MINERVA: A facile strategy for SARS-CoV-2 whole genome deep sequencing of clinical samples

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Abstract

The novel coronavirus disease 2019 (COVID-19) pandemic poses a serious public health risk. Analyzing the genome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from clinical samples is crucial for the understanding of viral spread and viral evolution, as well as for vaccine development. Existing sample preparation methods for viral genome sequencing are demanding on user technique and time, and thus not ideal for time-sensitive clinical samples; these methods are also not optimized for high performance on viral genomes. We have developed MetagenomIc RNA EnRichment VirAl sequencing (MINERVA), a facile, practical, and robust approach for metagenomic and deep viral sequencing from clinical samples. This approach uses direct tagmentation of RNA/DNA hybrids using Tn5 transposase to greatly simplify the sequencing library construction process, while subsequent targeted enrichment can generate viral genomes with high sensitivity, coverage, and depth. We demonstrate the utility of MINERVA on pharyngeal, sputum and stool samples collected from COVID-19 patients, successfully obtaining both whole metatranscriptomes and complete high-depth high-coverage SARS-CoV-2 genomes from these clinical samples, with high yield and robustness. MINERVA is compatible with clinical nucleic extracts containing carrier RNA. With a shortened hands-on time from sample to virus-enriched sequencing-ready library, this rapid, versatile, and clinic-friendly approach will facilitate monitoring of viral genetic variations during outbreaks, both current and future.

Introduction

As of April 24, 2020, the ongoing COVID-19 viral pandemic has affected more than 2.6 million people in over 200 countries and territories around the world, and has claimed more than 180 thousand lives. Closely monitoring the genetic diversity and distribution of viral strains at the population level is essential for epidemiological tracking, and for understanding viral evolution and transmission; additionally examining the viral heterogeneity within a single individual is imperative for diagnosis and treatment. The disease-causing pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified from early disease cases and its draft genome sequenced within weeks, thanks to the rapid responses from researchers around the world(*1*, *2*). The initial SARS-CoV-2 draft genome was obtained independently from the same early COVID-19 patient samples using various conventional RNA-seq sequencing library construction methods. Although these library construction methods successfully generated a draft genome, several drawbacks hinder the use of these methods for routine viral genome sequencing from the surge of clinical samples during an outbreak.

One direct library construction approach which was used to generate the SARS-CoV-2 draft genome(*1*, *2*) essentially captures each sample's entire metatranscriptome, in which SARS-CoV-2 is just one species among many. The abundance of SARS-CoV-2 in clinical swabs, sputum, and stool samples is often low(*3*, *4*), therefore this catch-all method requires deeper sequencing of each sample in order to obtain sufficient coverage and depth of the whole viral genome, which increases the time and cost of sequencing. Target enrichment with spiked-in primers can improve SARS-CoV-2 genome coverage(*5*), but the reliance on specific primers inherently limits this approach for the profiling of fast evolving viruses such as coronaviruses. The same limitation applies to multiplex RT-PCR-based strategies(*6*). Additionally, once the sample is subject to targeted amplification during the initial reverse transcription (RT) steps, its metatranscriptomic information is lost forever.

Currently, the most comprehensive strategy is the combination of metatranscriptomics profiling with post-library SARS-CoV-2 target enrichment(*6*). However, in most

conventional RNA-seq methods, the double-strand DNA ligation (dsDL) portion of the protocol is usually the most demanding on hands-on time and user technique(*7*). When superimposed on the target enrichment process, these labor intensive and lengthy protocols become impractical for routine use in the clinic, much less for the timely monitoring of viral genetics and evolution on large volumes of samples during an outbreak. Furthermore, due to the low molecular efficiency of dsDL, these protocols also require a high amount of input material, further restricting their application on clinical samples.

Summarily, although next generation sequencing platforms are high-throughput and have short turn-around time, library construction from samples – whether including targeted enrichment or not – remains a major bottleneck. To broadly apply viral sequencing on clinical samples, especially during outbreaks when biomedical resources are already limited, a rapid, simple, versatile, and scalable sample library construction method that does not compromise on performance is urgently needed.

Recently, we reported a new RNA-seq library construction strategy that aims to address some of these challenges: SHERRY avoids the problematic dsDL step in library construction by taking advantage of the newly discovered Tn5 tagmentation activity on RNA/DNA hybrids, to directly tag RNA/cDNA fragments with sequencing adapters (*7*). As such, SHERRY has minimal sample transfers and greatly reduced hands-on time, making it simple, robust, and suitable for inputs ranging from single cells to 200 ng total RNA. We now combine the advantages of SHERRY with a simplified post-library target enrichment protocol to enable rapid sequencing of high-quality viral genomes from clinical samples. MetagenomIc RNA EnRichment VirAl sequencing or MINERVA, is an easy-to-use, versatile, scalable, and cost-effective protocol that yields high-coverage high-depth viral genomes, while preserving the sample's rich metatranscriptomic profile. The hands-on time required from clinical sample to sequencing-ready library using conventional approaches without enrichment is 190 mins; MINERVA requires only 100 mins hands-on time, and if deep viral coverage is desired, an additional 90 mins for post-library enrichment, totaling 190 mins for the entire workflow (**Fig. S1**), making MINERVA practical for high-volume, routine clinical use. We applied MINERVA to various types of COVID-19

samples and successfully obtained up to 10,000-fold SARS-CoV-2 genome enrichment. This strategy will facilitate all studies regarding SARS-CoV-2 genetic variations in the current pandemic, and can also be applied to other pathogens of interest.

Results

Metagenomic RNA enrichment viral sequencing (MINERVA). To analyze both metagenomics and SARS-CoV-2 genetics from COVID-19 patient samples, we developed a two-stage metagenomic RNA enrichment viral sequencing strategy termed MINERVA (**Fig. 1**). First, we employed a SHERRY-based RNA-seq pipeline for metagenomic analysis. Since clinical samples may contain DNA, RNA, and possibly carrier RNA, MINERVA starts with ribosomal RNA (rRNA) removal and optional simultaneous carrier RNA removal, followed by DNase I treatment. The remaining RNA is then subject to standard SHERRY. Previously we observed 3' bias in SHERRY libraries; to address this, we used 10 ng mouse 3T3 cell total RNA as starting material, and tested whether adding random decamers (N10) during RT could improve coverage evenness (**Fig. S2**). Compared with the standard SHERRY protocol, which uses 1 μM T30VN primer during RT, the supplement of 1 μM N10 indeed improves gene body coverage evenness, presumably by improving the RT efficiency. When the N10 concentration was further increased to 10 μM, we observed almost no coverage bias in the gene body. The high N10 concentration can result in an increased rRNA ratio in the product, sometimes as high as 90%, but MINERVA employs rRNA removal as the first step prior to RT, thus negating this problem. We also performed enzyme titration with homemade and commercial Tn5 transposomes. Based on these N10 and Tn5 titration results, we used 10 μM N10 during RT and 0.5 μl V50 for each 20-μl tagmentation reaction in all following experiments. The whole procedure from nucleic acid to metagenomic sequencing-ready library, including wait time, takes 5.5 hours (**Fig. S1**).

For target enrichment, we first quantified SARS-CoV-2 abundance in each metagenomic sequencing library using an N gene qPCR assay, and pooled eight libraries based on quantification results. Then we performed standard in-solution hybridization on the pooled library with biotinylated RNA probes covering the whole viral genome. The enrichment

procedure takes 19 hours; the entire MINERVA pipeline can be completed within 24 hours.

MINERVA achieves better SARS-CoV-2 genome coverage compared to conventional dsDL strategies. To evaluate its performance on clinical samples, we applied MINERVA on 85 pharyngeal swabs, sputum, or stool samples, collected from 72 COVID-19 patients. These patients were admitted to Ditan Hospital within a two-month period from January to March 2020, presenting different symptom severity (**Fig. 2A and Fig. S3**). Some patients were re-sampled longitudinally to investigate temporal and intra-host viral heterogeneity. We also used these samples to benchmark MINERVA against conventional dsDL strategies. Samples Y1-Y6 were excluded from dsDL processing as this processing strategy is incompatible with samples containing carrier RNA. On average, we sequenced 1-3 Gbp for each MINERVA library pre- and post-enrichment, and nearly 100 Gbp for each dsDL library (**Fig. 2B**).

