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# Pathogen-Oriented Low-cost Assembly & Re-sequencing (POLAR): A highly sensitive and high-throughput SARS-CoV-2 diagnostic based on whole genome sequencing

Forked from nCoV-2019 sequencing protocol (single sample)

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1 Works for me dx.doi.org/10.17504/protocols.io.bearjad6

Coronavirus Method Development Community



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## ABSTRACT

Here, we introduce a low-cost, high-throughput method for diagnosis of SARS-CoV-2 infection, dubbed Pathogen-Oriented Low-Cost Assembly & Re-Sequencing (POLAR), that enhances sensitivity by aiming to amplify the entire SARS-CoV-2 genome rather than targeting particular viral loci, as in typical RT-PCR assays. To achieve this goal, we combine a SARS-CoV-2 enrichment method developed by the ARTIC Network (<https://artic.network/>) with short-read DNA sequencing and *de novo* genome assembly. For details on our computational pipeline for automated data processing, including documentation and test set, please visit our Github at <https://github.com/aidenlab/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/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

NAME	CATALOG #	VENDOR
Q5 Hot Start High-Fidelity DNA Polymerase - 100 units	M0493S	New England Biolabs
Deoxynucleotide Solution Set - 25 umol of each	N0446S	New England Biolabs
Random hexamers	N8080127	Thermo Fisher Scientific
Nextera XT DNA Sample Preparation Kit, 96 samples	FC-131-1096	illumina
Qubit dsDNA HS (High sensitivity) Assays	Q32851	Thermo Fisher Scientific
SuperScript™ IV Reverse Transcriptase	18090050	Thermo Fisher Scientific
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher
SuperPlate PCR Plate, 96-well, semi-skirted	AB2400	Thermo Fisher
100mM DTT	18090050	Thermo Fisher Scientific
SuperScript™ IV Reverse Buffer (5X)	18090050	Thermo Fisher Scientific
Q5 Reaction Buffer (5X)	M0493S	New England Biolabs

NAME ▾	CATALOG # ▾	VENDOR ▾
sparQ PureMag Beads	95196-060	Quantabio
Nuclease-Free Water	129115	Qiagen
Quick-RNA Viral 96 Kit	R1041	Zymo Research
Agencourt SPRIPlate 96R Ring Super Magnet Plate	A32782	Beckman Coulter
Zymo-Spin I-96 Plate	C2004	Zymo Research
Nextera XT Index Kit v2 Set A (96 Indexes 384 Samples)	FC-131-2001	illumina
10mM Tris-HCl (pH 8.0)		
NextSeq500 v2.5 Mid-Output Kit	20024904	illumina
IDT primers Custom ordered from IDT created by ARTIC Network v3	Custom picked from ARTIC	IDT
0.1 N NaOH		
100% Ethanol	E7023	Sigma Aldrich

## RNA extraction 41m

- Collect 96 patient samples and record sample data. Add **200 µl** of the samples into each well of the Zymo-Spin™ I-96 plate. Keep plate **On ice** while preparing reagents in Step 2. 5m
- Prepare the following reagents before starting viral RNA extraction: 2m
  - Add beta-mercaptoethanol (user supplied) to the Viral RNA Buffer to a final dilution of **0.5 % volume** i.e., **500 µl** per 100 ml.
  - Add **192 ml** of 100% ethanol (or **204 ml** of 95% ethanol) to the **48 ml** Viral Wash Buffer concentrate.
- Add **400 µl** of Viral RNA Buffer to each sample well. Mix well by pipetting. 2m
- Mount the Zymo-Spin™ I-96 plate on a Collection Plate and centrifuge at **3000 x g** - **5000 x g** for **00:05:00**. Discard the flow-through from the collection plate. 7m
- Add **500 µl** Viral Wash Buffer to each well and centrifuge at **3000 x g** - **5000 x g** for **00:05:00**. Discard the flowthrough from the collection plate. Repeat this step. 12m
- Add **500 µl** ethanol (95-100%) to each well and centrifuge at **3000 x g** - **5000 x g** for **00:05:00**. 6m

- 7 To elute RNA, mount a new 96-well plate between the Zymo-Spin™ I-96 plate and an empty Collection Plate. Add **15 µl** DNase/RNase-Free Water directly to the column matrix of the Zymo-Spin™ I-96 plate wells and centrifuge at **3000 x g** - **5000 x g** for **00:05:00**. The eluted RNA extract will be collected in the middle 96-well plate. 7m

