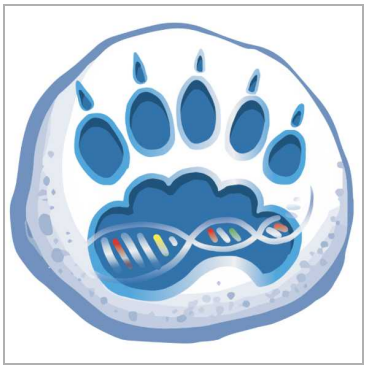

VERSION 2

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Pathogen-Oriented Low-cost Assembly & Re-sequencing on Opentrons (POLARtron): An automation-friendly highly sensitive and high-throughput SARS-CoV-2 diagnostic based on whole genome sequencing V.2

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XPRIZE Rapid Covid Testing

Per A. Adastra

Protocol status: In development
We are still developing and optimizing this protocol

Created: Mar 15, 2023

Last Modified: Mar 15, 2023

PROTOCOL integer ID: 78863

DISCLAIMER

ABSTRACT

An automation-friendly variant of POLAR.

GUIDELINES

SARS-CoV-2 Specific Primer Set

The ARTIC Network designed and tested[1] the primer set used in this protocol and must be custom ordered prior to experiments. Details on their primer set can be found on their Github page https://github.com/artic-network/artic-ncov2019.

1. Artic Network. https://artic.network/resources/ncov/ncov-amplicon-v3.pdf.

The World Health Organization: Dos and Don'ts for Molecular Testing (https://www.who.int/malaria/areas/diagnosis/molecular-testing-dos-donts/en/)

Molecular detection methods have the ability to produce a large volume of nucleic acid through the amplification of trace quantities found in samples. While this is beneficial for enabling sensitive detection, it also introduces the possibility of contamination through the spreading of amplicon aerosols in the laboratory environment. When conducting experiments, measures can be undertaken to avoid the contamination of reagents, laboratory equipment, and bench space, as such contamination may generate false-positive (or false-negative) results.
To help reduce the likelihood of contamination, Good Laboratory Practice should be exercised at all times. Specifically, precautions should be taken regarding the following points:

Handling reagents

- Briefly centrifuge reagent tubes before opening to avoid the generation of aerosols.
- Aliquot reagents to avoid multiple freeze-thaw and the contamination of master stocks.
- Clearly label and date all reagent and reaction tubes and maintain logs of reagent lot and batch numbers used in all experiments.
- Pipette all reagents and samples using filter tips. Prior to purchase, it is advisable to confirm with the manufacturer that the filter tips fit the brand of the pipette to be used.

Organization of workspace and equipment

The workspace *should be organized to ensure that the flow of work occurs in one direction, from clean areas (pre-PCR) to dirty areas (post-PCR). The following general precautions will help to reduce the chance of contamination.*

Have separate designated rooms, or at minimum physically separate areas, for:
1. master mix preparation,
2. nucleic acid extraction and DNA template addition

In some settings, having 4 separate rooms is difficult. A possible but less desirable option is to do the master mix preparation in a containment area, e.g. a laminar flow cabinet. In the case of nested PCR amplification, the preparation of the master mix for the second round reaction should be prepared in the 'clean' area for master mix preparation, but the inoculation with the primary PCR product should be done in the amplification room, and if possible in a dedicated containment area (e.g. a laminar flow cabinet).

Each room/area needs a separate set of clearly labeled pipettes, filter tips, tube racks, vortexes, centrifuges (if relevant), pens, generic lab reagents, lab coats, and boxes of gloves that will remain at their respective workstations.
Hands must be washed and gloves and lab coats changed when moving between the designated areas. Reagents and equipment should not be moved from a dirty area to a clean area. Should an extreme case arise where a reagent or piece of equipment needs to be moved backward, it must first be decontaminated with 10% sodium hypochlorite, followed by a wipe down with sterile water

Ideally, staff should abide by the unidirectional workflow ethos and not go from dirty areas (post-PCR) back to clean areas (pre-PCR) on the same day. However, there may be occasions when this is unavoidable. When such occasion arises, personnel

must take care to thoroughly wash hands, change gloves, use the designated lab coat and not introduce any equipment they will want to take out of the room again, such as lab books. Such control measures should be emphasized in staff training on molecular methods.