The metagenomic composition of SHERRY and dsDL libraries were comparable: total virus, fungus and bacteria ratios were highly concordant between the two methods (**Fig. S4**); bacterial heterogeneity revealed by entropy is also correlated between the two strategies. In both MINERVA and dsDL data, we detected low yet significant levels of SARS-CoV-2 sequences. The viral ratio is between 10^{-7} and 10^{-1}. It is worth noting that the SARS-CoV-2 sequence ratio is higher in MINERVA data than in dsDL data (**Fig. 2C and 2D**), suggesting that MINERVA libraries capture more SARS-CoV-2 sequences. This phenomenon was more prominent for low viral load samples. Though SARS-CoV-2 genome coverage and depth was not high in SHERRY results due to low viral ratio and low sequencing depth, performing MINERVA subsequently can enrich the SARS-CoV-2 sequence ratio up to 10,000-fold (**Fig. 2E and S5**). As a result, MINERVA gives more complete and deeper coverage of SARS-CoV-2 genomes (**Fig. 2F and 2G**), despite sequencing dsDL libraries to two orders of magnitude more depth (**Fig. 2B**). We achieved even higher quality of MINERVA data by scaling up the reaction volume (**Fig. S5**). Using the same samples and the same amount of sequencing data, more input in a higher reaction volume resulted in deeper SARS-CoV-2 genome coverage.

178 The superior quality of MINERVA data became clearer when we included clinical RT-
179 qPCR results. Both dsDL and MINERVA libraries detect SARS-CoV-2 sequences for
180 samples with various Ct values, but MINERVA produced more complete and deeper
181 genome coverage than dsDL methods (**Fig. 2H and 2I**), and this advantage is more
182 pronounced for low viral load samples, including two samples with negative qPCR results.
183
184 Carrier RNA, which is widely used in viral DNA/RNA extraction before RT-qPCR assays,
185 severely impacts high-throughput sequencing analysis. Therefore, most RT-qPCR
186 positive clinical samples are not amenable to further viral genetic studies. We explored
187 the effect of adding polyT oligos during the rRNA removal step to simultaneously remove
188 spike-in polyA RNA and carrier RNA. By incorporating this step in MINERVA, we
189 successfully avoided the overwhelming representation of unwanted RNA sequences
190 while retaining desired human and viral sequences (**Fig. 2J**).
191
192 **MINERVA can be used to investigate multiple facets of COVID-19 biology.** Among
193 the three sample types, pharyngeal swabs, sputum, and stool samples, MINERVA
194 showed the biggest improvement over dsDL for stool samples (**Fig. 2H**), which prompted
195 us to investigate these samples further. By assessing the relative compositions of
196 bacterial species and viral species in each sample type, we noted that the bacteria largely
197 account for the difference between stool samples and the other two samples types, not
198 viruses (**Fig. 3A and S6**). This is likely due to the unique relationship between the
199 microbiome and its environment, subject to host-specific variations. In the stool samples,
200 the bacterial abundance and diversity are highest, and are most likely to dominate and
201 obscure signals coming from viral species. Since dsDL approaches are less sensitive and
202 require more input, this may explain why MINERVA outperforms dsDL most evidently in
203 stool samples.
204
205 Following this line of investigation based on metagenomic composition, we further
206 performed principle component analysis of specifically the bacterial sequences and
207 confirmed clear separation between stool sample and the other two sample types along
208 PC1 (**Fig. 3B**). Stool samples contained more *Bacteroides*, whereas the pharyngeal and

209 sputum samples were rich in *Streptococcus* (**Fig. 3C and S7**). There also appears to be
210 separation between samples by COVID-19 symptom severity along PC2 (**Fig. 3B**);
211 specifically, we found samples from severe and critical condition patients to be abundant
212 in *Comamonas* rather than *Streptococcus*, which is abundant in mild and severe condition
213 samples (**Fig. 3C**). SARS-CoV-2 mapping ratio of SHERRY libraries further confirmed
214 that virus is more abundant in samples from critically ill patients (**Fig. 3D and S8**).
215
216 As a novel virus, little known about the evolutionary features of SARS-CoV-2. Recent
217 studies have identified genetic variations and raised the possibility that multiple variants
218 could co-exist in the same host individual. We evaluated the performance of MINERVA in
219 detecting SARS-CoV-2 genetic variations. Aided by deep coverage of the viral genome
220 (**Fig. 3E**), MINERVA detects genetic variations more effectively than dsDL. Using 85
221 samples, we constructed a SARS-CoV-2 mutational profile (**Fig. 3E**), which was distinct
222 from the Guangdong profile(*8*). A few mutation sites, including the two linked to S and L
223 strains, were found in multiple samples. Surprisingly, despite high linkage between these
224 two positions, there were 6 samples in which we detected only the T28144C mutation but
225 not the C8782T mutation. Further investigation showed that the T28144C mutant fractions
226 in these 6 samples were all <87%, in contrast to >93% in the other 7 double-mutant
227 samples. These results, together with the intermediate mutation fractions in many other
228 sites, support the co-existence of multiple variants in the same individual. Further
229 investigation is required to understand this phenomenon.
230
231 In summary, MINERVA effectively converts SARS-CoV-2 genomes into sequencing
232 libraries with a simple and quick experimental pipeline, and subsequent target enrichment
233 can further improve SARS-CoV-2 genome coverage and genetic variation detection.
234 MINERVA can facilitate the study of SARS-CoV-2 genetics, and be easily implemented to
235 fight future RNA pathogen outbreaks.
236
237 **Discussion**
238 As of today, our knowledge of SARS-CoV-2 is still preliminary and much of it extrapolated
239 from past studies of other beta coronaviruses such as SARS and MERS. However, the

240 epidemiology, physiology, and biology of COVID-19 are evidently unique(*9*). To speed up
241 our investigation of this virus and the disease it causes, a practical protocol for viral
242 genome research of clinical samples is urgently needed. Currently, methods for
243 transforming clinical samples into sequencing libraries are laborious and painstaking,
244 while clinical personnel at the frontlines are already strained for time and energy.
245 MINERVA minimizes the need for expert technique and hands-on operation; we believe
246 it will be pivotal in accelerating clinical research of SARS-CoV-2.
247
248 Recent evolutionary tracing studies suggest the emergence of multiple novel, evolved
249 subtypes of SARS-CoV-2(*10*), including the S/L-subtypes(*11*) and the A/B/C-variants(*12*).
250 New variants will likely continue to emerge as the virus mutates, and to uncover them
251 requires deep, complete coverage of viral genomes from a large number of patients. With
252 the existence of asymptomatic carriers(*13*) and possible recurrent infections in the same
253 individual(*14*), longitudinal re-sampling of patients is also important to uncover intra-host
254 viral heterogeneity, but as viral load decreases with time(*15*), the sensitivity of the sample
255 processing method becomes critical. These studies all require processing large volumes
256 of clinical samples with a highly robust and scalable method that does not compromise
257 on sensitivity. We have demonstrated that MINERVA libraries from clinical samples can
258 generate deep and complete coverage of SARS-CoV-2 genomes that can be used for
259 evolutionary tracing and variant characterization research.
260
261 It is well-established now that SARS-CoV-2 can infect multiple organ systems, tissue
262 compartments, and cell types(*3*, *4*, *16*, *17*). In our profiling of COVID-19 clinical samples
263 from multiple body sites of the same patient, we found that the viral load and viral
264 subtypes vary across different body sites, possibly affected by interactions between
265 microbial and other viral species as well as overall metagenomic diversity present in
266 different microenvironments of each body site. The effects of metatranscriptomic diversity
267 and inter-compartment heterogeneity on SARS-CoV-2 biology and COVID-19 symptom
268 severity are also not understood. In particular, it is difficult to obtain high-quality unbiased
269 metatranscriptomes using conventional library construction methods from low-quantity
270 samples, as well as samples such as stool in which bacteria dominate the

metatranscriptomes, as conventional methods are not sufficiently sensitive. The versatility of MINERVA as a two-part protocol integrating SHERRY and post-library virus enrichment provides flexibility for sample processing that uses one standard sample pipeline for both highly sensitive metatranscriptomic analysis and targeted deep sequencing of specific transcripts.

MINERVA was not created to be a rapid diagnostic assay; rather, we hope its ease-of-use, versatility, scalability, sensitivity, and cost-effectiveness will drive adoption of routine sequencing of COVID-19 clinical samples, and thereby facilitate multiple areas of much-needed SARS-CoV-2 and COVID-19 research for clinicians and researchers.

Author contributions

C.C., Y.C., X.S.X., H.Z., Y.H. and J.W. conceived the project; J.L., P.D., Q.L. and C.S. conducted experiments; C.C., L.D., Q.J., J.L., Y.H. and J.W. analyzed the data; C.C., J.L., L.D., Q.J., A.R.W., Y.H. and J.W. wrote the manuscript with the help from all other authors.

Conflict of interest statement

The authors declare no conflict of interest.

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348

349

Figure Caption

Figure 1. Scheme of MINERVA. RNA extracted from pharyngeal swabs, sputum and stool samples undergo rRNA and DNA removal before a SHERRY processing pipeline metagenomic sequencing library construction. Multiple libraries were then pooled for SARS-CoV-2 sequence enrichment.