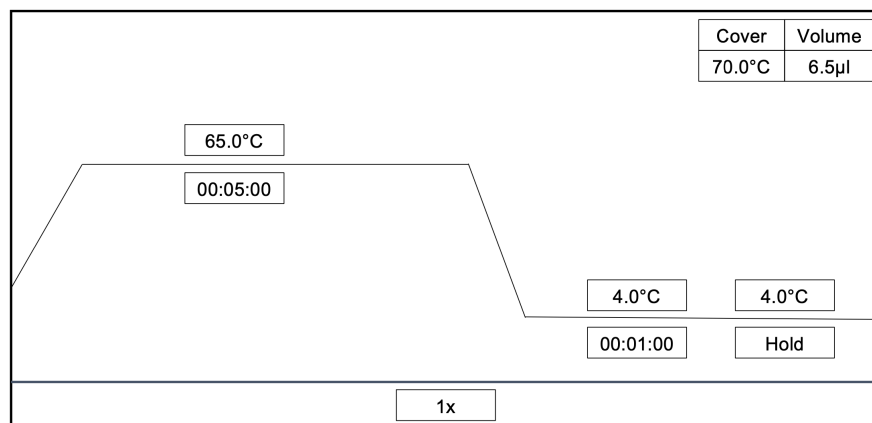
#### cDNA preparation 1h 8m

- 8 Make a mastermix of the dNTPs and Random Hexamers for 96 samples (account for pipette error), pipette to mix and add **1 µl** from mastermix to each well in a 96-well plate. 5m

To each well, add **5.5 µl** of RNA extract eluted in Step 7.

Reagent	Amount
RNA Extract	5.5µl
10mM dNTPs Mix (NEB, N0446S)	0.5µl
50µM Random Hexamers (ThermoFisher, N8080127)	0.5µl
Total	6.5µl

- 9 Set-up and run the following program on a thermal cycler to incubate reaction: 6m



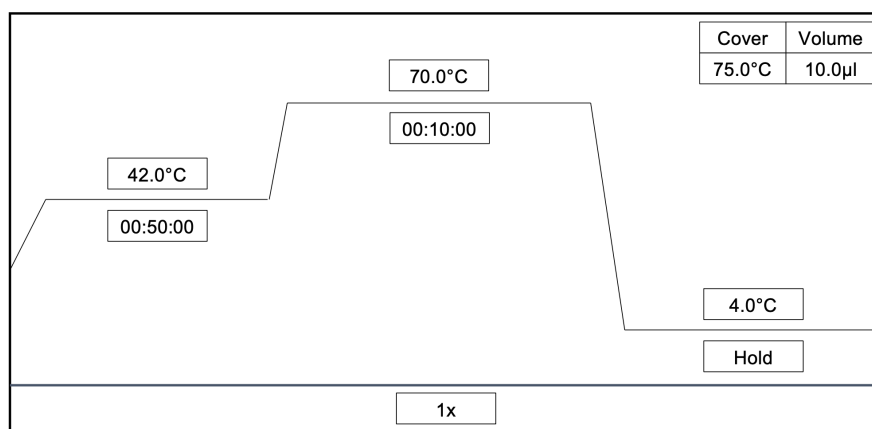
- 10 Make a mastermix of the following components for 96 samples (account for pipette error) and mix by pipetting. Add **3.5 µl** of mastermix to each well with the annealed template RNA from Step 9.

5m

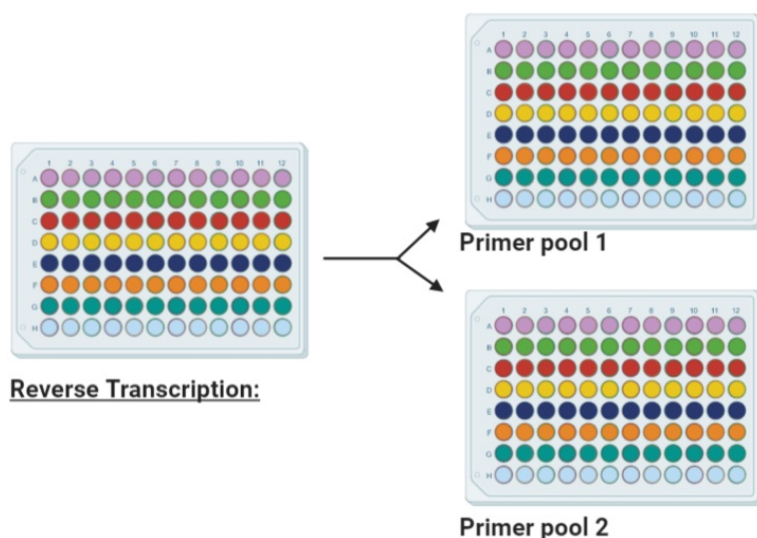
Reagent	Amount
5X SuperScript™ IV Reverse Buffer (ThermoFisher, 18090050)	2.0µl
SuperScript™ IV Reverse Transcriptase (200U/µl) (ThermoFisher 18090050)	0.5µl
100mM DTT (ThermoFisher 18090050)	0.5µl
RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher, 10777-019)	0.5µl
Total	3.5µl