After use, bench spaces should be cleaned with 10% sodium hypochlorite (followed by sterile water to remove residual bleach), 70% ethanol, or a validated commercially available DNA-destroying decontaminant. Ideally, ultra-violet (UV) lamps should be fitted to enable decontamination by irradiation. However, the use of UV lamps should be restricted to closed working areas, e.g. safety cabinets, in order to limit the laboratory staff's UV exposure. Please abide by manufacturer instructions for UV lamp care, ventilation, and cleaning in order to ensure that lamps remain effective.

If manufacturer instructions permit it, pipettes should be routinely sterilized by autoclave. If pipettes cannot be autoclaved, it should suffice to clean them with 10% sodium hypochlorite (followed by a thorough wipe down with sterile water) or with a commercial DNA-destroying decontaminant followed by UV exposure.

All equipment needs to be calibrated regularly according to the manufacturer-recommended schedule. A designated person should be in charge of ensuring that the calibration schedule is adhered to, detailed logs are maintained, and service labels are clearly displayed on equipment.

Use and cleaning advice for the designated molecular space

- **Pre-PCR: Reagent aliquoting / mastermix preparation**

This should be the cleanest of all spaces used for the preparation of molecular experiments and should ideally be a designated laminar flow cabinet equipped with a UV light.
Samples, extracted nucleic acid, and amplified PCR products must not be handled in this area.
Amplification reagents should be kept in a freezer (or refrigerator, as per manufacturer recommendations) in the same designated space, ideally next to the laminar flow cabinet or pre-PCR area.
Gloves should be changed each time upon entering the pre-PCR area or laminar flow cabinet.
The pre-PCR area or laminar flow cabinet should be cleaned before and after use as follows: Wipe down all items in the cabinet, e.g. pipettes, tip boxes, vortex, centrifuge, tube racks, pens, etc. with 70% ethanol or a commercial DNA-destroying decontaminant, and allow to dry. In the case of a closed working area, e.g. a laminar flow cabinet, expose the hood to UV light for 30 minutes.

- **Pre-PCR: Nucleic acid extraction/template addition**

Nucleic acid must be extracted and handled in a second designated area, using a separate set of pipettes, filter tips, tube racks, fresh gloves, lab coats, and other equipment.
This area is also for the addition of template, controls, and trendlines to the master mix tubes or plates. To avoid contamination of the extracted nucleic acid samples that are being analyzed, it is recommended to change gloves prior to handling positive controls or standards and to use a separate set of pipettes.
PCR reagents and amplified products must not be pipetted in this area.
Samples should be stored in designated fridges or freezers in the same area.
The sample workspace should be cleaned in the same way as the master mix space.

- **Post-PCR: Amplification and handling of the amplified product**

This designated space is for post-amplification processes and should be physically separate from the pre-PCR areas. It usually contains thermocyclers and real-time platforms, and ideally should have a laminar flow cabinet for adding the round 1 PCR product to the round 2 reaction, if nested PCR is being performed.
PCR reagents and extracted nucleic acid must not be handled in this area since the risk of contamination are high.
This area should have a separate set of gloves, lab coats, plate and tube racks, pipettes, filter tips, bins, and other equipment.
Tubes must be centrifuged before opening.
The sample workspace should be cleaned in the same way as the master mix space.