Figure 2. Direct comparison between MINERVA and the conventional dsDL strategy. (A) COVID-10 sample profiles. (B) Amount of sequencing data for different libraries. (C) SARS-CoV-2 mapping ratio statistics of SHERRY and dsDL libraries. (D) Comparison of SARS-CoV-2 mapping ratios between SHERRY and dsDL libraries. (E) Comparison of SARS-CoV-2 mapping ratios between SHERRY and MINERVA libraries. (F and G) SARS-CoV-2 genome coverage and depth statistics of MINERVA and dsDL libraries. (H and I) Comparison of SARS-CoV-2 sequencing results between MINERVA and dsDL libraries. (J) Removal of carrier RNA.

Figure 3. MINERVA could facilitate COVID-10 and SARS-CoV-2 research. (A) Total viral and bacterial ratios in different sample types. (B) Principle component analysis of bacteria composition. (C) Bacterial composition features of each sample. (D) SARS-CoV-2 map ratio of SHERRY libraries from patients with different severity. (E) SARS-CoV-2 mutation profiles.

Figure S1. Comparison of workflow between MINERVA and the conventional dsDL strategy.

Figure S2. Optimization of SHERRY protocol. (A-C) Effect of N10 primer during reserve transcription and Tn5 amount on detected gene number, ribosomal rate and insert size. (D-F) Effect of N10 primer during reserve transcription and Tn5 amount on gene body coverage evenness.

Figure S3. Sample collection profile.

381

382 Figure S4. Comparison between SHERRY and dsDL libraries on total viral ratio (A), total
383 fungal ratio (B), total bacterial ratio (C), and bacterial entropy (D).

384

385 Figure S5. SARS-CoV-2 genome sequencing results of MINERVA libraries. (A) SARS-
386 CoV-2 mapping ratio statistics of MINERVA libraries. (B and C) SARS-CoV-2 genome
387 coverage and depth statistics of SHERRY libraries. (D) Effect of sample input and reaction
388 volume on SARS-CoV-2 genome sequencing depth. (E) Results of carrier RNA removal.

389

390 Figure S6. Metagenomic profiles and the relationship between SARS-CoV-2 map ratio
391 and RT-qPCR Ct values.

392

393 Figure S7. Individual bacterial ratio in samples.

394

395 Figure S8. SARS-CoV-2 map ratio of dsDL libraries from patients with different severity.

Material and Methods

Ethics approval

This study was approved by the Ethics Committee of Beijing Ditan Hospital, Capital Medical University (No. KT2020-006-01).

Optimization of SHERRY protocol

We used the total RNA extracted from 3T3 cells to optimize experimental protocols. RNA extraction was performed using RNeasy Mini Kit (Qiagen, Cat.No.74104). DNA was then removed through DNase I (NEB, Cat.No.M0303) digestion. The resulting total RNA was concentrated by RNA Clean & Concentrator-5 kit (Zymo Research, Cat R1015), and its quality was assessed by the Fragment Analyzer Automated CE System (AATI). Its quantification was done by Qubit 2.0 (Invitrogen). To optimize the SHERRY protocol, different amount of random decamer (N10) (0, 10, or 100 pmol) was used to set up reverse transcription reactions. Titration of Tn5 transposome (0.2, 0.5, or 1.0 μl Vazyme V50; 0.05 or 0.25 μl home-made pTXB1) was performed in tagmentation procedure. In all tests, 10 ng 3T3 total RNA was used, and all reagents except for N10 or Tn5 transposome remain unchanged. All libraries were sequenced on Illumina NextSeq 500 with 2x75 paired-end mode. Clean data was aligned to GRCm38 genome and known transcript annotation using Tophat2 v2.1.1. Ribosome-removed aligned reads were proceeded to calculate FPKM by Cufflinks v2.2.1 and gene body coverage by RSeQC v.2.6.4.

Patients and clinical samples

From January 23, 2020 to March 20, 2020, 72 patients were enrolled in this study according to the 7th guideline for the diagnosis and treatment of COVID-19 from the National Health Commission of the People's Republic of China. All patients, diagnosed with COVID-19, were hospitalized in Beijing Ditan Hospital and classified into four severity degrees, mild, moderate, severe, and critical illness, according to the guideline. We collected 85 samples (38 pharyngeal swabs, 34 sputum samples, and 13 stool samples) from these patients.

427

428 **RNA extraction and rRNA removal**

429 For all the clinical samples, nucleic acids extraction was performed in a BSL-3 laboratory.
430 Samples were deactivated by heating at 56°C for 30 min before extraction. Total RNA
431 was extracted using QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's
432 instructions. In most samples (79 out of 85) we specifically omitted the use of carrier RNA
433 due to its interference on the most prevalent sample preparation protocols for high-
434 throughput sequencing. After nucleic acids extraction, rRNA was removed by rDNA probe
435 hybridization and RNase H digestion, followed by DNA removal through DNase I digestion,
436 using MGIEasy rRNA removal kit (BGI, Shenzhen, China). The final elution volume was
437 12-20 μl for each sample. For carrier RNA removal tests, 1.7 μg polyA carrier RNA was
438 spiked into 18 μl of elute from QIAamp Viral RNA Mini Kit. To remove the carrier RNA from
439 these spike-in samples and other samples extracted with carrier RNA, 2 μg poly(T) 59-
440 mer (T59) oligo was added during the rDNA hybridization step.

441

442 **dsDL Metagenomic RNA library construction and sequencing**

443 The libraries were constructed using MGIEasy reagents (BGI, China) following
444 manufacture's instruction. The purified RNA, after rRNA depletion and DNA digestion,
445 underwent reverse transcription, second strand synthesis, and sequencing adaptor
446 ligation. After PCR amplification, DNA was denatured and circularized before being
447 sequenced on DNBSEQ-T7 sequencers (BGI, China).

448

449 **MINERVA library preparation**

450 Totally, 2.7 μl RNA from rRNA and DNA removal reaction was used for standard SHERRY
451 reverse transcription, with the following modifications: 1) 10 pmol random decamer (N10)
452 was added to improve coverage; 2) initial concentrations of dNTPs and oligo-dT (T30VN)
453 were increased to 25 mM and 100 μM, respectively. For 5.4 μl and 10.8 μl input, the entire
454 reaction was simply scaled up 2 and 4 folds, respectively. The RNA/DNA hybrid was
455 tagmented in TD reaction buffer (10 mM Tris-Cl pH 7.6, 5 mM $MgCl_2$, 10% DMF)
456 supplemented with 3.4% PEG8000 (VWR Life Science, Cat.No.97061), 1 mM ATP (NEB,
457 Cat.No. P0756), and 1U/μl RNase inhibitor (TaKaRa, Cat.No. 2313B). The reaction was

458 incubated at 55°C for 30 min. 20 µl tagmentation product was mixed with 20.4 µl Q5 High-
459 Fidelity 2X Master Mix (NEB, Cat.No. M0492L), 0.4 µl SuperScript II reverse transcriptase,
460 and incubated at 42°C for 15 min to fill the gaps, followed by 70°C for 15 min to inactivate
461 SuperScript II reverse transcriptase. Then index PCR was performed by adding 4 µl 10
462 µM unique dual index primers and 4 µl Q5 High-Fidelity 2X Master Mix, with the following
463 thermo profile: 98°C 30 s, 18 cycles of [98°C 20 s, 60°C 20 s, 72°C 2 min], 72°C 5 min.
464 The PCR product was then purified with 0.8x VAHTS DNA Clean Beads (Vazyme, Cat.
465 No. N411). These SHERRY libraries were sequenced on Illumina NextSeq 500 with 2x75
466 paired-end mode for metagenomic analysis.
467 For preparing MINERVA libraries through SARS-CoV-2 enrichment, 1µl SHERRY
468 metagenomic library was first quantified with N gene using quantitative PCR (F:
469 GGGGAACTTCTCCTGCTAGAAT, R: CAGACATTTTGCTCTCAAGCTG) after 1:200
470 dilution, then multiple libraries were pooled together based on qPCR results and
471 processed with TargetSeq One Cov Kit (iGeneTech, Cat.No.502002-V1) following
472 manufacturer's instruction. The iGeneTech Blocker was replaced by the IDT xGen
473 Universal Blockers (NXT). These MINERVA libraries were sequenced on Illumina
474 NextSeq 500 with 2x75 paired-end mode for deep SARS-CoV-2 analysis.
475
476 **Data processing**
477 For metagenomic RNA-seq data, raw reads were quality controlled using BBmap (version
478 38.68) and mapped to the human genome reference (GRCh38) using STAR (version
479 2.6.1d) with default parameters. All unmapped reads were collected using samtools
480 (version 1.3) for microbial taxonomy assignment by Centrifuge (version 1.0.4). Custom
481 reference was built from all complete bacterial, viral and any assembled fungal genomes
482 downloaded from NCBI RefSeq database (viral and fungal genomes were downloaded
483 on February 4th, 2020, and bacterial genomes were downloaded on November 14th,
484 2018). There were 11,174 bacterial, 8,997 viral, and 308 fungal genomes respectively.
485 Bacterial Shannon diversity (entropy) was calculated at species level, and the species
486 abundance was measured based on total reads assigned at the specific clade normalized
487 by genome size and sequencing depth. Bacterial genus composition was analyzed based
488 on reads proportion directly assigned by Centrifuge. For dsDL sequencing data, sub-

