

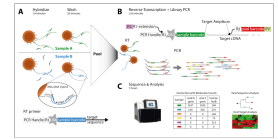
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SARS-CoV-2 detection with ApharSeq V.3

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We use this protocol and it's working

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Abstract

The global SARS-CoV-2 pandemic led to a steep increase in the need for viral detection tests worldwide. Most current tests for SARS-CoV-2 are based on RNA extraction followed by quantitative reverse-transcription PCR assays that involve a separate RNA extraction and qPCR reaction for each sample with a fixed cost and reaction time. While automation and improved logistics can increase the capacity of these tests, they cannot exceed this lower bound dictated by one extraction and reaction per sample. Multiplexed next generation sequencing (NGS) assays provide a dramatic increase in throughput, and hold the promise of richer information on viral strains and host immune response.

Here, we establish a significant improvement of existing RNA-seq detection protocols. Our workflow, **ApharSeq (Amplicon Pooling by Hybridization And RNA-Seq)**, includes a fast and cheap RNA capture step, that is coupled to barcoding of individual samples, followed by sample-pooling prior to the reverse transcription, PCR and massively parallel sequencing. Thus, only one step is performed before pooling hundreds of barcoded samples for subsequent steps and further analysis. Considering these improvements, our proposed workflow is estimated to reduce costs by 10-50 fold, labor by 5-100 fold, automated liquid handling by 5-10 fold, and reagent requirements by 100-1000 fold compared to existing methods.



Attachments



[ApharSeq_oligos.xlsx](#)

26KB

Guidelines

This protocol was tested on samples derived by a swab into a standard **Viral Transport Medium** collection tube, and then mixed 1:1 **with Zymo RNA/DNA shield** for lysis.

The first part of this protocol, before samples are pooled, can be performed manually or robotically and was tested on an a Tecan / Evoware platform.



Materials

MATERIALS

⊗ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

⊗ Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #KK2612**

⊗ Water, nuclease free

⊗ 1M Tris pH 7.5 **Merck MilliporeSigma (Sigma-Aldrich)**

⊗ Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL **GE Healthcare Catalog #65152105050250**

⊗ 20% Sodium dodecyl sulfate (SDS)

⊗ High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #5067-5584**

⊗ 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #15568025**

⊗ Ethanol

⊗ Qubit 1X dsDNA High Sensitivity Assay Kit **Thermo Fisher Scientific Catalog #Q33230**

⊗ Ethylenediaminetetraacetic acid (EDTA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #EDS**

⊗ Lithium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L4632**

⊗ 1M DL-Dithiothreitol solution (DTT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #646563**

⊗ Magnesium chloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M8787-5ML**

⊗ Dimethyl sulfoxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D4540**

⊗ SMARTScribe™ Reverse Transcriptase **Takara Bio Inc. Catalog #639538**

⊗ Proteinase K **Lucigen Catalog #MPRK092**

Beads conjugation

Materials for this step are listed in [manufacturer conjugation protocol](#)

Binding buffer

100 mM Tris-HCl, pH 7.5

500 mM LiCl

10 mM EDTA, pH 8.0

5 mM DTT

Wash buffer A

10 mM Tris-HCl, pH 7.5

150 mM LiCl

1 mM EDTA, pH 8.0

0.1 % SDS

Wash buffer B



10 mM Tris-HCl, pH 7.5

150 mM LiCl

Proteinase K (PK) buffer

10mM Tris pH8

5mM EDTA

1% SDS

RT reaction mix - make fresh before RT reaction

1x SmartScribe buffer

1.2 mM dNTPs

2 mM DTT

6 mM MgCl₂

5% DMSO

100 U SmartScribe enzyme per reaction

Primers

As an example, for the N1 amplicon the following primers were used (barcodes in **bold**):

RT -

GCGTCAGATGTGTATAAGAGACAGNNNN**CTGAC**NNNN**CGGCA**NNNTCTGGTTACTGCCAGTTGAATCTG

PCR F -

AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

PCR R -

CAAGCAGAAGACGGCATACGAGAT**TGATACGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACCCCAAAATC
AGCGAAA

See complete primer list in attached file, and review "methods" section in accompanying paper for more details.

Safety warnings



A risk assessment should be made when working with potentially infectious specimen. Be sure that samples were properly handled and deactivated by certified personnel. Consult your local bio-safety staff.



Before start

- Take out beads and buffers and bring to room temp
- Pre-warm 72C heating block
- Prepare fresh 70% EtOH
- Prepare poly dT beads: Use commercial polyT beads (ThermoFisher dynabeads cat# **61002**), or prepare oligo dT beads by conjugating an amine-C12 5' modified 25 dT oligonucleotide to carboxylate coated beads (GE healthcare Sera-Mag SpeedBeads cat# 65152105050250), and follow the **manufacturer conjugation protocol**
- Clinical samples are assumed to have been collected in viral transport media and inactivated by 1:1 mix with lysis buffer
- Before using RT primers for the first time we recommend testing them for cross-contamination and performance variation. We do so by pooling (e.g.) every other column to two pools and preparing libraries using these oligo pools. If a barcode appears in a pool where it was not used there is contamination that should be accounted for. In case of significant variance or contamination between primers either discard the extreme primers, or consider pooling several primers together (e.g. every sample will have 3 barcodes) to reduce sample-to-sample variability.



