).
- f. Add the following components to the tube. If doing the cDNA synthesis in batch, make a master mix and add 14 μl of the mixture to each tube.
 - 8 μl of 5× buffer
 - 2 μl of DTT (100 mM)
 - 2 μl of RNaseOUT (40 U/μl)
 - 2 μl of SSIII reverse transcriptase (200 U/μl)
- g. Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the cDNA synthesis.
 - 55 °C for 1 h
 - 50 °C for 1 h
 - 70 °C for 15 min
- h. To each tube, add 0.5 μl (1U) Ribonuclease H, incubate at 37 °C for 20 min. After this step, the cDNA can be handled on a regular workbench (BSL1).
- i. Purify cDNA with RNAClean XP.
 - i. Resuspend the beads by vortexing and take an aliquot out. Keep in room temperature for at least 30 min before use.
 - ii. Resuspend the beads thoroughly by vortexing. Add 28 μl of RNAClean XP beads to 1.5 ml tubes. Transfer all of the cDNA reaction (40.5 μl) to each tubes with beads.
 - iii. Mix the RNAClean XP and sample thoroughly by pipette mixing 15 times. Vortexing is

- not recommended. Let the tube incubate at room temperature for 20 min. Spin down briefly if samples touches the upper part of the tube.
- iv. Place the tube onto the magnetic tube rack for 5 min to separate the beads from solution.
 - v. Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.
 - vi. Dispense 400 μ l of 70% ethanol into the tube and incubate for 30 s at room temperature. Aspirate out the ethanol and discard. Repeat for a total of three washes. It is important to perform these steps with the tube situated on the magnetic rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
 - vii. Let the reaction tube air-dry up to 10 min on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. However, over drying the sample may result in a lower recovery.
 - viii. Remove tube from magnetic rack and resuspend beads in 24 μ l DNase/RNase-free water by pipetting up and down. Place tube back on rack and leave for 3 min.
 - ix. Pipette the eluent (23.5 μ l) from the tube while it is situated on the magnetic tube rack.
2. First round PCR amplification with KAPA2G Robust PCR kit.
- In the first round PCR, we amplify all the cDNA we make from the previous step using a mixture of forward primers targeting multiple regions of the genome.
- a. Make forward primer working mix from the 100 μ M primer stock.
 - i. Determine regions of primer binding based on the initial choice of placement of the cDNA primers.
 - ii. Add 10 μ l of each forward primer (no more than 5 primers) to an Eppendorf microcentrifuge tube, and add DNase/RNase-free water to a total volume of 100 μ l.
 - iii. Increase the amount of primers and DNase/RNase-free water proportionally if larger volume of primer mix is needed.
 - b. Completely thaw, vortex and spin down reagents (except for enzyme) before use.
 - c. Use the following recipe to make one first round PCR mix (total volume of 50 μ l).
 - 10 μ l of 5 \times Buffer A
 - 10 μ l of Enhancer
 - 1 μ l of dNTPs
 - 2.5 μ l of Forward primer mix
 - 2.5 μ l of adapter R primer
 - 0.5 μ l of KAPA Robust polymerase
 - 23.5 μ l of template cDNA
 - d. Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the first round PCR amplification.
 - i. 95 $^{\circ}$ C for 1 min