MATERIALS

MATERIALS

- Nuclease-Free Water, 150ml **Promega Catalog #P1195**
- Luna Universal Probe One-Step RT-qPCR Kit - 2,500 rxns **New England Biolabs Catalog #E3006E**
- isopropyl alcohol **Sigma Catalog #W292907**
- 2-Mercaptoethanol **Sigma Aldrich Catalog #M3148**
- Proteinase K **New England Biolabs Catalog #P8107S**
- 200 Proof Ethanol pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E7023**
- Random Hexamer Primer **Thermo Fisher Catalog #SO142**
- NEBNext Q5U Master Mix – 50 rxns **New England Biolabs Catalog #M0597S**
- Magnesium Chloride Solution 1 M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028**
- sparQ PureMag Beads **Quantabio Catalog #95196-060**

- Nextera DNA Flex Library Prep Kit **Illumina, Inc.**
- NN-Dimethylformamide **Sigma Aldrich Catalog #227056-1L**
- TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**
- Corning 10% SDS (Sodium Dodecyl Sulfate) **Fisher Scientific Catalog #MT-46040CI**
- IDT for Illumina DNA/RNA UD Indexes **Illumina, Inc. Catalog #20027213**
- Quick-DNA/RNA Viral Magbead **Zymo Research Catalog #R2141**
- NN-Dimethylformamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #227056-100ML**
- EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**
- NaCl - 1Kg **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3014-1KG**

STEP MATERIALS

- 200 Proof Ethanol pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E7023**
- Mineral Oil **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M5904**
- Corning 10% SDS (Sodium Dodecyl Sulfate) **Fisher Scientific Catalog #MT-46040CI**
- Quick-DNA/RNA Viral Magbead **Zymo Research Catalog #R2141**
- Nextera DNA Flex Library Prep Kit **Illumina, Inc.**
- NN-Dimethylformamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #227056-100ML**
- Magnesium Chloride Solution 1 M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028**
- Poly(ethylene glycol) 8000 **Merck MilliporeSigma (Sigma-Aldrich)**
- EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**
- TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**
- EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**
- TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**
- EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**
- Poly Ethylene Glycol (PEG) 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #89510-250G-F**
- NaCl - 1Kg **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3014-1KG**
- Quick-DNA/RNA Viral Magbead **Zymo Research Catalog #R2141**
- Nuclease-Free Water, 150ml **Promega Catalog #P1195**
- TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**
- Luna Universal Probe One-Step RT-qPCR Kit - 2,500 rxns **New England Biolabs Catalog #E3006E**
- Random Hexamer **Thermo Fisher Scientific Catalog ##SO142**

sparQ PureMag Beads **Quantabio Catalog #95196-060**

IDT for Illumina DNA/RNA UD Indexes **Illumina, Inc. Catalog #20027213**

NEBNext Q5U Master Mix – 50 rxns **New England Biolabs Catalog #M0597S**

sparQ PureMag Beads **Quantabio Catalog #95196-060**

Quick-DNA/RNA Viral MagBead **Zymo Research Catalog #R2141**

Bio-rad Hard-shell low-profile 96 well skirted PCR plates **Contributed by users Catalog #HSP9601**

Proteinase K **New England Biolabs Catalog #P8107S**

Quick-DNA/RNA Viral MagBead **Zymo Research Catalog #R2141**

2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3148**

BEFORE START INSTRUCTIONS

- Add 500 µl beta-mercaptoethanol per 100 ml Viral DNA/RNA Buffer (final concentrationof 0.5% (v/v)) from the Quick-DNA/RNA Viral MagBead.
- Add 80 ml (R2141) of isopropanol to the MagBead DNA/RNA Wash 1 concentrate from the Quick-DNA/RNA Viral MagBead.
- Add 120 ml (R2141) of isopropanol to the MagBead DNA/RNA Wash 2 concentrate from the Quick-DNA/RNA Viral MagBead.

RNA Extraction

1h

1 For each saliva sample recieved add equal volume saliva and 2X DNA/RNA Shield from the Quick-DNA/RNA Viral magbead kit and vortex. Centrifuge the samples at 500 rpm, 00:05:00 to bring down debris.