Apharseq

14h

1 Prepare poly dT beads

1. Use 5 µl poly dT beads/sample
2. Wash beads twice in binding buffer:
 - 2.1 Resuspend in binding buffer
 - 2.2 Magnetize and remove buffer
3. Resuspend beads in 320 µl binding buffer

2 Hybridization to beads

1m

1. Add 320 µl inactivated viral sample to 320 µl beads in binding buffer
2. Incubate 00:10:00 at Room temperature while mixing
3. Magnetize and remove supernatant

3 Primer annealing

15m

1. Resuspend beads in 50 µl 1:1 mix of binding buffer and 10 µM RT primer(s)
2. Heat sample to 72 °C for 00:02:00 then incubate On ice for at least 00:02:00
2. Incubate samples 00:10:00 at Room temperature . Mix by rotating or by pipetting every 5 minutes
3. Magnetize beads and discard supernatant

4 Wash and Pool

15m

1. Resuspend beads in 450 µl wash buffer A
2. Magnetize and remove 380 µl
2. Resuspend beads in remaining 70µl and pool samples.
3. Wash beads twice in wash buffer B:
 - 2.1 Resuspend in 500 µl buffer B
 - 2.2 Magnetize and carefully remove buffer

Note

Pool can be stored in **RNA later** at 4 °C for a week. If stored, prior to step 5, wash twice in Wash buffer B

5 Proteinase K (PK) treatment

1h 15m

1. Wash with 500 µl PK buffer
2. Resuspend in 500 µl PK mix (0.4mg/ml PK in PK bufer)
3. Incubate 01:00:00 at Room temperature while rotating.
4. Magnetize and wash three times in 1ml wash buffer A



5. Magnetize and wash twice in 1ml wash buffer B

Note

This step may need calibration to shorten incubation time at RT or 37C

6 Reverse Transcription

1h 30m

1. Magnetize PCR tube and wash pooled beads in 1x RT buffer:

1.1 Resuspend in 50 µl 1xRT buffer

1.2 Magnetize and remove buffer

2. Resuspend in 50 µl RT reaction mix

3. Incubate

⌚ 00:15:00 🌡 25 °C

⌚ 00:45:00 🌡 42 °C

⌚ 00:15:00 🌡 70 °C

Note

We also found a standard RT-PCR kit to work on the beads (**PrimeScript™ One Step RT-PCR**). Importantly we execute the RT part of the reaction, and only then add the PCR primers.

7 cDNA elution

3m

1. Incubate the beads for ⌚ 00:02:00 at 🌡 98 °C

2. Magnetize and transfer the supernatant immediately to a new tube

8 Cleanup by 2x SPRI

20m

1. Add 100 µl SPRI beads and mix well

2. Incubate ⌚ 00:04:00

3. Magnetize: keep on magnet for ⌚ 00:04:00 and remove supernatant

4. Keep on magnet and wash twice with 70% ethanol

4.1 Add 100 µl 70% ethanol

4.2 Incubate ⌚ 00:00:30

4.3 Remove the ethanol without disturbing the beads

5. Air dry to remove traces of ethanol ⌚ 00:04:00

6. Resuspend in 23 µl 10 mM Tris 8.0

7. Incubate ⌚ 00:02:00

8. Magnetize and transfer 21 µl to new tube



9 Library PCR

1. Add to each sample pool
 - 25 µl Kapa RM
 - 2 µl 10 µM PCR-primer F
 - 2 µl 10 µM PCR-primer R
2. PCR program:
 - 98°C 3 minutes
 - 30 x {95°C 20sec, 59°C 15sec, 72°C 50sec};
 - 72°C 10 min
 - 10°C

10 Cleanup by 1x SPRI (see step 8)

Elute in 15 µl 10 mM Tris 8.0

11 Library assessment

1. Determine library concentration (e.g. by Qubit) and size (by tape/gel)
2. If necessary, clean primer dimers by running on gel or by 1x SPRI

12 Sequence

On an Illumina machine.

Assign 5 million reads per 100 sample pool.

Assign at least 20 cycles to R1 (barcode + UMI) and either additional 30 cycles to R1 or 30 cycles to R2

13 Data Analysis Outline

1. Demultiplex pool indices with the bcl2fastq script (Illumina)
2. Process each fastq file independently
 - 2.1 Split reads based on RT primer target (E/N1/ActB) and barcode (R1:4-8 + R1:13-17, inclusive)
 - 2.2 Filter reads that do not exhibit their expected sequence
 - i.e. read with E primer should have the E sequence immediately after the primer
 - 2.2 Collect UMI sequence per read (R1:1-4 + R1:9-12 + R1:18-19)
 - 2.3 Cluster UMI sequences (e.g. by hamming distance and/or with UMI-tools)
 - 2.4 Report unique molecules per target/sample