- ii. 25 cycles of:
 - 95 °C for 15 s
 - 58 °C for 60 s
 - 72 °C for 30 s
 - iii. 72 °C for 3 min
 - e. Purify PCR products using AMPure XP cleanup beads.
 - i. Vortex the 1 ml aliquot of AMPure XP cleanup beads and remove the needed volume. Keep in room temperature for at least 30 min before use.
 - ii. Transfer the first round of PCR reactions into 1.5 ml DNase-free tubes.
 - iii. Resuspend the beads. Add 35 µl AMPure XP beads to each PCR reaction.
 - iv. Mix the AMPure XP and sample thoroughly by vortexing. Let the tube incubate at room temperature for 5 min before proceeding to the next step (incubate off the rack).
 - v. Place the tube onto the magnetic tube rack for 5 min to separate the beads from solution.
 - vi. Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the tube is situated on the rack. Do not disturb the magnetic beads, which have formed a spot on the side of the tube.
 - vii. Dispense 400 µl of 70% ethanol into the tube and incubate for 30 s at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes. It is important to perform these steps with the tube situated on the rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
 - viii. Let the reaction tube air-dry up to 10 min on the rack with the cap open. The tube(s) should air-dry until the last visible traces of ethanol evaporate. Over drying the sample may result in a lower recovery.
 - ix. Remove tube from rack and resuspend beads in 50 µl DNase-free water by pipetting up and down. Place tube back on rack and leave for 3 min.
 - x. Pipette the 45 µl eluent from the tube while it is situated on the magnetic tube rack. Transfer the eluent to a new 1.5 ml microcentrifuge tube.
- 3. Second round of PCR amplification with KAPA2G HiFi PCR kits.
 - a. Complete thaw, vortex and spin down reagents (except for enzyme) before use.
 - b. Use the following recipe to make one second round PCR mix (total volume of 25 µl).
 - 10 µl of 5× KAPA HiFi Buffer
 - 1 µl of dNTPs
 - 1 µl of Universal Adapter
 - 1 µl of indexed Adapter
 - 0.5 µl of KAPA HiFi polymerase
 - 2 µl of purified DNA from the first round PCR.
 - c. Mix well by pipetting up and down for a few times. Transfer the tube(s) to a thermal cycler. Use the following thermal cycler program for the cDNA synthesis.

- i. 95 °C for 2 min
 - ii. 35 cycles of:
 - 98 °C for 20 s
 - 63 °C for 15 s
 - 72 °C for 30 s
 - iii. 72 °C for 3 min
4. Gel purification of PCR products using QIAGEN MinElute Gel extraction kit.
 - a. Before gel extraction, run 2 µl PCR products on 1% agarose gel to check if the library has the right size.
 - b. Make 1.2% (w/v) agarose gel with ethidium bromide (final concentration of 0.2-0.5 µg/ml).
 - c. Load all the second round PCR products on the 1.2% agarose gel. Adjust the voltage based on the size of the gel. The ideal electric field strength is 4 V/cm. Run the gel for 30 min.
 - d. Excise target DNA fragments of the right size (500 bp to 1 kb) on a non-UV blue light transilluminator.
 - e. Weigh the gel; add 3 volumes of Buffer QG to 1 volume of gel.
 - f. Incubate at 50 °C for 10 min to completely dissolve the gel fragment. Vortex every 2-3 min to help dissolve.
 - g. Check the color of the gel solution (should be yellow, otherwise add 10 µl 3 M sodium acetate of pH 5.0).
 - h. Apply the sample to the MinElute column and centrifuge at maximum speed for 1 min.
 - i. Transfer the column to a new collection tube, discard the filtrate, add 500 µl buffer QG and centrifuge at maximum speed for 1 min.
 - j. Transfer the column to a new collection tube, discard the filtrate, add 0.75 ml buffer PE, incubate for 5 min at room temperature, centrifuge at maximum speed for 1 min.
 - k. Transfer the column to a new collection tube, discard the filtrate, centrifuge at maximum speed for additional 3 min.
 - l. Put the column in a new 1.5 ml tube, add 10 µl buffer EB. Stand for 4 min, centrifuge at maximum speed for 2 min.
5. Quantification, pooling and QA/QC.
 - a. Quantify each gel-purified library using Qubit dsDNA BR Assay kit, following the [manufacturer's protocol](#). Use 1 µl of the library for each quantification.
 - b. Pool the quantified individual libraries in equal amounts. We suggest pooling a total number of 20-24 libraries, each with 100 to 150 ng DNA per library. Use the maximum available volume of libraries if the calculated volume exceeds the actual volume.
 - c. Purify the pool of libraries with AMPure XP beads following the protocol above in the First Round PCR section. The volume of AMPure beads to use equals the volume of pooled libraries multiplied by 0.7.
 - d. Quantify the purified pooled libraries using Qubit dsDNA BR Assay kit, following the manufacturer's protocol.

