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Version 2

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



DOI

dx.doi.org/10.17504/protocols.io.81wgbp8jqvpk/v2

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XPRIZE Rapid Covid Tes...



### Per A. Adastra

**Baylor College of Medicine** 

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Protocol status: In development

We are still developing and optimizing this protocol

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Keywords: polartron, pathogen, throughput sar, genome, whole genome, sar, friendly variant of polar, opentron



### Disclaimer

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### Abstract

An automation-friendly variant of POLAR.



### Guidelines

### **SARS-CoV-2 Specific Primer Set**

The ARTIC Network designed and tested<sup>1</sup> 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/articncov2019.

1. Artic Network. <a href="https://artic.network/resources/ncov/ncov-amplicon-v3.pdf">https://artic.network/resources/ncov/ncov-amplicon-v3.pdf</a>.

### 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, ultraviolet (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 Merck MilliporeSigma (Sigma-Aldrich) Catalog #W292907
- 🔯 2-Mercaptoethanol Merck MilliporeSigma (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
- X 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.
- X NN-Dimethylformamide Merck MilliporeSigma (Sigma-Aldrich) Catalog #227056-1L
- X TRIS 1M pH 8.0 VWR International (Avantor) Catalog #E199-500mL
- Corning 10% SDS (Sodium Dodecyl Sulfate) Fisher Scientific Catalog #MT-46040CI
- X 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

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- 🔯 Bio-rad Hard-shell low-profile 96 well skirted PCR plates Catalog #HSP9601
- Proteinase K New England Biolabs Catalog #P8107S
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  - SparQ PureMag Beads Quantabio Catalog #95196-060
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  - XX EDTA 100mL Thermo Fisher Scientific Catalog #AM9260G

### Troubleshooting

### Before start

- Add 500 µl beta-mercaptoethanol per 100 ml Viral DNA/RNA Buffer (final concentration of 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

- 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
  - 🔀 Bio-rad Hard-shell low-profile 96 well skirted PCR plates Catalog #HSP9601

Briefly mix by using a plate shaker at 1300 rpm, 25°C for 00:00:10 and incubate 00:015:00 at 8 Room temperature.

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

X Proteinase K New England Biolabs Catalog #P8107S



Remove  $\Delta$  5  $\mu$ L 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  $\Delta$  100  $\mu$ L 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.

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

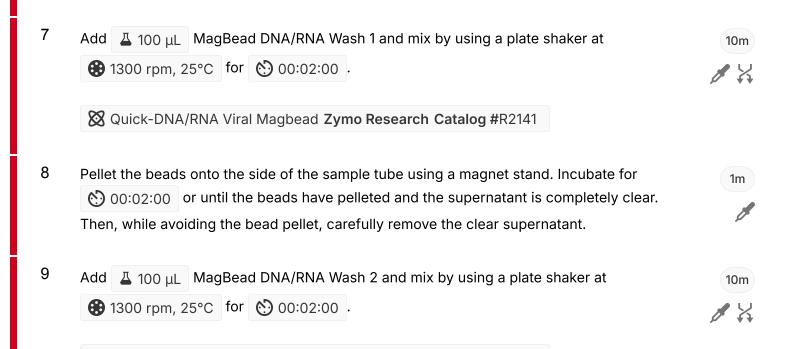
Pellet the beads onto the side of the sample tube using a magnet stand. Incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, carefully remove the clear supernatant.







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



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



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

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

11 Add 4 150 uL 95-100% ethanol and mix by using a plate shaker at

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

10m





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.





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

13 and repeat once.

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





(300 rpm, 25°C for (50 00:01:00)

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



# Equipment

ThermoMixer® C

NAME

**Eppendorf** 

BRAND

Catalog No. 2231000680

SKU

LINK

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



15 Pellet the beads and transfer  $\perp$  7  $\mu$ L of supernatant into a new tube. The eluted DNA/RNA can be used immediately or stored frozen at 4 -80 °C.

### RT-PCR

2h

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

Luna Universal Probe One-Step Reaction Mix (2X)



 $\perp$  1  $\mu$ L Luna WarmStart RT Enzyme Mix (20X)

Δ 1 μL hCoV-2019/nCoV-2019 (V3) Primer Set mix (Primer pool 1 & 2) (100μm)

 $\perp$  1  $\mu$ L Random Hexamers (100  $\mu$ M)

↓ 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 4 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.

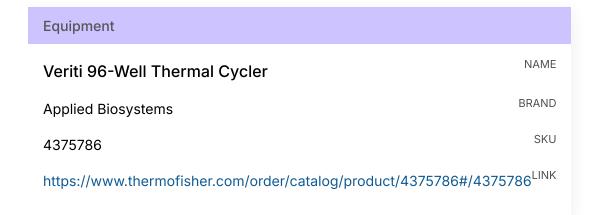
1h 30m

- 1. Reverse transcription: (5) 00:10:00 at 🖁 55 °C
- 2. Initial PCR activation: 60 00:01:00 at \$\mathbb{8}\$ 95 °C
- 3. 2-step PCR cycling (25X):

Denaturation: 60 00:00:15 at \$ 95 °C

Annealing & Extension: (5) 00:03:00 at \$ 63 °C

4. Hold: 4 °C



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  $\perp$  20  $\mu$ L 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.