Quick-DNA/RNA Viral MagBead **Zymo Research Catalog #R2141**

2 Without disturbing the pellet, transfer 25 µL of saliva sample and 1X DNA/RNA to the bottom of a well in a 96-well deep-plate of each sample to a new tube

Quick-DNA/RNA Viral MagBead **Zymo Research Catalog #R2141**

Bio-rad Hard-shell low-profile 96 well skirted PCR plates **Contributed by users Catalog #HSP9601**

3 Add 25 µL 1X DNA/RNA Shield supplemented with 2.5 µL of Proteinase K (20mg/mL) to each sample.
Briefly mix by using a plate shaker at 1300 rpm, 25°C for 00:00:10 and incubate

00:15:00 at Room temperature .

Equipment

ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

Proteinase K **New England Biolabs Catalog #P8107S**

4 Remove 5 µL of MagBinding Beads per sample of the stock and place on a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completly clea. Then while avoiding the bead pellet, carefully remove the clear supernatnat. Resuspend the beads in 100 µL of Viral DNA/RNA Buffer (2-Mercaptoethanol 0.5% (v/v)) per sample and vortex to fully resuspend

Safety information

2-Mercaptoethanol is toxic, causing irritation to the nasal passageways and respiratory tract upon inhalation, irritation to the skin, vomiting and stomach pain through ingestion, and potentially death if severe exposure occurs.

5 Add 100 µL of the Viral DNA/RNA Buffer (2-Mercaptoethanol 0.5% (v/v)) and MagBinding Beads misture to each 50 µL sample in 1X DNA/RNA Shield. Mix by using a plate shaker at 1300 rpm, 25°C for 00:10:00 .

Safety information

2-Mercaptoethanol is toxic, causing irritation to the nasal passageways and respiratory tract upon inhalation, irritation to the skin, vomiting and stomach pain through ingestion, and potentially death if severe exposure occurs.

2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3148**

6 1m

Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant.

Equipment

SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

7 10m

Add 100 µL MagBead DNA/RNA Wash 1 and mix by using a plate shaker at 1300 rpm, 25°C for 00:02:00 .

Quick-DNA/RNA Viral Magbead **Zymo Research Catalog #R2141**

8 1m

Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant.

9 10m

Add 100 µL MagBead DNA/RNA Wash 2 and mix by using a plate shaker at

1300 rpm, 25°C for 00:02:00 .

Quick-DNA/RNA Viral Magbead **Zymo Research Catalog #R2141**

Equipment	
ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

10 Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant. 1m

Equipment	
SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

11 Add 150 µL 95-100% ethanol and mix by using a plate shaker at 1300 rpm, 25°C for 10m

00:02:00 .

200 Proof Ethanol pure **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E7023**

12 Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant. 1m

Equipment	
SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

13 and repeat once.

14 To elute DNA/RNA from the beads, add 9 µL DNase/RNase-Free Water and mix using a plate shaker at 5m

1300 rpm, 25°C for 00:01:00

Nuclease-Free Water, 150ml **Promega Catalog #P1195**

Equipment

ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

15 Pellet the beads and transfer 7 µL of supernatant into a new tube. The eluted DNA/RNA can be used immediately or stored frozen at -80 °C.

RT-PCR

2h

16 Combine the following components into a thin-walled PCR tube. 1m

- 10 µL Luna Universal Probe One-Step Reaction Mix (2X)
- 1 µL Luna WarmStart RT Enzyme Mix (20X)
- 1 µL hCoV-2019/nCoV-2019 (V3) Primer Set mix (Primer pool 1 & 2) (100µm)
- 1 µL Random Hexamers (100 µM)
- 7 µL Viral RNA/DNA extract

Luna Universal Probe One-Step RT-qPCR Kit - 2,500 rxns **New England Biolabs Catalog #E3006E**