- e. Run the pooled libraries with the Experion Automated Electrophoresis System with DNA 12k analysis kit following the [manufacturer's protocol](#). An example of gel image of Experion is shown in Figure 4.
- f. Check the size of DNA peaks on the Experion gel image. Make sure there is no visible peak around 100 bp (primer dimer). If it is present, purify the pooled libraries with AMPure XP beads again and repeat the quantification and Experion electrophoresis.
- g. Calculate the average DNA size (bp) of the library pool based on the Experion molarity (nM) and Experion concentration (ng/μl) using the following equation. Do NOT use the Qubit concentration to calculate the average DNA size.

$$\text{avg. DNA size} = \frac{\text{Experion Concentration}}{\text{Experion Molarity} \times 650} \times 10^6$$

Notes:

1. *It is important to set up the cDNA reaction in a separate room from where the PCR amplification takes place to avoid potential contamination from PCR products.*
2. *Viral RNA in this protocol that have very little quantity may not be measurable. qRT-PCR is usually recommended to confirm the copy numbers of viral RNA before library prep. We recommend at least 1,000 copies of input viral RNA for each library prep. However, this protocol may still work with less than 1,000 copies of input viral RNA, with compromised sampling depth.*
3. *Primer mix is only needed when multiplexed regions are sequenced. It is possible to use one set of cDNA/forward primers at 10 μM concentration when sequencing only one region.*
4. *Multiplexed regions normally should not overlap as this may inhibit the efficiency of priming and/or amplification.*
5. *Include one negative control and possibly one positive control for each batch of library prep.*
6. *It is recommended to aliquot the RNAClean XP beads and the AMPure XP beads into 1 ml aliquots. It is very important to warm the beads at room temperature for 30 min before use.*
7. *The ratio of the volume of beads to cDNA/DNA is 0.7. It is optimized to remove long Primer ID cDNA primers or PCR primers.*
8. *We recommend a second round of cDNA purification for some clinical specimens, especially plasma samples, to remove inhibitors of the downstream amplification. After the cDNA is eluted in water (in this case, use 40 μl water), add 28 μl of new RNAClean XP beads and purify the cDNA for a second time.*
9. *Residual beads in the cDNA or PCR products eluent won't affect the downstream amplification.*
10. *First and second round PCR amplifications use two different KAPA PCR kits. The enzyme and buffer tubes look similar. It is very important to use the right buffer with the right enzyme for PCR 1 and PCR 2.*
11. *Save the first round PCR product in the 1.5 ml tube in -20 °C, in case it is needed to repeat PCR2.*

12. Make sure to excise the fragment of the right size during gel extraction. Occasionally larger fragments (1.5-3 kb) can be present on a gel, which should not be extracted.
13. Library concentration normally should be at least 20 ng/μl. Discard libraries with concentration less than 10 ng/μl as they are less likely to have high quality sequences.
14. Qubit 2.0 fluorometer has been discontinued. Qubit 4.0 fluorometer is available at the time this protocol is written.
15. Qubit dsDNA HS Assay kit is not recommended to quantify the libraries. The HS kits have an upper detection limit of 100 ng/μl. In some cases the library concentration can be higher than 100 ng/μl.
16. The Bio-Rad Experion system is used in this protocol but it is close to obsolete. A better substitution is Agilent TapeStation System (Agilent, catalog number: G2991AA). The TapeStation system can directly measure the average DNA size of the library pool.