- 11 Set-up and run the following program on a thermal cycler to incubate reaction:

1h



- 12 The following steps are performed in plates prepared according to the schematic below:



Two 96-well plates are prepared for PCR such that each contains a mastermix with the corresponding primer pool and cDNA from each biological sample.

- 13 Make a Pool #1 and Pool #2 master mix with the following reagents for 96 samples (account for pipette error) and mix by <sup>10m</sup> pipetting. In two 96-well plates, labeled Pool #1 or Pool #2, aliquot **22.5 µl** of the corresponding Pool master mix into each well.

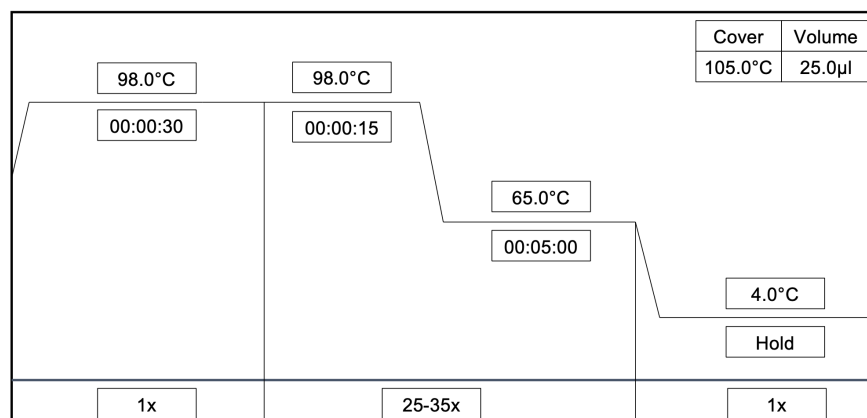
Reagent	Pool #1	Pool #2
5X Q5 Reaction Buffer (NEB, M0493S)	5µl	5µl
10 mM dNTPs (NEB, N0446S)	0.5µl	0.5µl
Q5 Hot Start DNA Polymerase (NEB, M0493S)	0.25µl	0.25µl
10µM Primer (Pool 1 or 2)	3.6µl	3.6µl
Nuclease-free water (Qiagen, 129115)	13.15µl	13.15µl
Total	22.5µl	22.5µl

- 14 Add **2.5 µl** of cDNA of each biological sample from from Step 11 to both Pool #1 and Pool #2 96-well plates.

2m

15 Set-up and run the following program on a thermal cycler to incubate reaction

5m 45s



#### Post PCR Clean-up

15m

16 Add an equal volume (1:1) of sparQ PureMag beads to each sample well in plates Pool #1 and Pool #2 and mix gently by either flicking or pipetting. For example, add **50 µl** sparQ PureMag beads to a **50 µl** reaction. Then pulse centrifuge to collect all liquid at the bottom of the tube.

17 Incubate for **00:05:00** at room temperature.

5m

18 Place the plates on the Agencourt SPRIPlate Super Magnet Plate 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.

3m



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

Agencourt A32782 [↗](#)

19 Keeping on magnetic plate, add **200 µl** of **Room temperature** **80 % volume (freshly made)** ethanol to the side of the well opposite to the pellet and let sit for **00:00:30**.