Random Hexamer **Thermo Fisher Scientific Catalog ##SO142**

17 Add 20 µL of mineral oil to each RT-PCR reaction to avoid evaporation and subsequent reaction failure.

Mineral Oil **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M5904**

18 Set up and run the following RT-PCR program. 1h 30m

1. Reverse transcription: 00:10:00 at 55 °C
2. Initial PCR activation: 00:01:00 at 95 °C
3. 2-step PCR cycling (25X):
   Denaturation: 00:00:15 at 95 °C
   Annealing & Extension: 00:03:00 at 63 °C
4. Hold: 4 °C

Equipment

Veriti 96-Well Thermal Cycler	NAME
Applied Biosystems	BRAND
4375786	SKU
https://www.thermofisher.com/order/catalog/product/4375786#/4375786	LINK

19 Add 0.7x volume of sparQ PureMag beads to each sample well in RT-PCR plate and mix gently by either flicking or pipetting. For example, add 14 µL sparQ PureMag beads to a 20 µL reaction. Note that the volume of oil is not taken into consideration given the oil is effectively inert. Then pulse centrifuge to collect all liquid at the bottom of the tube. 1m

sparQ PureMag Beads **Quantabio Catalog #95196-060**

20 Incubate for 00:05:00 at Room temperature. 5m

21 Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant. 2m

Equipment	
SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

22 Keeping on the magnetic plate, add 150 µL of Room temperature freshly made [M] 80 % (v/v) ethanol to the side of the wall opposite to the pellet and let sit for 00:00:30. 30s

23 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for 00:00:10 then remove any remaining ethanol. 10s

24 and repeat once.

25 Add 11 µl of [M] 10 mM Tris-HCl (Ph 8.0) and pipette to mix well. Incubate for 00:01:00 at 37 °C. 1m

TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**

Equipment

ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

26 Separate beads on the Agencourt SPRIPlate Super Magnet Plate for 00:02:00 or until the beads have pelleted. 2m

Equipment

SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

27 Pellet the beads and transfer 10 µl of supernatant containing SARS-CoV-2 amplicons into a new tube. The eluted DNA can be used immediately or stored frozen at -20 °C.

Hackflex Library Preparation 2h

28 Combine the following components into a thin-walled PCR tube.

4 µL 5X Hacklfex Buffer (20mM Tris, 20 mM MgCl, 50% DMF)

5.5 µL Nuclease-Free Water

0.5 µL Enrichment Bead-Linked Transposomes (eBLT)

10 µL SARS-CoV-2 amplicons

Nextera DNA Flex Library Prep Kit **Illumina, Inc.**

NN-Dimethylformamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #227056-100ML**

Magnesium Chloride Solution 1 M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028**

29 Set up and run the following thermocycler program. 15m

1. Tagmentation: 00:05:00 at 55 °C
2. Hold: 10 °C

Equipment

ThermoMixer® C	NAME
Eppendorf	BRAND
Catalog No. 2231000680	SKU
https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html	LINK

30 Add 5 µL of Hackflex Stop Buffer (0.2% SDS) to the sample. 1m

Corning 10% SDS (Sodium Dodecyl Sulfate) **Fisher Scientific Catalog #MT-46040CI**

31 Set up and run the following thermocycler program. 15m

1. Tagmentation: 00:05:00 at 25 °C
2. Hold: 10 °C

Equipment

ThermoMixer® C — NAME

Eppendorf — BRAND

Catalog No. 2231000680 — SKU

https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html — LINK

32 Place the plates on the Agencourt SPRIPlate Super Magnet Plate and incubate for 00:05:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant.

Equipment

SPRIPlate 96R Ring Super Magnet Plate — NAME

96-well Magnet Plate — TYPE

Agencourt — BRAND

A32782 — SKU

https://www.beckman.com/supplies/plates/a32782 — LINK

33 Keeping on the magnetic plate, add 30 µL of Room temperature Hackflex Wash Buffer (10% PEG 8000, 0.25 M NaCl, 10mM Tris-HCL pH 8.0, 0.1mM EDTA) gently mix and then let sit for 30s

00:00:30 .