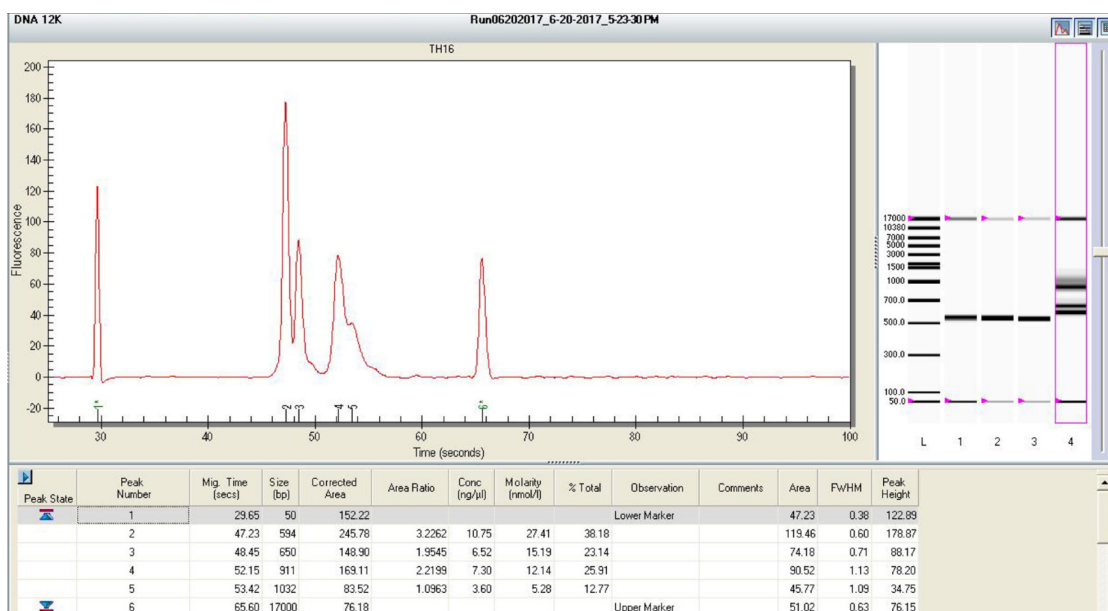


Figure 4. An example of Experion gel image

C. MiSeq sequencing run

The MiSeq is an integrated instrument that performs clonal amplification, DNA sequencing based on sequencing-by-synthesis, and data analysis with base calling, alignment, variant calling, and reporting in a single run. In our protocol, we use the MiSeq reagent kit V3 (600 cycle) for sequencing. Figure 5 shows the MiSeq reagent cartridge and content by well numbers. This step is modified based on the MiSeq V3 protocol.

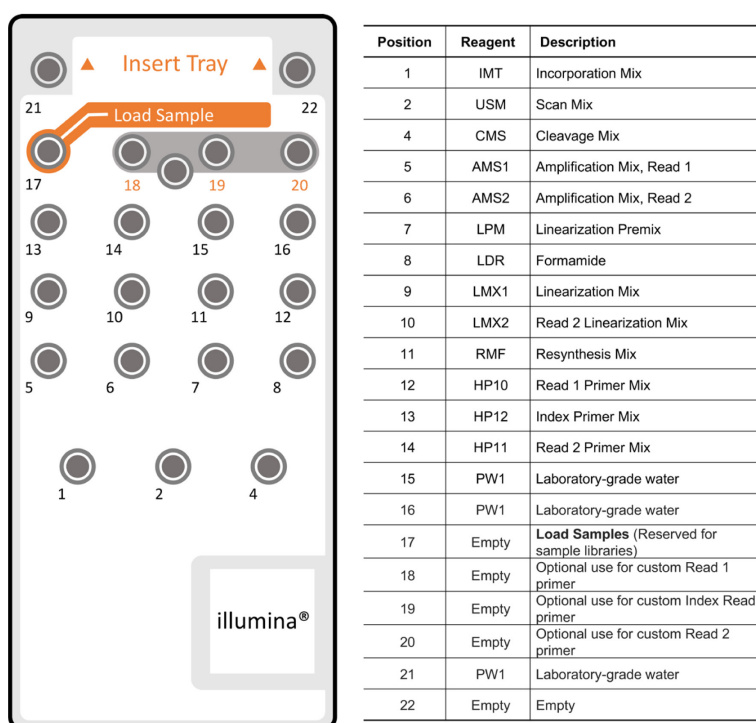


Figure 5. MiSeq Reagent Cartridge and content by well numbers

1. Prepare the reagent cartridge.
 - a. Remove the reagent cartridge from -20 °C storage.
 - b. Place the reagent cartridge in a water bath containing only enough room temperature, deionized water to submerge the base of the reagent cartridge. Do not allow the water to exceed the maximum water line printed on the reagent cartridge.
 - c. Allow the reagent cartridge to thaw in the room temperature water bath for approximately 60 min or until completely thawed.
 - d. Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge, if necessary.
 - e. Invert the reagent cartridge ten times to mix the thawed reagents, and visually inspect that all positions are thawed.
 - f. Visually inspect the reagent marked IMT (incorporation mix) to make sure that it is fully mixed and free of precipitates.
 - g. Gently tap the cartridge on the bench to dislodge water from the base of the cartridge and reduce air bubbles in the reagents.
Note: The MiSeq sipper tubes go to the bottom of each plate well to aspirate the reagents so it is important that there are no air bubbles stuck at the bottom of the reagent wells.
 - h. Place the reagent cartridge on ice or set aside at 2 °C to 8 °C until you are ready to load the sample onto the reagent cartridge and set up the run.
2. Prepare the libraries.
 - a. Denature DNA