489 sampling was performed for each sample to obtain ~12M paired-end nonhuman reads,
490 which is the median of SHERRY datasets. Same workflow was performed as described
491 above for the removal of human reads and microbial taxonomy assignment.
492 For SARS-CoV-2 genome analysis, raw reads were trimmed to remove sequencing
493 adaptors and low-quality bases with Cutadapt v1.15. BWA 0.7.15-r1140 was used to align
494 reads to the SARS-CoV-2 reference genome (NC_045512.2). Then we removed
495 duplicates from the primary alignment with Picard Tools v2.17.6. We used mpileup
496 function in samtools v1.10 to call SNP and InDel with parameter -C 50 -Q 30 -q 15 -E -d
497 0. We called mutation if the depth >=10 and strand bias >0.25. The strand bias is defined
498 as the value that minimum of positive strand depth and negative strand depth divided by
499 the maximum.
500
501
502 **Data deposition**
503 The datasets generated during this study (related to Figure 2 and 3) are not publicly
504 available due to ethical concerns. Partial access to the datasets is available from the
505 corresponding author on reasonable request.

Figure 1

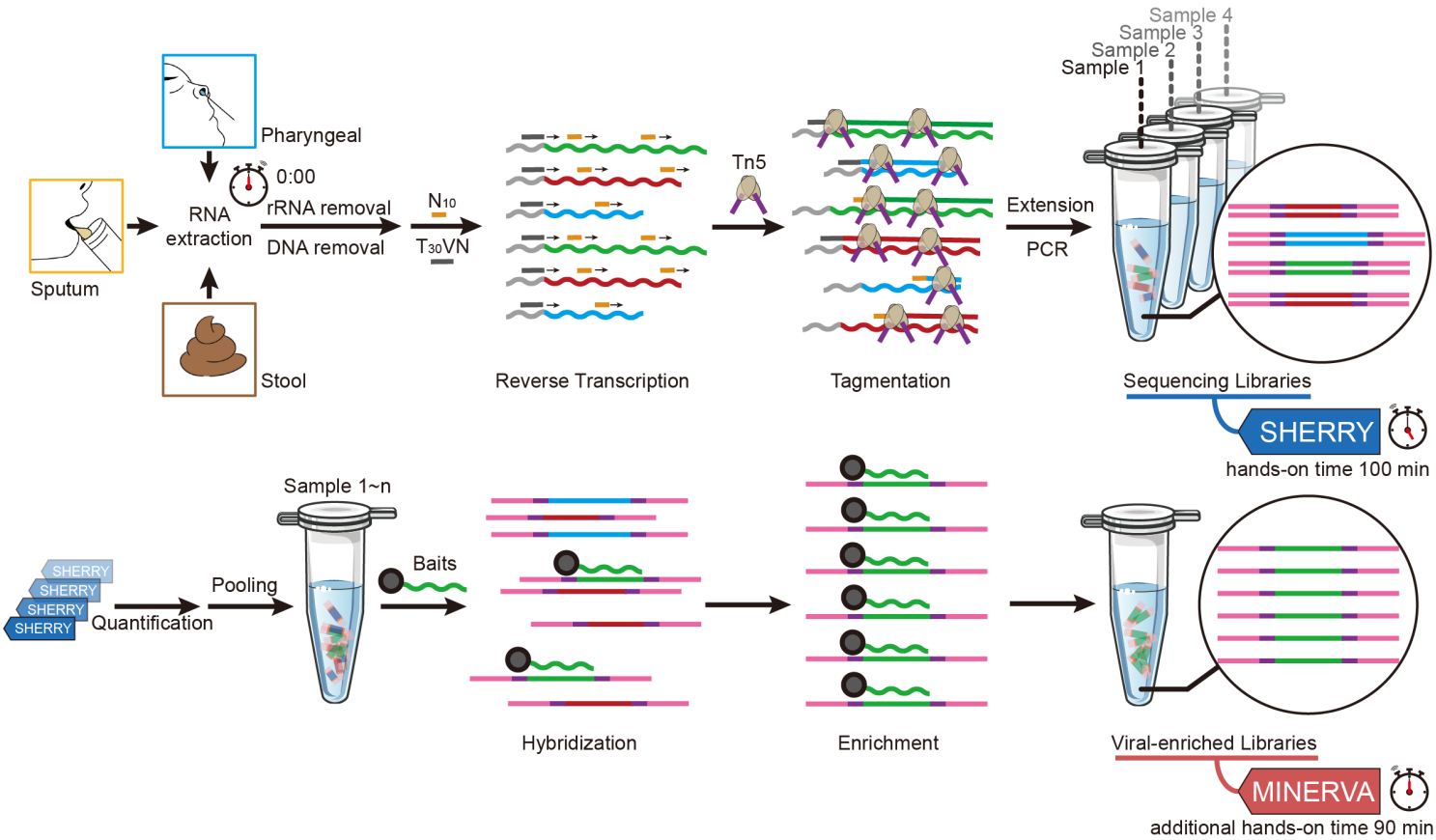

Pharyngeal
0:00
Sputum
RNA extraction
rRNA removal
DNA removal
N10
T30VN
Stool
Reverse Transcription
Tn5
Tagmentation
Extension
PCR
Sample 4
Sample 3
Sample 2
Sample 1
Sequencing Libraries
SHERRY
hands-on time 100 min
SHERRY
SHERRY
SHERRY
SHERRY
Quantification
Pooling
Sample 1~n
Baits
Hybridization
Enrichment
Viral-enriched Libraries
MINERVA
additional hands-on time 90 min

