Supplementary Appendix - Protocol

Protocol: MINERVA – a rapid library construction method to sequence SARS-CoV-2 gRNA

Version 1.1

April 23, 2020

Materials

REAGENTS

- RNaseZap (Ambion, Cat. No. AM9780)
- DNA-OFF (Takara Bio, Cat. No. 9036)
- QIAamp Viral RNA Mini Kit (Qiagen, Cat. No. 52906)
- MGIEasy rRNA removal kit (BGI, Cat. No. 1000005953)
- DNase I (RNase-free) (NEB, Cat.No.M0303)
- Tris-HCl (1M, pH 7.6; ROCKLAND, Cat. No. MB-003)
- $MgCl_2$ (1 M; Invitrogen, Cat. No. AM9530G)
- N,N-Dimethylformamide (for molecular biology, ⩾99%; Sigma, Cat. No. D4551)
- DPEC-treated water (Invitrogen, Cat. No. AM9915G)
- Recombinant RNase Inhibitor (40 U/μl; Takara, Cat. No. 2313)
- Deoxynucleotide (dNTP) Solution Set (NEB, Cat. No. N0446S)
- Superscript II reverse transcriptase (Invitrogen, Cat. No. 18064014)
- DTT (0.1M; Invitrogen, Cat. No. 18064014)
- Betaine solution (5 M; Sigma, Cat. No. B0300)
- TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme, Cat. No. TD501)
- PEG8000 (VWR Life Science, Cat.No.97061)
- ATP (10 mM; NEB, Cat. No. P0756)
- Q5 High-Fidelity 2x Master Mix (NEB, Cat. No. M0492)
- ChamQ SYBR qPCR master mix (Vazyme, Cat. No. Q311-02)
- VAHTS DNA Clean Beads (Vazyme, Cat. No. N411)
- Ethanol (200 proof, for molecular biology; Sigma, Cat. No. E7023)
- TargetSeq One Cov Kit (iGeneTech, Cat. No. 502002-V1)
- xGen Universal Blockers (IDT, Cat.No. 1079586)
- All oligos were acquired from Sangon.

SUPPLIES

- Millex-GP Syringe Filter Unit (0.22 μm, polyethersulfone; Millipore, Cat. No. SLGP033RB)
- 0.2 mL Thin Wall PCR Tubes (Axygen, Cat. No. PCR-02-C)

EQUIPMENT

- Thermo cycler
- Magnetic stand
- Vortexer
- Real-Time PCR machine
- A compatible Illumina DNA sequencing instrument

REAGENT SETUP

- **SARS-Cov-2 infected samples**

SARS-Cov-2 infected samples, including pharyngeal swabs, sputum samples or stool samples are collected following clinical guidelines. All of the samples, or their viral transfer media, must be deactivated at 56 °C for 30 min before nucleic acid extraction.

IMPORTANT NOTE: All the procedure should be operated in a BSL-3 laboratory before the total RNA is ready.

- **Total RNA**

  Total RNA can be extracted from SARS-Cov-2 infected samples by QIAamp Viral RNA Mini Kit, while omitting the addition of carrier RNA if possible. Then use MGIEasy rRNA removal kit to remove the ribosomal RNA, and DNase I to remove the DNA. The final elution volume is 12-20 μl for each sample.

  IMPORTANT NOTE: All the procedure should be operated in a BSL-3 laboratory before the total RNA is ready.

- **Oligo dT primer**

  Oligo dT primer (5'-T_{30}VN-3') anneals to all the RNAs containing a poly(A) tail. 'N' is any base and 'V' is either A, C or G. Dissolve the oligonucleotide in DPEC-treated water, to a final concentration of 100 μM. The oligo solution can be stored at −20 °C for at least 6 months.

- **Random decamer primer**

  Random decamer primer (5'-N_{10}-3') anneals to the RNA randomly. 'N' represents any base. Dissolve the oligonucleotide in DPEC-treated water, to a final concentration of 100 μM. The oligo solution can be stored at −20 °C for at least 6 months.

- **dNTP mix**

  Combine equal volume of dATP, dTTP, dCTP, dGTP in Deoxynucleotide (dNTP) Solution Set.

- **5X TD Buffer**

  50 mM Tris-HCl, 25 mM $MgCl_2$, 50% N,N-Dimethylformamide (DMF). This buffer can be stored at 4℃ for at least 6 months.

- **N-ch-F primer**

  N-ch-F primer (5'-GGGGAACTTCTCCTGCTAGAAT-3') is the forward primer used to amplify the N region of SARS-Cov-2 in qPCR. Dissolve the oligonucleotide in DPEC-treated water, to a final concentration of 100 μM. The oligo solution can be stored at −20 °C for at least 6 months.

- **N-ch-R primer**

  N-ch-R primer (5'-CAGACATTTTGCTCTCAAGCTG-3') is the reverse primer that used to amplify the N region of SARS-Cov-2 in qPCR. Dissolve the oligonucleotide in DPEC-treated water, to a final concentration of 100 μM. The oligo solution can be stored at −20 °C for at least 6 months.

Procedure

1) Clean the work space, including the hood and pipettes, with DNA-off and RNase-Zap. Filter the self-made buffer with 0.22 μm filter. Use a thermal cycler with a heated lid set to 105 °C for all incubations throughout this protocol. All reaction mixes should be set up on ice.

IMPORTANT NOTE: All the procedure should be operated in a BSL-3 laboratory before the total RNA is ready.