- i. Prepare a fresh dilution of 0.2 N NaOH on the day of sample loading. This is essential in order to completely denature samples for cluster generation on the MiSeq. Always start with a concentrated stock of NaOH that is 10 N or higher, or you may get a decrease in cluster density.
 - ii. Calculate the molarity of the pooled library based on the Qubit concentration and average DNA size, dilute the pooled library to 4 nM.
 - iii. Combine the following volumes of sample DNA and 0.2 N NaOH in a microcentrifuge tube:
 - 4 nM sample DNA (5 μ l)
 - 0.2 N NaOH (5 μ l)
 - iv. Vortex briefly to mix the sample solution, and then briefly centrifuge the sample solution.
 - v. Incubate for five minutes at room temperature to denature the DNA into single strands.
 - vi. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA to result in a 20 pM denatured library:
 - Denatured DNA (10 μ l)
 - Pre-chilled HT1 (990 μ l) (Shipped frozen in the MiSeq reagent cartridge box)
 - vii. Place the denatured DNA on ice until you are ready to proceed to final dilution.
- b. Dilute Denatured DNA
- i. Dilute the denatured DNA to 8 pM concentration using the following volumes:
 - 20 pM DNA (400 μ l)
 - Pre-chilled HT1 (600 μ l)
 - ii. Vortex briefly to mix the DNA solution.
 - iii. Pulse centrifuge the DNA solution.
 - iv. Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge directly or after adding the PhiX control.
- c. Denature and Dilute PhiX control
- i. PhiX is used as an internal sequencing control provided by Illumina. Use the following instructions to denature and dilute the 10 nM PhiX library to 8 pM. This should result in a cluster density of 750-850 K/mm².
 - ii. Combine the following volumes to dilute the PhiX library to 2 nM:
 - 10 nM PhiX library (2 μ l)
 - 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20 (8 μ l)
 - iii. Combine the following volumes of 2 nM PhiX library and 0.2 N NaOH in a microcentrifuge tube to result in a 1 nM PhiX library:
 - 2 nM PhiX library (10 μ l)
 - 0.2 N NaOH (10 μ l)
 - iv. Vortex briefly to mix the 1 nM PhiX library solution.
 - v. Briefly centrifuge the template solution.
 - vi. Incubate for five minutes at room temperature to denature the PhiX library into single

- strands.
- vii. Add the following volume of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library.
 - Denatured PhiX library (20 μ l)
 - Pre-chilled HT1 (980 μ l)
 - viii. Dilute the denatured 20 pM PhiX library to 8 pM as follows:
 - 20 pM denatured PhiX library (400 μ l)
 - Pre-chilled HT1 (600 μ l)
 - d. Mix Sample Library and PhiX Control
 - i. Combine the following volumes of denatured PhiX control library and your denatured sample library to result in a 15% volume ratio:
 - 8 pM PhiX control library (105 μ l)
 - Denatured sample library (595 μ l)
 - ii. Set the combined sample library and PhiX control aside on ice until you are ready to load it onto the MiSeq reagent cartridge.
 3. Load Sample Libraries onto Cartridge
 - a. Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir labeled Load Samples, position 17 on the reagent cartridge.
 - b. Pipette 700 μ l of your sample libraries into the Load Samples reservoir.
 4. Load Old Nextera Primer onto Cartridge.
 - a. Old Nextera Primer is a custom Read 1 primer that gets spiked into position 12 on the reagent cartridge. Old Nextera Primer sequence can be found in Table 1.
 - b. In a new microcentrifuge tube, add 3.8 μ l of 100 μ M Old Nextera Primer, and place on ice.
 - c. Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir position 12 on the reagent cartridge.
 - d. Pipette approximately 500 μ l of the Read 1 Primer Mix out of the cartridge using a 1 ml serological pipette.
 - e. Add the Read 1 Primer Mix to the tube with the Old Nextera Primer.
 - f. Vortex briefly to mix the primer solution, and then briefly centrifuge the solution.
 - g. Pipette the entire primer solution back into position 12 on the reagent cartridge using a 1 ml pipette tip.