Figure 2

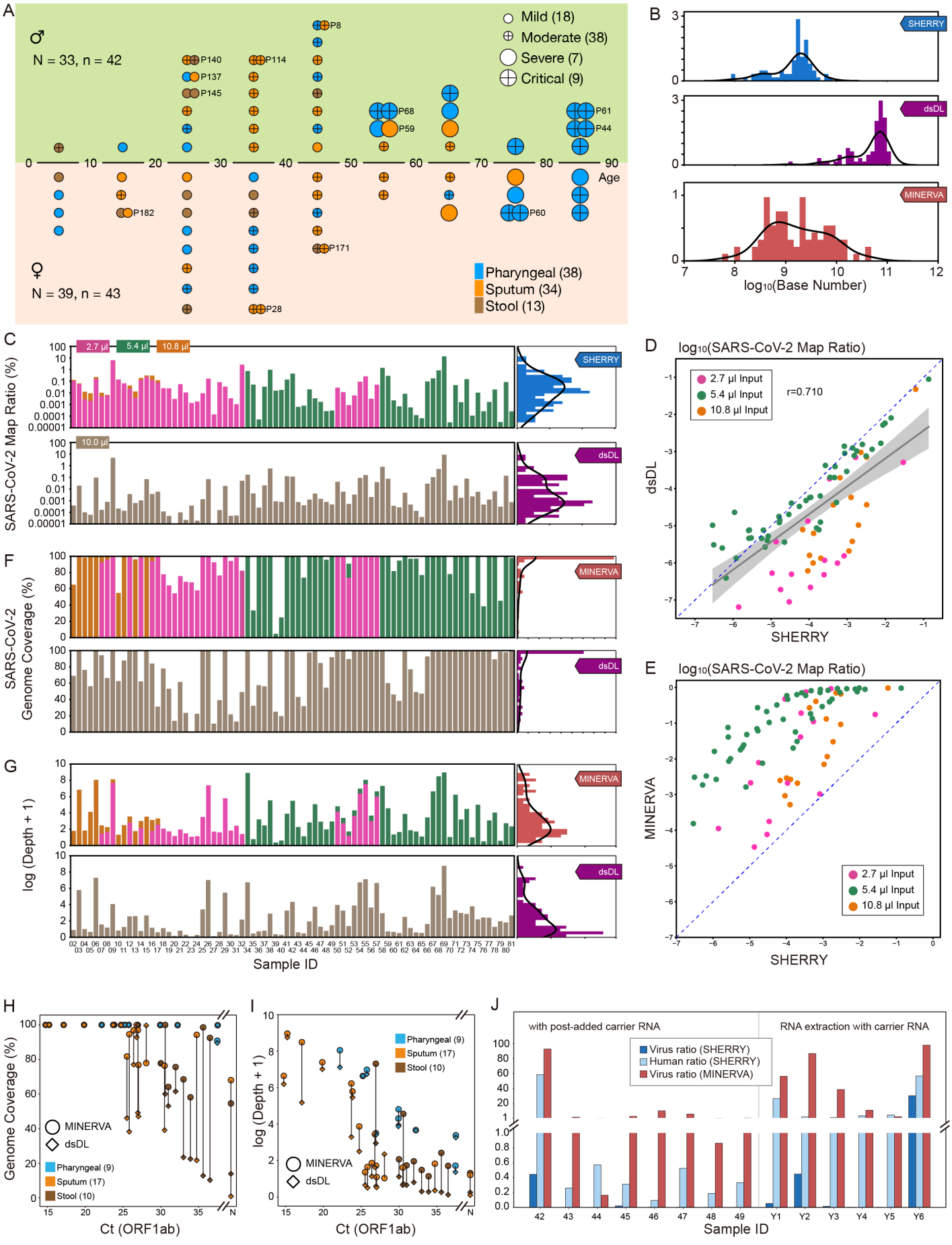
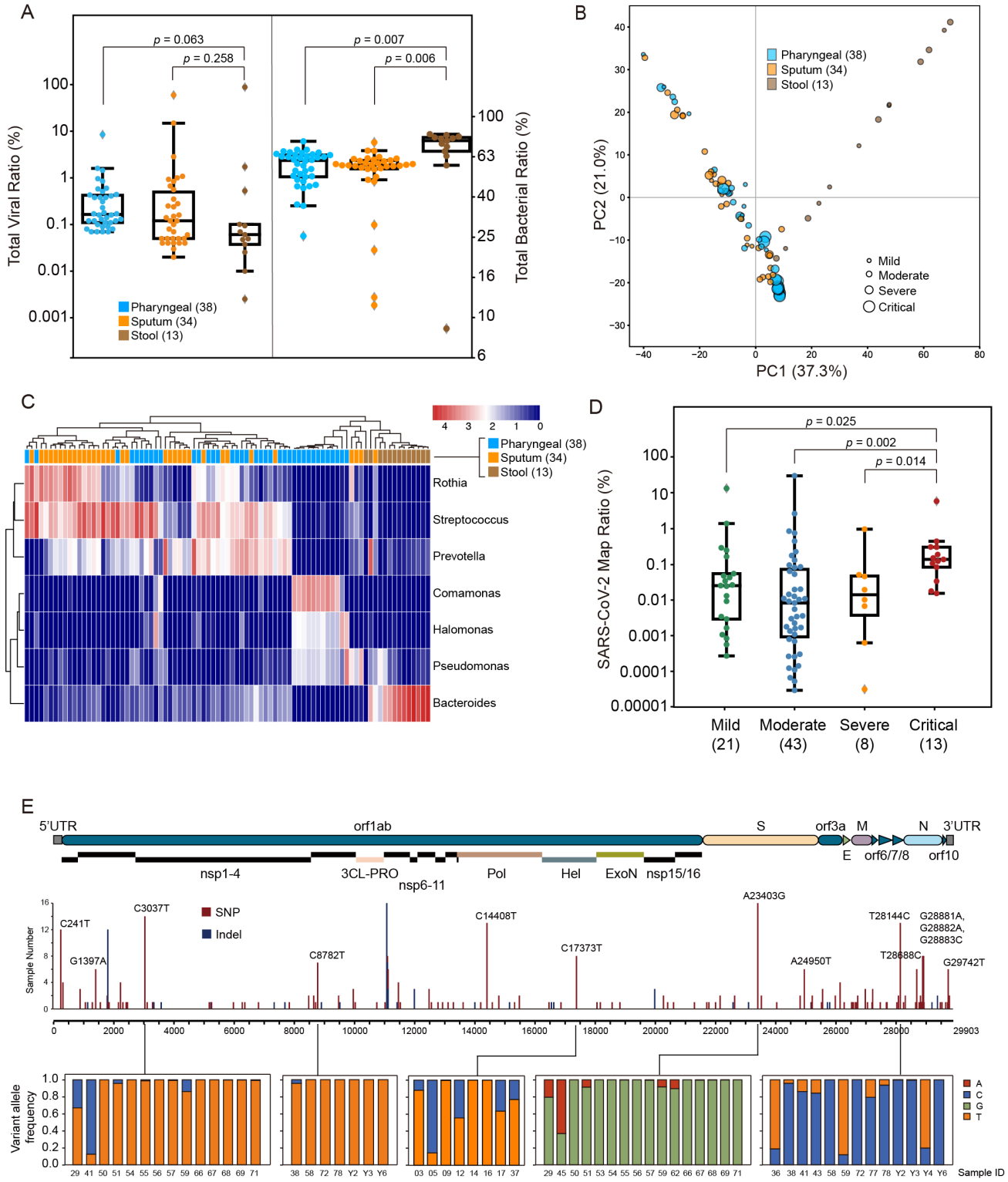
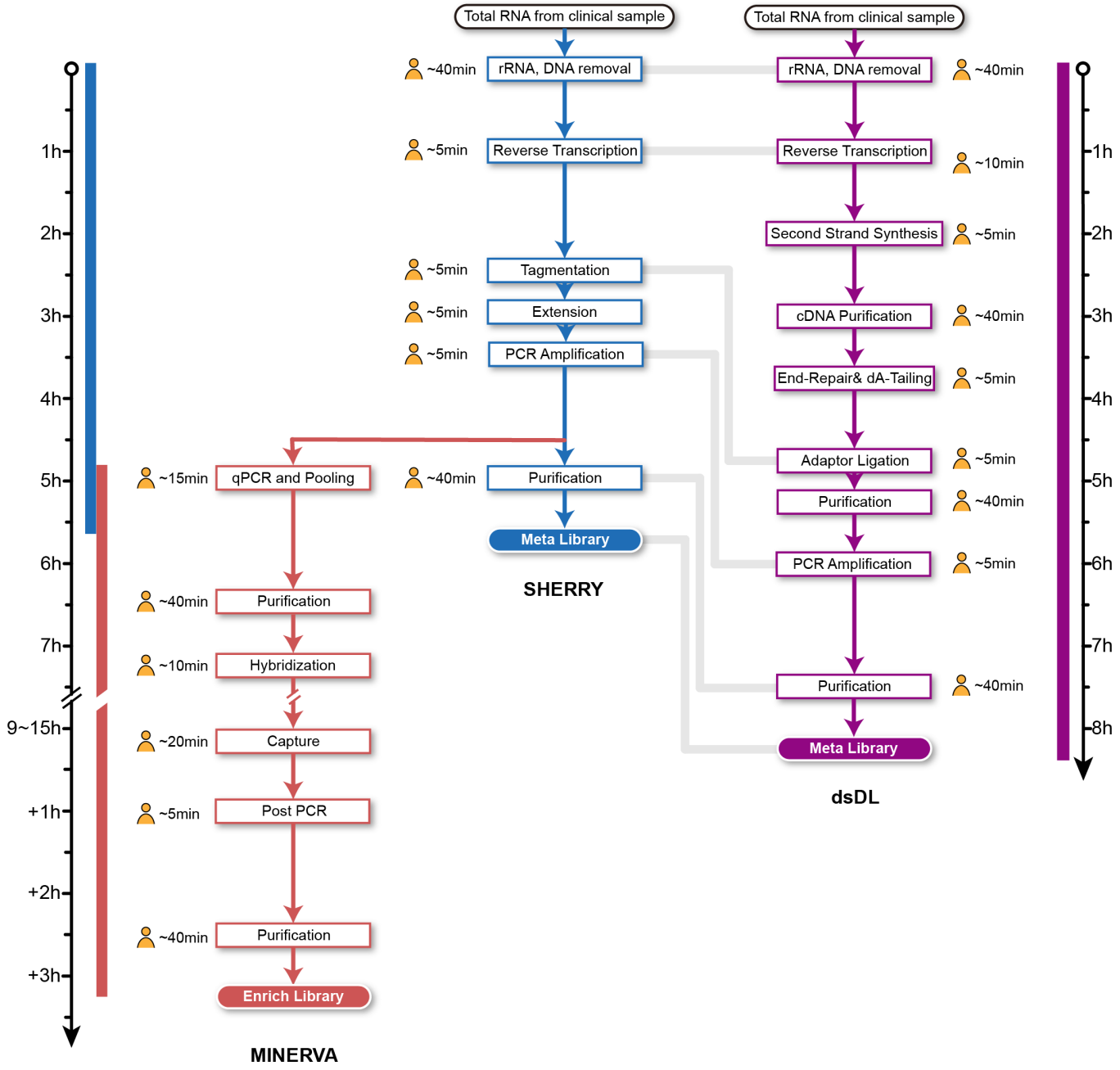