2m



- 20 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for ⌚ 00:00:10 then remove any remaining ethanol. 50s
- 21 🔗 go to step #19 and repeat ethanol wash. 2m
- 22 Add 🧴 20 µl of [M]10 Milimolar (mM) Tris-HCl (Ph 8.0) and pipette to mix well. Incubate for ⌚ 00:02:00 at 🌡 37 °C . 3m
- 23 Separate beads on the Agencourt SPRIPlate Super Magnet Plate for ⌚ 00:02:00 or until the beads have pelleted. 3m
- 24 Transfer supernatant from each biological sample in the two plates (Pool #1 and Pool #2) and combine into a single well per sample in a new 96-well plate. 2m 30s

Quantification of DNA concentration using a Qubit® High Sensitivity Kit: 1h

- 25 Quantify the DNA concentration using the Qubit® High Sensitivity DNA kit from 🧴 1 µl of each product using Steps 26-35.
- 26 Set up the required number of 0.5-mL tubes for standards and samples and label lids accordingly. 7m
- 27 Prepare a working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. For example, add 🧴 199 µl of buffer and 🧴 1 µl of dye per sample to a tube and vortex. 1m
- 28 Add 🧴 190 µl of Qubit® working solution and 🧴 10 µl of the standard to each of the standards tubes and vortex. 1m
- 29 For each sample, add 🧴 199 µl of Qubit® working solution and 🧴 1 µl of the sample in each 0.5-mL sample tube and vortex. 10m
- 30 Allow all tubes to incubate at room temperature for ⌚ 00:02:00 2m
- 31 On the Home screen of the Qubit® 3.0 Fluorometer, press DNA, select dsDNA High Sensitivity, and press Read Standards to proceed. 2s
- 32 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~ ⌚ 00:00:03 ), remove Standard #1. 3s
- 33 Repeat Step 32 with Standard #2. 3s
- 34 To quantify samples, Press Run samples and select the sample volume ( 🧴 1 µl ) and units. 3s

35 Repeat Step 34 for all samples.

6m

#### Nextera Library Preparation

17m

- 36 Make a master mix of the Tagment mix and Tagment Buffer for 96 samples (account for pipette error) and pipette to mix well.<sup>4m</sup>  
Aliquot **6 µl** of the master mix into each well of a new 96-well plate and add **4 µl** of 1ng Amplicon DNA.

Reagent	Amount
1ng of Amplicon DNA	4.0µl
Amplicon Tagment Mix (Illumina, FC-131-1096)	1.0µl
Tagment DNA Buffer (Illumina, FC-131-1096)	5.0µl
Total	10µl

- 37 Incubate at **55 °C** for **00:05:00**, then lower to **10 °C**. Once at 10°C, immediately add **2.5 µl** of Neutralizing Tagment Buffer to stop the reaction. Mix by pipetting up and down.<sup>5m</sup>

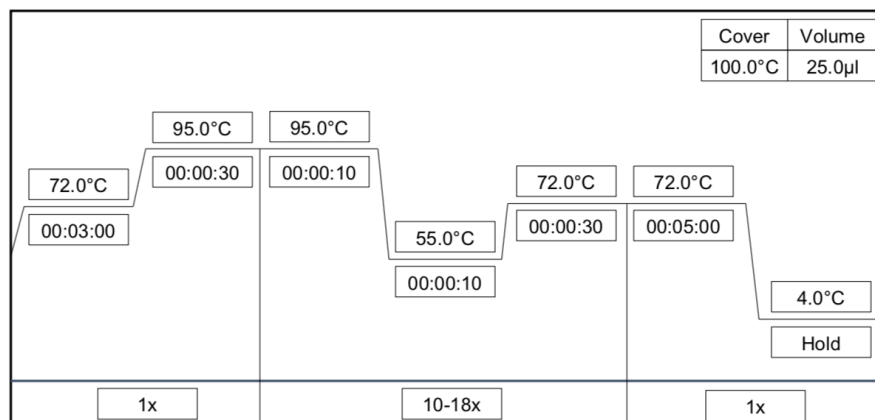
- 38 Incubate at **Room temperature** for **00:05:00**. Centrifuge at **280 x g** for **00:01:00**.

- 39 Make a mastermix of the Nextera PCR Master Mix and Index Primers 1 and 2 for 96 samples (account for pipette error) and mix by pipetting.<sup>1m</sup> Aliquot **12.5 µl** of this mastermix into each well of a new 96-well plate and add **12.5 µl** of tagmented amplicon DNA from Step 35.