Reverse transcription

2) Prepare preRT mix on ice by adding 0.2 µl RNase Inhibitor (40U/µl), 0.2 µl Oligo dT (100 µM), 2 µl $(N)_{10}$ random primer (100 µM), 0.8 µl dNTP mix (25 mM) to 5.4 µl total RNA.
NOTE: If the volume of the RNA sample is more or less than 5.4 µl, please scale up or down the whole reaction volume proportionally.

3) Mix the reaction gently and thoroughly without bubbles and incubate it at 72°C for 3 min, then quickly cool it on ice.

4) Prepare RT mix by combining the reagents in the table below.

Component	Volume (µl)	Final concentration
SuperScript II reverse transcriptase (200U/µl)	1.00	200 U
RNase Inhibitor (40U/µl)	0.50	20 U
SuperScript II first strand buffer (5x)	4.00	1x
DTT (0.1M)	1.00	5 mM
Betaine (5M)	4.00	1 M
$MgCl_2$ (1M)	0.12	6 mM
DPEC water	0.78	-
Total Volume	11.40	-

5) Add 11.40 µl RT mix to the samples from step 3 and gently pipette without bubbles. Incubate the reaction at 42°C for 1.5 hour for reverse transcription, and followed by 70°C 15min to inactivate SuperScript II reverse transcriptase. Then put the sample on ice.

Tagmentation

6) Dissolve PEG8000 powder to DPEC-treated water with concentration of 40% (w/w), and filter the solution by 0.22 µm filter.

7) Prepare the tagmentation mix by combining the reagents listed below.

Component	Volume (µl)	Final concentration
V50 (TruePrep DNA Library Prep Kit V2)	1.00	-
5xTD buffer	8.00	1x
RNase Inhibitor (40U/µl)	1.00	40 U
40% PEG8000	3.40	3.4%
ATP (10 mM)	4.00	1.00 mM
DPEC water	2.60	-
Total Volume	20.00	-

8) Add the tagmentation mix to the sample from step 5. Pipette the reaction gently and thoroughly. Incubate the reaction at 55°C for 30 min, then cool it on ice.

Amplification and Sample Pooling

9) Add 0.8 µl SuperScript II reverse transcriptase and 40.8 µl Q5 High-Fidelity 2x Master Mix to the tagmentation products.

10) Mix the reaction well without forming bubbles. Incubate the reaction at 42°C for 15 min to fill the 9 bp gap left by Tn5 transposome, followed by 70°C 15 min to inactivate SuperScript II reverse transcriptase.

11) Prepare the PCR mix by combining 4 µl N6xx index primer (10 µM), 4 µl N8xx index primer (10 µM) and 8 µl Q5 High-Fidelity 2x Master Mix. The final concentration of each index primer is around 0.4 µM.

12) Add PCR mix to the sample from step 10 and pipette thoroughly. Perform PCR as detailed below.

Cycle	Denature	Anneal	Extension	Hold
1	98°C, 30 s	-	-	
2-19	98°C, 20 s	60°C, 20 s	72°C, 2 min	
20	-	-	72°C, 5 min	
21	-	-	-	4°C

13) For targeted SARS-CoV-2 deep sequencing, continue with **qPCR and Sample Pooling.** For metagenomic sequencing, pool 8-16 libraries in equal volumes and go to **Step 18**.

qPCR and Sample Pooling

14) Take 1 µl PCR product and dilute it 200-fold with nuclease-free water.

15) Prepare qPCR mix by adding 0.05 µl N-ch-F primer (100 µM), 0.05 µl N-ch-R primer (100 µM), 5 µl ChamQ SYBR qPCR master mix and 3.9 µl nuclease-free water to 1 µl diluted template.

16) The program of qPCR is performed at 95°C for 60s, followed by 40 cycles of [95°C 5s, 60°C 15s].

17) Pool 8-16 libraries together according to their Ct values as following:

Ct	Volume taken (µl)
>28	50.0
24-28	16.0
20-24	3.0
<20	0.5

Purification

18) Place VATHS DNA clean beads at room temperature for 15 min, then vortex violently.

19) Add equal volume of beads (1x) to the pooled samples and vortex violently. Incubate the mixture at room temperature for 5 min.

20) Transfer the tube to compatible magnetic stand until the solution is clear.

21) Carefully remove the solution without disturbing beads.

22) Wash beads with 200 µl 80% ethanol (freshly prepared) and incubate for 30 s, then remove the ethanol. Repeat this step one more time.

23) Dry the beads on magnetic stand with cap open until the color of beads gets light. Add 52

μl nuclease-free water and close the cap, vortex violently to wash DNA off.

24) Incubate the tube at room temperature for 5 min off the magnet stand.

25) Quickly spin down the tube then place it on magnetic stand until the solution is clear.

26) Carefully aspirate 50 μl supernatant to a clean tube without disturbing beads. The library can be restored at -20°C for 6 months.

IMPORTANT NOTE: The equal-volume mixed libraries are now ready for sequencing.

SARS-Cov-2 Sequence Enrichment

27) Subject the purified Ct-adjusted library pool to one round of SARS-Cov-2 sequence capture following the instruction of TargetSeq One Cov Kit. Replace the iGeneTech Blocker with the IDT xGen Universal Blockers.

High-Throughput Sequencing

28) Both the metagenomic library and the SARS-Cov-2 targeted library can be sequenced on any Illumina platform with paired-end mode. 10-20 million reads are typically collected.