A
♂
N = 33, n = 42
♀
N = 39, n = 43
Mild (18)
Moderate (38)
Severe (7)
Critical (9)
Pharyngeal (38)
Sputum (34)
Stool (13)
Age
B
SHERRY
dsDL
MINERVA
log10(Base Number)
C
2.7 µl
5.4 µl
10.8 µl
10.0 µl
SARS-CoV-2 Map Ratio (%)
D
log10(SARS-CoV-2 Map Ratio)
2.7 µl Input
5.4 µl Input
10.8 µl Input
r=0.710
dsDL
SHERRY
E
log10(SARS-CoV-2 Map Ratio)
MINERVA
SHERRY
F
SARS-CoV-2 Genome Coverage (%)
G
log (Depth + 1)
Sample ID
H
SARS-CoV-2 Genome Coverage (%)
MINERVA
dsDL
Pharyngeal (9)
Sputum (17)
Stool (10)
Ct (ORF1ab)
I
log (Depth + 1)
Pharyngeal (9)
Sputum (17)
Stool (10)
MINERVA
dsDL
Ct (ORF1ab)
J
with post-added carrier RNA
RNA extraction with carrier RNA
Virus ratio (SHERRY)
Human ratio (SHERRY)
Virus ratio (MINERVA)
Sample ID

Figure 3

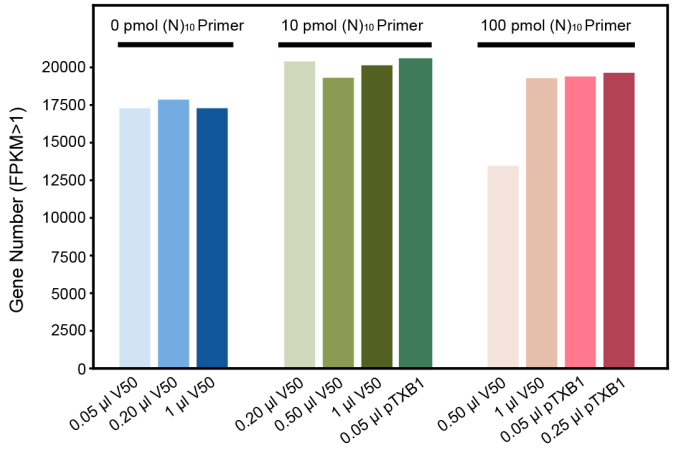

A
p = 0.063
p = 0.258
p = 0.007
p = 0.006
Total Viral Ratio (%)
Total Bacterial Ratio (%)
Pharyngeal (38)
Sputum (34)
Stool (13)
B
PC2 (21.0%)
PC1 (37.3%)
Mild
Moderate
Severe
Critical
C
Rothia
Streptococcus
Prevotella
Comamonas
Halomonas
Pseudomonas
Bacteroides
D
p = 0.025
p = 0.002
p = 0.014
SARS-CoV-2 Map Ratio (%)
Mild (21)
Moderate (43)
Severe (8)
Critical (13)
E
5'UTR
orf1ab
S
orf3a
M
N
3'UTR
E
orf6/7/8
orf10
nsp1-4
3CL-PRO
nsp6-11
Pol
Hel
ExoN
nsp15/16
SNP
Indel
Sample Number
C241T
C3037T
G1397A
C8782T
C14408T
C17373T
A23403G
A24950T
T28144C
G28881A, G28882A, G28883C
T28688C
G29742T
Variant allele frequency
Sample ID