Reagent	Amount
Tagment Amplicon DNA	12.5µl
Nextera PCR Master Mix (FC-131-1096)	7.5µl
Index 1 primer (i7) (Illumina, FC-131-2001)	2.5µl
Index 2 primer (i5) (Illumina, FC-131-2001)	2.5µl
Total	25µl

40 Set-up and run the following program on a thermal cycler to incubate reaction:

9m 20s



Final Library Cleanup

27m

41 After PCR, bring up sample volume in each well to **50 µl** with nuclease free water.



















42 Add a 1:1.8 ratio of sparQ PureMag beads to each well and mix gently pipetting. For example, add **90 µl** sparQ PureMag beads to a **50 µl** reaction. 30s

43 Incubate for **00:05:00** at room temperature. 5m

44 Place the plate on the Agencourt SPRIPlate Super Magnet Plate 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





45 While keeping on magnetic plate, add **200 µl** of **Room temperature 80 % volume (fresh made)** ethanol to the side of the well opposite of the pellet and let sit for **00:00:30**. 1m

46 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for **00:00:10** then remove any remaining ethanol. 20s

- 47 Add  **100 µl** of **[M]10 Milimolar (mM)** Tris-HCl (pH 8.0) , vortex, and pulse centrifuge to collect all liquid at the bottom of the tube. Incubate for  **00:02:00** at  **37 °C** . 2m
- 48 Separate beads on the Agencourt SPRIPlate Super Magnet Plate and transfer  **100 µl** of supernatant to a new plate for a second round of cleanup.
- 49 Add an equal volume (1:1) of sparQ PureMag beads to each well and mix gently by pipetting. For example add  **100 µl** <sup>30s</sup> sparQ PureMag beads to a  **100 µl** reaction.
- 50 Incubate for  **00:05:00** at room temperature. 5m
- 51 Separate beads on the Agencourt SPRIPlate Super Magnet Plate 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
- 52 While keeping on magnetic plate, add  **200 µl** of  **Room temperature** **[M]80 % volume (fresh made)** ethanol to the side of the well opposite of the pellet and let sit for  **00:00:30** . 1m
- 53 Avoid disturbing the bead pellet, carefully remove and discard ethanol. Wait for  **00:00:10** then remove any remaining ethanol. 10s
- 54  **go to step #52** and repeat ethanol wash. 1m 30s
- 55 Add  **20 µl** of **[M]10 Milimolar (mM)** Tris-HCl (Ph 8.0) and mix well by pipetting. Incubate for  **00:02:00** at  **37 °C** 2m
- 56 Place the plate on the Agencourt SPRIPlate Super Magnet Plate and incubate for  **00:02:00** or until the beads have pelleted and the supernatant is completely clear. Then, while avoiding the bead pellet, transfer the  **20 µl** final library to a new plate for subsequent steps and storage. 2m

#### Library Normalization

30m

- 57 Transfer  **10 µl** supernatant from each well of the PCR plate to the corresponding well of a new midi plate.
- 58 Combine the following volumes in a  **15 ml** conical tube to prepare the LN master mix. Multiply each volume by the number of samples being processed:  **23 µl** of LNA1 and  **4 µl** of LNA2.

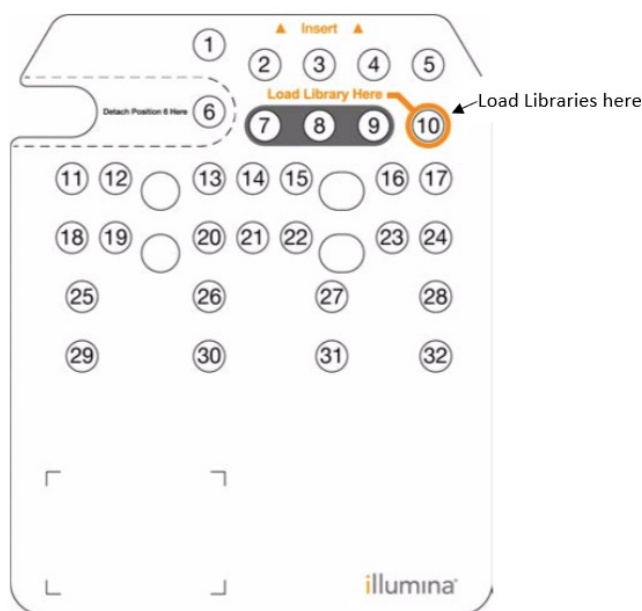
- 59 Pipette 10 times to mix and pour the LN master mix into a trough.
- 60 Use a p200 multichannel pipette to transfer **22.5 µl** LN master mix to each sample well.
- 61 Seal the plate, and then use a plate shaker at **1800 rpm** for **00:30:00**.
- 62 Place on the magnetic stand and wait until the liquid is clear (~2 minutes). Without disturbing the beads, discard all the supernatant.
- 63 Wash two times as follows:  
Add **22.5 µl** LNW1 to each well. Seal the plate, and then use a plate shaker at **1800 rpm** for **00:05:00**. Separate on a magnetic plate and wait until the liquid is clear (~2 minutes). Without disturbing the beads, remove and discard all supernatant.
- 64 Add **15 µl** 0.1 N NaOH to each well. Seal the plate, and then use a plate shaker at **1800 rpm** for **00:05:00**.
- 65 Add **15 µl** LNS1 to each well of a new 96-well PCR plate labeled SGP.
- 66 After the **00:05:00** elution completes, make sure that all samples in the midi plate are resuspended. If they are not, resuspend as follows:
  - 66.1 Pipette 10 times to mix or lightly tap the sample plate on the bench. Seal the plate, and then use a plate shaker at **1800 rpm** for **00:05:00**. Place on a magnet rack and transfer **15 µl** supernatant from each well of the midi plate to the corresponding well of the SGP plate.
  - 66.2 Seal the plate, and then centrifuge at **1000 x g** for **00:01:00**.
- 67 Place on a magnetic stand and wait until the liquid is clear ~ **00:02:00**.
- 68 Transfer **15 µl** supernatant from each well of the midi plate to the corresponding well of the SGP plate.
- 69 Seal the sample plate, and then centrifuge at **1000 x g** for **00:01:00**.

