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POLAR Express: Pathogen-Oriented Low-cost Assembly & Re-sequencing

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1 Works for me dx.doi.org/10.17504/protocols.io.bhv5j686

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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.

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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

NAME	CATALOG #	VENDOR
Nuclease-Free Water, 150ml	P1195	Promega
Luna Universal Probe One-Step RT-qPCR Kit - 2,500 rxns	E3006E	New England Biolabs
isopropyl alcohol	W292907	Sigma
2-Mercaptoethanol	M3148	Sigma Aldrich
Proteinase K	P8107S	New England Biolabs
200 Proof Ethanol pure	E7023	Sigma Aldrich
Random Hexamer Primer	SO142	Thermo Fisher
NEBNext Q5U Master Mix – 50 rxns	M0597S	New England Biolabs
Magnesium Chloride Solution 1 M	M1028	Sigma Aldrich
sparQ PureMag Beads	95196-060	Quantabio
Nextera DNA Flex Library Prep Kit		Illumina, Inc.
NN-Dimethylformamide	227056-1L	Sigma Aldrich
TRIS 1M pH 8.0	E199-500mL	VWR Scientific
Corning 10% SDS (Sodium Dodecyl Sulfate)	MT-46040CI	Fisher Scientific
IDT for Illumina DNA/RNA UD Indexes	20027213	illumina
Quick-DNA/RNA Viral Magbead	R2141	Zymo Research
NN-Dimethylformamide	227056-100ML	Sigma Aldrich
EDTA 100mL	AM9260G	Thermo Fisher Scientific
NaCl - 1Kg	S3014-1KG	Sigma Aldrich

STEPS MATERIALS

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NAME	CATALOG #	VENDOR
200 Proof Ethanol pure	E7023	Sigma Aldrich
Mineral Oil	M5904	Millipore Sigma
Corning 10% SDS (Sodium Dodecyl Sulfate)	MT-46040CI	Fisher Scientific
Quick-DNA/RNA Viral Magbead	R2141	Zymo Research
Nextera DNA Flex Library Prep Kit		Illumina, Inc.
NN-Dimethylformamide	227056-100ML	Sigma Aldrich
Magnesium Chloride Solution 1 M	M1028	Sigma Aldrich
Poly(ethylene glycol) 8000		Sigma Aldrich
EDTA 100mL	AM9260G	Thermo Fisher Scientific
TRIS 1M pH 8.0	E199-500mL	VWR Scientific
Poly Ethylene Glycol (PEG) 8000	89510-250G-F	Sigma Aldrich
NaCl - 1Kg	S3014-1KG	Sigma Aldrich
Nuclease-Free Water, 150ml	P1195	Promega
Luna Universal Probe One-Step RT-qPCR Kit - 2,500 rxns	E3006E	New England Biolabs
Random Hexamer	#SO142	Thermo Fisher Scientific
sparQ PureMag Beads	95196-060	Quantabio
IDT for Illumina DNA/RNA UD Indexes	20027213	illumina
NEBNext Q5U Master Mix – 50 rxns	M0597S	New England Biolabs
Quick-DNA/RNA Viral MagBead	R2141	Zymo Research
Bio-rad Hard-shell low-profile 96 well skirted PCR plates	HSP9601	
Proteinase K	P8107S	New England Biolabs
2-Mercaptoethanol	M3148	Sigma Aldrich

EQUIPMENT

NAME	CATALOG #	VENDOR
SPRIPlate 96R Ring Super Magnet Plate	A32782	
ThermoMixer® C	Catalog No. 2231000680	
Magnetic Stand	MR02	
MiSeq	SY-410-1003	
Veriti 96-Well Thermal Cycler	4375786	Thermo Fisher Scientific

BEFORE STARTING

- 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
by Zymo Research
Catalog #: R2141

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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
by Zymo Research
Catalog #: R2141

Bio-rad Hard-shell low-profile 96 well skirted PCR plates
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** .

ThermoMixer® C

Eppendorf	Catalog No.
	2231000680

Proteinase K
by 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

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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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** .

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
by 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.

SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt **A32782**

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
by Zymo Research
Catalog #: R2141

8  1m

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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
by Zymo Research
Catalog #: R2141

ThermoMixer® C
Eppendorf | Catalog No. 2231000680

10 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.

SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt A32782

11 10m

Add **150 µl** 95-100% ethanol and mix by using a plate shaker at **1300 rpm, 25°C** for **00:02:00** .

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200 Proof Ethanol pure
by Sigma Aldrich
Catalog #: E7023

12 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.

SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt **A32782**

13 **go to step #11** and repeat once.

14 5m

To elute DNA/RNA from the beads, add **9 µl** DNase/RNase-Free Water and mix using a plate shaker at **1300 rpm, 25°C** for **00:01:00**

Nuclease-Free Water, 150ml
by Promega
Catalog #: P1195

ThermoMixer® C
Eppendorf **Catalog No.** **2231000680**

Citation: Brian Glenn St Hilaire (09/08/2020). POLAR Express: Pathogen-Oriented Low-cost Assembly & Re-sequencing. https://dx.doi.org/10.17504/protocols.io.bhv5j686

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 1m

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

- **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
by New England Biolabs
Catalog #: E3006E

Random Hexamer
by 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
by Millipore Sigma
Catalog #: M5904

18 1h 30m

Set up and run the following RT-PCR program.

1. Reverse transcription: **00:10:00** at **55 °C**
2. Initial PCR activation: **00:01:00** at **95 °C**

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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**

Veriti 96-Well Thermal Cycler

Applied Biosystems **4375786**

19 1m

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.

sparQ PureMag Beads

by Quantabio

Catalog #: 95196-060

20 5m

Incubate for **00:05:00** at **Room temperature** .

21 2m

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**

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22 30s

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

23 10s

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

24 **go to step #22** and repeat once.

25 1m

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

TRIS 1M pH 8.0
by VWR Scientific
Catalog #: E199-500mL

ThermoMixer® C
Eppendorf — Catalog No. 2231000680

26 2m

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

SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt A32782

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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
by Illumina, Inc.

NN-Dimethylformamide
by Sigma Aldrich
Catalog #: 227056-100ML

Magnesium Chloride Solution 1 M
by Sigma Aldrich
Catalog #: M1028

29 15m

Set up and run the following thermocycler program.

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

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ThermoMixer® C

Eppendorf	Catalog No.
	2231000680

30 1m

Add **5 µl** of Hackflex Stop Buffer (0.2% SDS) to the sample.

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

31 15m

Set up and run the following thermocycler program.

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

ThermoMixer® C

Eppendorf	Catalog No.
	2231000680

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.

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SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt A32782

33 30s

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 **00:00:30** .

Poly(ethylene glycol) 8000
by Sigma Aldrich
CAS Number: 25322-68-3

EDTA 100mL
by 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

SPRIPlate 96R Ring Super Magnet Plate
96-well Magnet Plate
Agencourt A32782

35 30s

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).

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Poly Ethylene Glycol (PEG) 8000
by Sigma Aldrich
Catalog #: 89510-250G-F

NaCl - 1Kg
by Sigma Aldrich
Catalog #: S3014-1KG

TRIS 1M pH 8.0
by VWR Scientific
Catalog #: E199-500mL

EDTA 100mL
by Thermo Fisher Scientific
Catalog #: AM9260G

36 **go to step #33** and repeat once.

37 1m

Resuspend beads in the following **20 µl** Indexing-PCR Master Mix

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
by New England Biolabs
Catalog #: M0597S

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IDT for Illumina DNA/RNA UD Indexes
by illumina
Catalog #: 20027213

38 20m

Set up and run the following Indexing-PCR program.

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**

Veriti 96-Well Thermal Cycler
Applied Biosystems **4375786**

39

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

40 1m

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 **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
by Quantabio
Catalog #: 95196-060

41 5m

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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, carefully remove the clear supernatant. 2m

Magnetic Stand
Magnetic Stand
Thermo Scientific MR02
Any magnetic rack that fits your tubes will suffice.

43 30s

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** .

44 10s

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

45 **go to step #43** and repeat once.

46 1m

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** .

TRIS 1M pH 8.0
by VWR Scientific
Catalog #: E199-500mL

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EDTA 100mL
by Thermo Fisher Scientific
Catalog #: AM9260G

Quantifying Pool and Sequencing

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

MiSeq
Sequencer
illumina SY-410-1003

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