Supplementary Figure 1

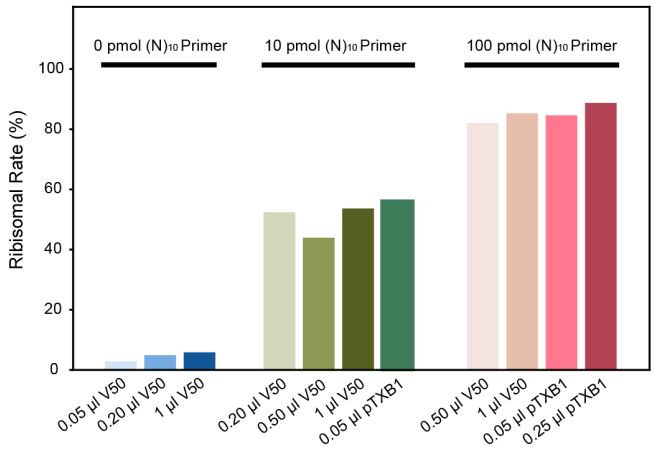


Supplementary Figure 2

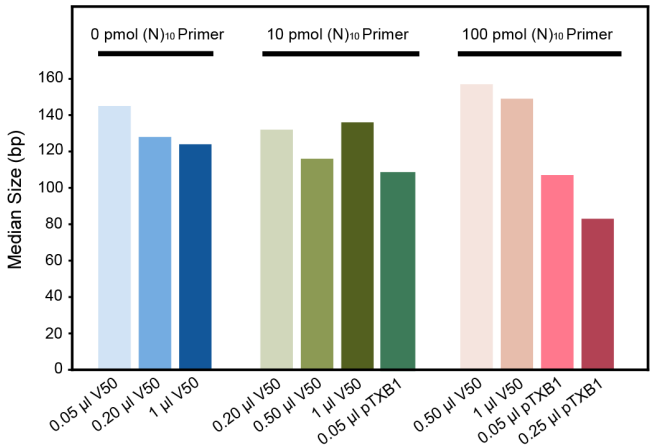
A



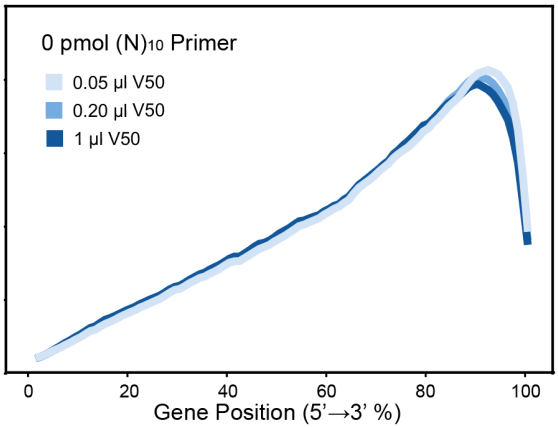
B



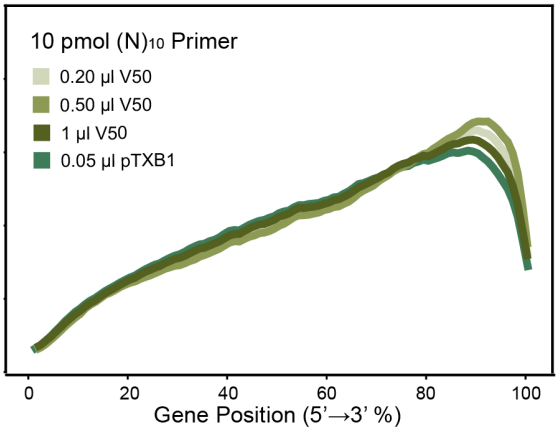
C



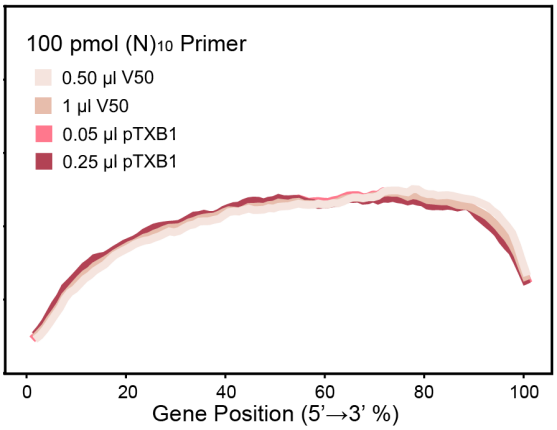
D



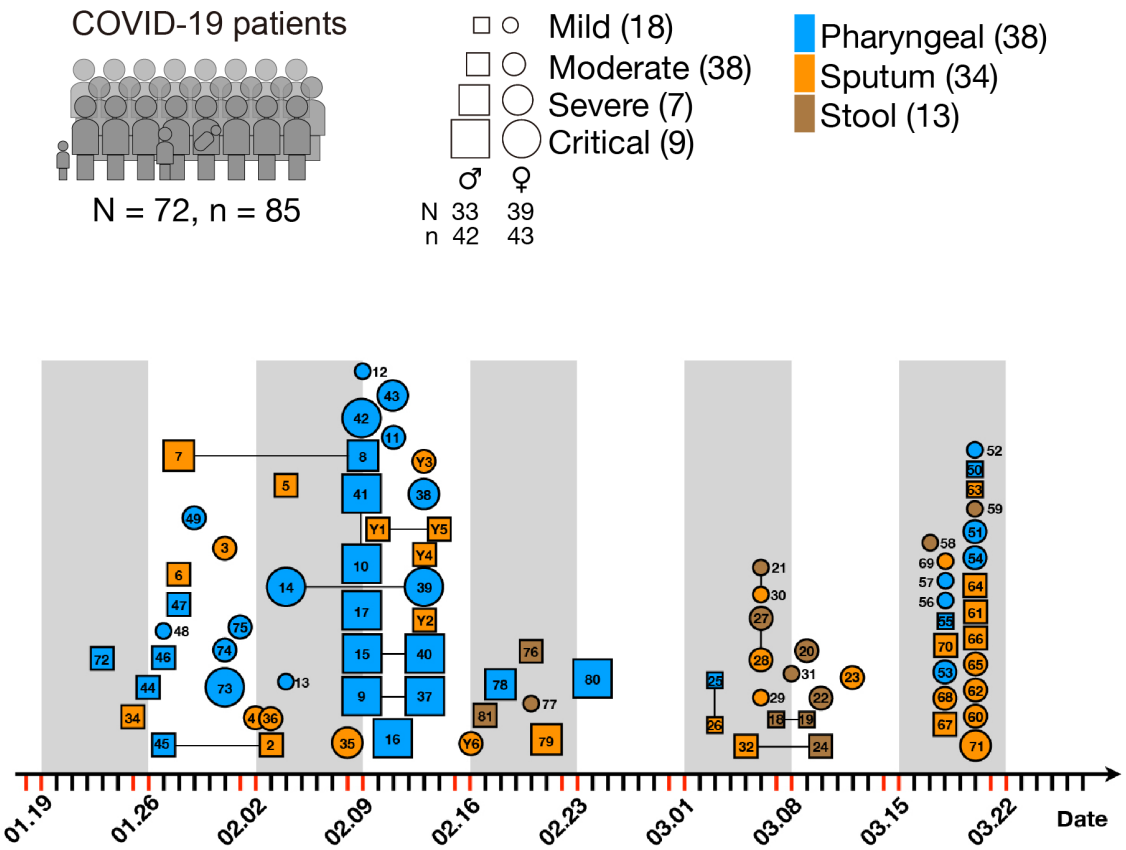
E



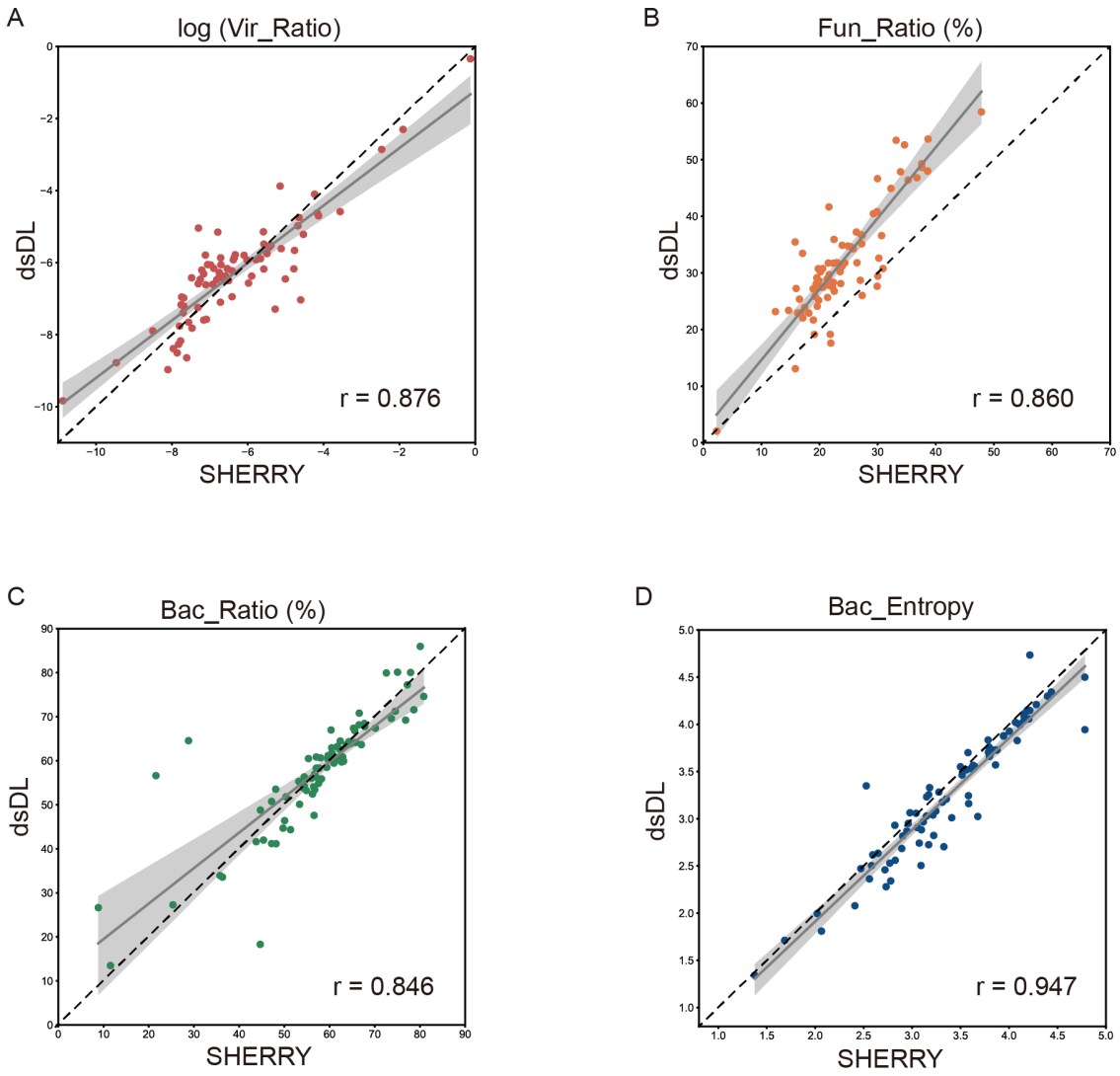