- 70 Remove a "Mid-Output Kit" reagent cartridge from -20°C freezer storage.
- 71 Place the cartridge right-side-up in a secondary container and fill the container with water at room temperature, but do not fully submerge the cartridge. Thaw cartridge in this water bath for about 🕒 **01:00:00** .
- 72 When the reagent cartridge has been thawing for 🕒 **00:30:00** , remove a "Mid-Output Kit" flow cell from the 4°C refrigerator but do not open the foil package. Place the flow cell on the bench for 🕒 **00:30:00** to warm up to 🌡 **Room temperature**
- 73 After the reagent cartridge is fully thawed, take it out of the water bath, tap on the bench to dislodge excess water, and dry with wipes.
- 74 Invert the cartridge to mix the reagents and make sure that the sequencing reagents in positions 29-32 are completely thawed. Then tap on the bench again to reduce air bubbles.
- 75 Remove the flow cell from its packaging and gently clean with a 70% ethanol wipe. Dry the glass with another wipe.
- 76 Inspect the flow cell to confirm: ports are free of obstructions, port gaskets are sealed and white plastic posts are visible, all 4 white retention clips are snapped over the edge of the black carrier plate and all 4 metal spring clips are laying flat against the black carrier plate.
- 77 Preheat an incubator to 🌡 **98 °C**
- 78 Remove a tube of Hybridization Buffer (HT1) from the -20°C freezer and thaw at 🌡 **Room temperature** . When thawed, place on ice.
- 79 In a fresh 1.5ml tube, combine an aliquot from each library pool such that the total volume is 📏 **5 µl** . For a Mid-Output kit, expect to obtain 150M reads in total.
- 80 Add 📏 **995 µl** of ice-cold Hybridization Buffer (HT1). Vortex briefly and centrifuge at 🌀 **300 x g** for 🕒 **00:01:00** .
- 81 Transfer 📏 **750 µl** of diluted library to a new tube and add 📏 **750 µl** of ice-cold Hybridization Buffer (HT1).

- 82 Vortex briefly and centrifuge at **300 x g** for **00:01:00**.
- 83 Place the tube on the **98 °C** heated incubator for **00:02:00** and immediately cool on ice. Leave on ice for **00:05:00**.
- 84 In a fresh 1.5ml tube, combine **1203 µl** of ice-cold Hybridization Buffer (HT1) and **97 µl** diluted library for a final concentration of **1.5 Picomolar (pM)**. Invert to mix and pulse centrifuge. Place on ice until ready to load onto the reagent cartridge.


### 85 Part 3: Load the Sequencer

Using a clean P1000 tip, poke a hole in the foil seal covering position 10 on the reagent cartridge (labeled "Load Library Here"). Avoid breaking any of the other seals on the cartridge:



Finalized libraries are loaded in hole 10 of reagent cartridge.

- 86 Add the entire 1.3ml of the **1.5 Picomolar (pM)** library dilution into this reservoir.
- 87 The reagent cartridge, flow cell, and buffer pack are now ready to be loaded onto the NextSeq500 for sequencing.

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