Note: Do not pierce any other reagent positions, they will be pierced when the cartridge is loaded on the instrument.
 - h. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface. Index sequences required for MCS can be found in Table 2.

Notes:

1. In the Illumina Experiment Manager (the software where you create the sample sheet) there is an option to select "Custom Primer for Read 1". Since we are spiking in the custom primer into

- position #12, we do **NOT** select that option.*
2. *Double check the primer sequence is correct and it is diluted to 100 μ M. It is important that the custom primer gets mixed well with the other primers in position #12. We recommend pipetting 3.8 μ l of primer into a new Eppendorf tube. Then remove the contents of position #12 and add them to the Eppendorf tube. Vortex to mix well. Then pipette the entire contents back into position #12 of the reagent cartridge. Tap the cartridge to make sure the liquid falls to the bottom of the tube.*
 3. *Do **NOT** add Old Nextera Primer into Positions 18 to 20 (custom sequencing primers).*
 4. *It is very important that the stock solution of NaOH is 10 N or higher. Some people try to use either 5 N or 2 N stock. For some reason these lower concentration stocks don't work as well for denaturation and the 0.2 N dilution has to be made on the day of loading samples on the MiSeq.*
 5. *The denatured 20 pM PhiX library can be stored up to three weeks if stored at -15 $^{\circ}$ C to -25 $^{\circ}$ C. After three weeks, cluster numbers tend to decrease.*

Data analysis

1. Use the Illumina bcl2fastq pipeline (v.2.20.0) for initial data processing. This pipeline will de-multiplex sequences based on their barcodes. In this experiment design, each barcoded library is a library constructed from an RNA specimen, either from the treatment group or from the control group. In this step, only the library barcodes are de-multiplexed. The region and Primer ID de-multiplexing will be done in the following steps.
2. Use TCS pipeline (v.1.3.8) to de-multiplexed each target region and create template consensus sequences. There are two ways to process it.
 - a. (Recommended) Use TCS web app (<https://tcs-dr-dept-tcs.cloudapps.unc.edu/>) to process the raw MiSeq sequences automatically. The web app asks for the information of each sequenced regions including a name, the cDNA primer sequence and the first round PCR forward primer sequence. Choose direct upload, UNC Longleaf sequence repo or Dropbox to upload the raw MiSeq sequences. The processed sequences will be returned to the email address provided by the user. The process time is from 20 min to 2 h, based on the size of files for each batch submitted.
 - b. Use the TCS pipeline script (<https://github.com/SwanstromLab/PID/blob/master/TCS.rb>) to process the MiSeq raw sequence data manually, following the instructions in the script. It is recommended to use the file-sorting and logging script in the TCS pipeline 'log_mutli.rb' (https://github.com/SwanstromLab/PID/blob/master/log_multi.rb) to sort the files after running the TCS pipeline. The script can be run on the Linux/MacOS platform using Terminal and Ruby without additional support, and in Windows platform with terminal and Ruby installed.
3. Use the 'end_join.rb' script (https://github.com/SwanstromLab/PID/blob/master/end_join.rb) in

the TCS pipeline to join the paired-end sequences.

4. The number of TCS is an important parameter that reveals the sampling depth of sequencing as the number of initial templates actually queried.
5. Use the RUBY package 'viral_seq' (1.0.8) to calculate the mutation rates of each specimen. An executable script can be found in the TCS pipeline (https://github.com/SwanstromLab/PID/blob/master/mut_table.rb). This script compares each individual sequence with a sample consensus sequence and generates a table listing frequencies of each type of substitutions.

Notes:

1. *The newer version of the TCS pipeline (version $\geq 2.0.0$) is included in the RUBY package 'viral_seq' (version $\geq 1.0.8$) as an executable command line tool. It has several major updates including the end-joining functions along with the TCS creation, thus it is recommended for future use.*
2. *The authors dedicated to improve the bioinformatics analysis pipelines. Users are welcome to contact the authors for any related questions.*

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Competing interests

The University of North Carolina is pursuing IP protection for Primer ID and R.S. has received nominal royalties.

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