Poly(ethylene glycol) 8000 **Merck MilliporeSigma (Sigma-Aldrich)**

EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**

34 Separate beads on the Agencourt SPRIPlate Super Magnet Plate for 00:02:00 or until the beads have pelleted. 2m

Equipment	
SPRIPlate 96R Ring Super Magnet Plate	NAME
96-well Magnet Plate	TYPE
Agencourt	BRAND
A32782	SKU
https://www.beckman.com/supplies/plates/a32782	LINK

35 Avoid disturbing the bead pellet, carefully remove and discard HWB (10% PEG 8000, 0.25 M NaCl, 10mM Tris-HCL pH 8.0, 0.1mM EDTA). 30s

Poly Ethylene Glycol (PEG) 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #89510-250G-F**

NaCl - 1Kg **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3014-1KG**

TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**

EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**

36 and repeat once.

37 Resuspend beads in the following 20 µL Indexing-PCR Master Mix 1m

10 µL NEBNext Q5U Master Mix (2X)
1 µL IDT for Illumina DNA/RNA UD Indexes
9 µL Nuclease-free Water

NEBNext Q5U Master Mix – 50 rxns **New England Biolabs Catalog #M0597S**

IDT for Illumina DNA/RNA UD Indexes **Illumina, Inc. Catalog #20027213**

38 Set up and run the following Indexing-PCR program. 20m

1. Initial Denaturation: 00:01:00 at 98 °C
2. 3-step PCR cycling (6X):
   Denaturation: 00:00:15 at 98 °C
   Annealing: 00:00:30 at 62 °C
   Extension: 00:00:30 at 65 °C
3. Final Extension: 00:01:00 at 65 °C
4. Hold: 4 °C

Equipment

Veriti 96-Well Thermal Cycler	NAME
Applied Biosystems	BRAND
4375786	SKU
https://www.thermofisher.com/order/catalog/product/4375786#/4375786	LINK

39 After PCR, pool 10 µL of each sample into a single tube and vortex to mix.

40 Add an equal volume (1:1) of sparQ PureMag beads to the library pool and mix gently by either 1m

flicking or pipetting. For example, add 25 µL sparQ PureMag beads to a 25 µL reaction. Then pulse centrifuge to collect all liquid at the bottom of the tube.

sparQ PureMag Beads **Quantabio Catalog #95196-060**

41 Incubate for 00:05:00 at Room temperature. 5m

42 Place the pool on a magnet and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant. 2m

Equipment

Magnetic Stand	NAME
Magnetic Stand	TYPE
Thermo Scientific	BRAND
MR02	SKU
https://www.thermofisher.com/order/catalog/product/MR02	LINK
Any magnetic rack that fits your tubes will suffice.	SPECIFICATIONS

43 Keeping the pool on the magnet, add 200 µL of Room temperature freshly made [M] 80 % (v/v) ethanol to the side of the wall opposite to the pellet and let sit for 00:00:30. 30s

44 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for 00:00:10 then remove any remaining ethanol. 10s

45 and repeat once.

46 Add 25 µl of [M] 10 mM TE Buffer (10mM Tris-HCL pH 8.0, 0.1mM EDTA) and pipette to mix well. Incubate for 00:01:00 at 37 °C. 1m

TRIS 1M pH 8.0 **VWR International Catalog #E199-500mL**

EDTA 100mL **Thermo Fisher Scientific Catalog #AM9260G**

Quantifying Pool and Sequencing

47 Quantify final pool with adapted libraries. Load onto sequencer using platform appropriate dilutions.

Equipment

MiSeq	NAME
Sequencer	TYPE
illumina	BRAND
SY-410-1003	SKU
https://www.illumina.com/systems/sequencing-platforms/miseq/order-miseq.html	LINK