F



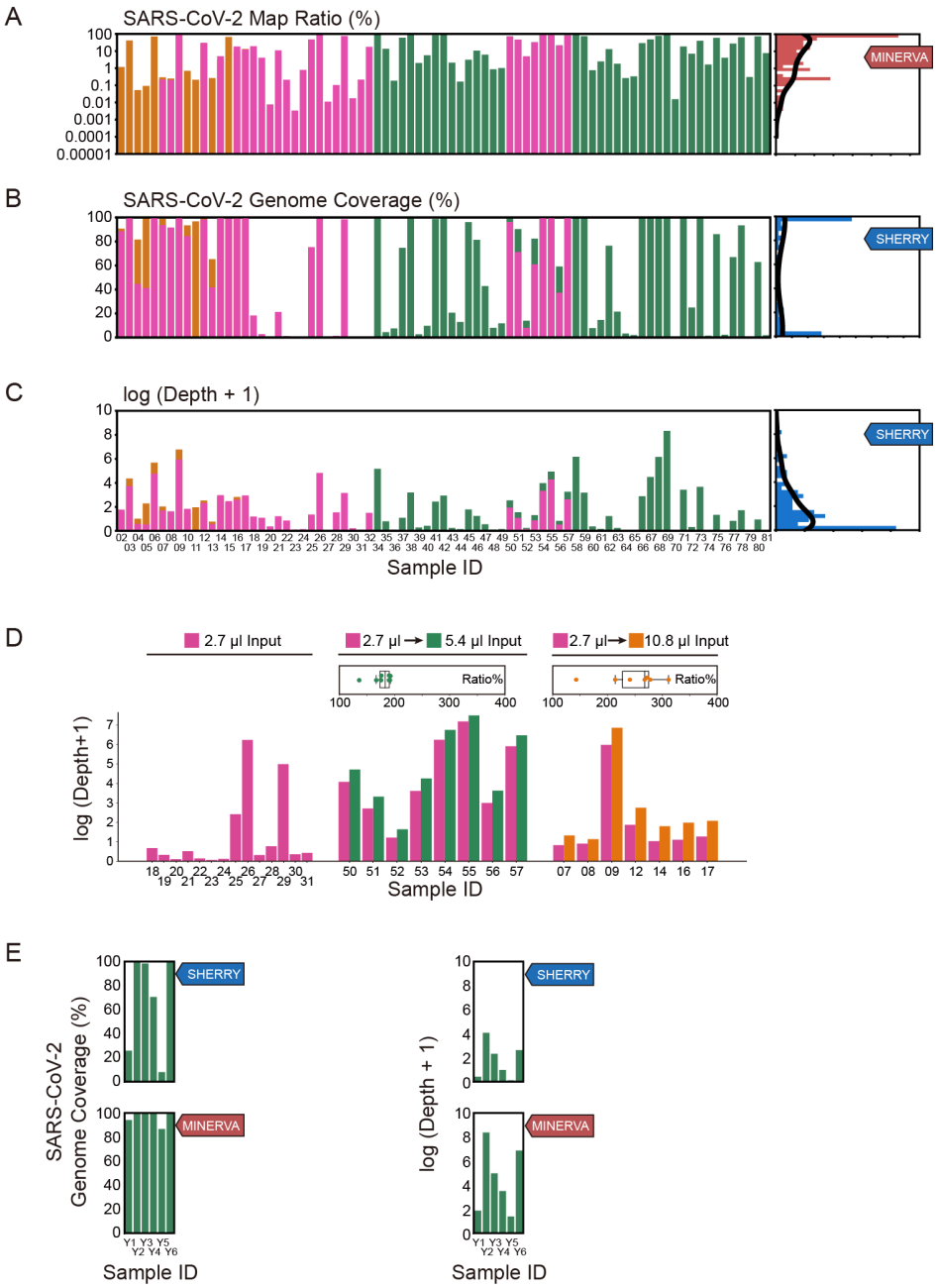
Supplementary Figure 3



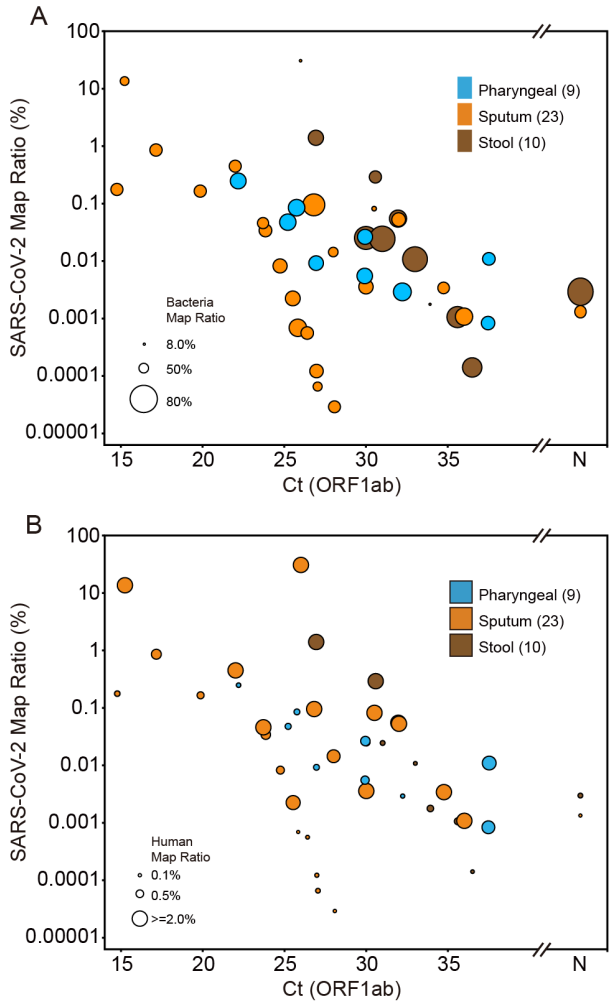
Supplementary Figure 4



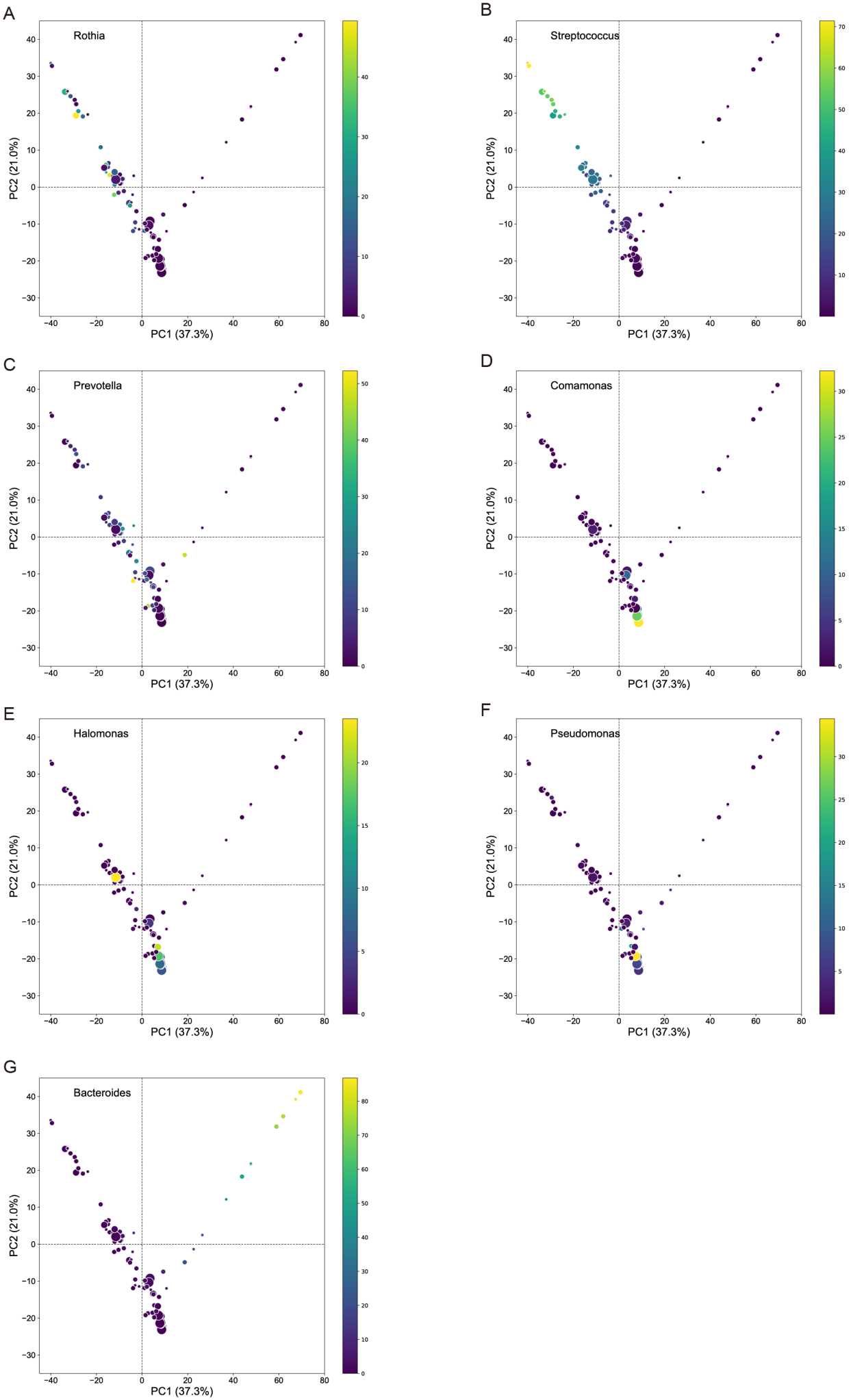
Supplementary Figure 5



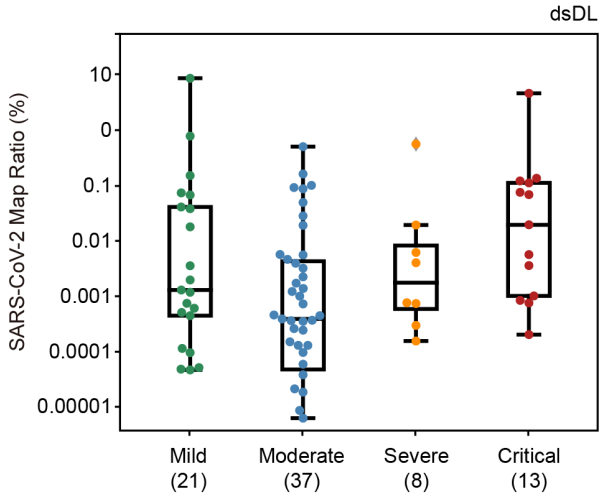
Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8



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.