SparQ PureMag Beads Quantabio Catalog #95196-060

20 Incubate for 00:05:00 at Room temperature

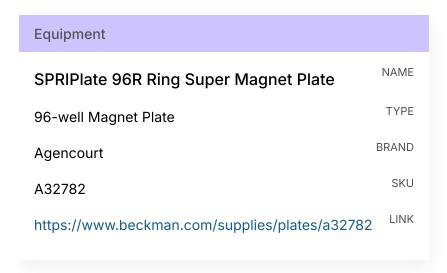
5m

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.
- 2m

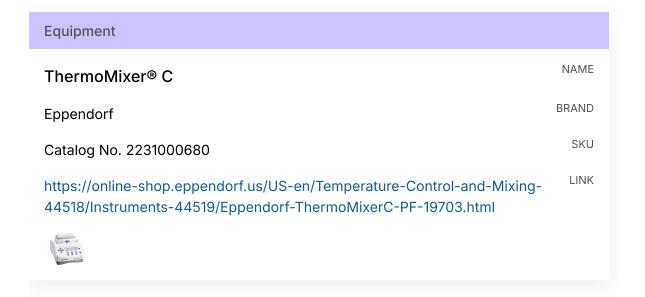


Then, while avoiding the bead pellet, carefully remove the clear supernatant.



- 22 Keeping on the magnetic plate, add  $\perp$  150  $\mu$ L of  $\parallel$  Room temperature freshly made 30s [M] 80 % (V/V) ethanol to the side of the wall opposite to the pellet and let sit for **(5)** 00:00:30 . 23 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for 10s 00:00:10 then remove any remaining ethanol. 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 .
  - X TRIS 1M pH 8.0 VWR International (Avantor) Catalog #E199-500mL





26 Separate beads on the Agencourt SPRIPlate Super Magnet Plate for 600:02:00 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/a327	82 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 2 -20 °C.



# **Hackflex Library Preparation**



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





Δ 4 μL 5X HackIfex Buffer (20mM Tris, 20 mM MgCl, 50% DMF) Δ 5.5 μL Nuclease-Free Water Δ 0.5 μL Enrichment Bead-Linked Transposomes (eBLT) Δ 10 μL SARS-CoV-2 amplicons

X 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: (5) 00:05:00 at \$\cdot 55 \cdot \cdot \cdot 55 \cdot \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  $\triangle$  5 µL of Hackflex Stop Buffer (0.2% SDS) to the sample.





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

31 Set up and run the following thermocycler program.

15m

1. Tagmentation: (5) 00:05:00 at \$\color{1}{25} \cdot \color{1}{25} \cdot \color{1}{25

2. Hold: 10 °C



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.





33 Keeping on the magnetic plate, add  $\perp$  30  $\mu$ L of  $\parallel$  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 00:00:30 .



30s

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 (5) 00:02:00 or until the beads have pelleted.

2m



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

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
- X TRIS 1M pH 8.0 VWR International (Avantor) Catalog #E199-500mL
- 🔀 EDTA 100mL Thermo Fisher Scientific Catalog #AM9260G
- 36 and repeat once.

1m



- Δ 1 μL IDT for Illumina DNA/RNA UD Indexes
- Δ 9 μL Nuclease-free Water



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

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

20m

宮

1. Initial Denaturation: 👏 00:01:00 at 🖁 98 °C

2. 3-step PCR cycling (6X):

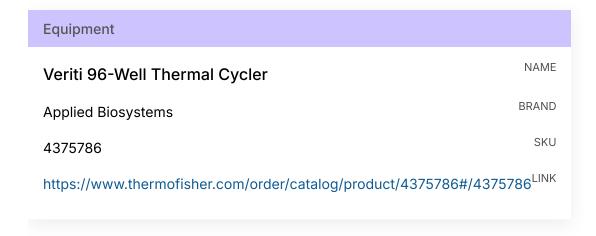
Denaturation: 00:00:15 at \$ 98 °C

Annealing: 00:00:30 at \$ 62 °C

Extension: 60 00:00:30 at 65 °C

3. Final Extension: (5) 00:01:00 at \$\mathbb{8}\$ 65 °C

4. Hold: 4 °C



39 After PCR, pool  $\stackrel{\perp}{\perp}$  10  $\mu$ L of each sample into a single tube and vortex to mix.

D.

Add an equal volume (1:1) of sparQ PureMag beads to the library pool and mix gently by either flicking or pipetting. For example, add Δ 25 μL sparQ PureMag beads to a

Δ 25 μL reaction. Then pulse centrifuge to collect all liquid at the bottom of the tube.

**4** ()

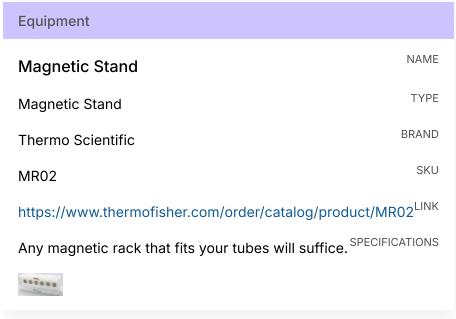
SparQ PureMag Beads Quantabio Catalog #95196-060

carefully remove the clear supernatant.



Incubate for 00:05:00 at Room temperature.

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,



Keeping the pool on the magnet, add Δ 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 .
Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for 00:00:10 then remove any remaining ethanol.

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 .



- X TRIS 1M pH 8.0 VWR International (Avantor) 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.

