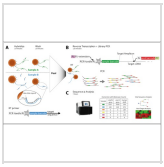

Version 3

Sep 04, 2020

SARS-CoV-2 detection with ApharSeq V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.bkdcks2w

XPRIZE Rapid Covid Testing

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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** (**A**mplicon **P**ooling by **H**ybridization **A**nd **R**NA-**S**eq), 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

DOI

dx.doi.org/10.17504/protocols.io.bkdcks2w

PROTOCOL CITATION

Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman 2020. SARS-CoV-2 detection with ApharSeq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bkdcks2w

KEYWORDS

SARS-CoV-2, covid19, RNA, Aphar-Seq, NGS, ApharSeq, test, diagnostics, virus

Citation: Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman (09/04/2020). SARS-CoV-2 detection with ApharSeq. https://dx.doi.org/10.17504/protocols.io.bkdcks2w

CREATED

Aug 27, 2020

LAST MODIFIED

Sep 04, 2020

PROTOCOL INTEGER ID

41092

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 roboticaly and was tested on an a Tecan / Evoware platform.

MATERIALS

NAME	CATALOG #	VENDOR
Agencourt AMPure XP	A63880	Beckman Coulter
Kapa HiFi Hotstart ReadyMix (2x)	KK2612	Kapa Biosystems
Water, nuclease free		
1M Tris pH 7.5		Sigma
Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL	65152105050250	Ge Healthcare
20% Sodium dodecyl sulfate (SDS)		
High Sensitivity D1000 ScreenTape	5067-5584	Agilent Technologies
1M Tris-HCl (pH 8.0)	15568025	Thermo Fisher Scientific
Ethanol		
Qubit 1X dsDNA High Sensitivity Assay Kit	Q33230	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	EDS	Sigma Aldrich
Lithium dodecyl sulfate	L4632	Sigma Aldrich
1M DL-Dithiothreitol solution (DTT)	646563	Sigma Aldrich
Magnesium chloride solution	M8787-5ML	Sigma Aldrich
Dimethyl sulfoxide	D4540	Sigma Aldrich
SMARTScribe™ Reverse Transcriptase	639538	Takara
Proteinase K	MPRK092	Lucigen

MATERIALS TEXT

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

Citation: Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman (09/04/2020). SARS-CoV-2 detection with ApharSeq. https://dx.doi.org/10.17504/protocols.io.bkdcks2w

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**NNTCTGGTTACTGCCAGTTGAATCTG
PCR F -
AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
PCR R -
CAAGCAGAAGACGGCATACGAGAT**TGATACGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACCCCAAAATCAGCGAAA

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 potenetially infectious specimen. Be sure that samples were properly handled and deactivated by certified personnal. Consult your local bio-safety staff.

BEFORE STARTING

- 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 prepararing 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 significnat variance or contmaination 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

Citation: Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman (09/04/2020). SARS-CoV-2 detection with ApharSeq. https://dx.doi.org/10.17504/protocols.io.bkdcks2w

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 carfully remove buffer

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

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**

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.

Citation: Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman (09/04/2020). SARS-CoV-2 detection with ApharSeq. https://dx.doi.org/10.17504/protocols.io.bkdcks2w

3m

7 cDNA elution

1. Incubate the beads for **00:02:00** at **98 °C**
2. Magnetize and transfer the supenatant immediately to a new tube

20m

8 Cleanup by 2x SPRI

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. Magnetzie and transfer 21 µl to new tube

1h 30m

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 Analasys 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

Citation: Daphna Strauss, Ayelet Rahat, Israa Sharkia, Alon Chappleboim, Miriam Adam, Daniel Kitsberg, Gavriel Fialkoff, Matan Lotem, Omer Gershon, Anna-Kristina Schmidtner, Esther Oiknine-Djian, Agnes Klochendler, Ronen Sadeh, Yuval Dor, Dana Wolf, Naomi Habib, Nir Friedman (09/04/2020). SARS-CoV-2 detection with ApharSeq. https://dx.doi.org/10.17504/protocols.io.bkdcks2w