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Probe-based target enrichment of SARS-CoV-2

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1 Works for me dx.doi.org/10.17504/protocols.io.bd5di826

Coronavirus Method Development Community COG-UK

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ABSTRACT

- Viral RNA library prep using **SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian** followed by a probe-based bait capture (SeqCap, Roche and xGen, IDT) to generate tagged enriched viral libraries from total RNA that retain directionality in the library.
- The **SMARTer Stranded** kit uses random primers, tailed with Illumina Read1 sequence, to start reverse transcription, and a Template Switching Oligo (TSO) to add Read2 sequence at the 3' of the synthesized cDNA. The 1st stranded cDNA is then amplified using Indexed primers to generate in one step 2nd strand cDNA and complete tagged Illumina libraries.
- Pooled libraries undergo target enrichment using custom virus-specific biotinylated probes to capture cDNA derived from viral RNA present in the sample.
- This method has been optimized for large scale viral sequencing projects, and up to 2 x 96-well RNA plates can be prepped in parallel.
- The protocol follows "**Option 2 (without fragmentation)**" workflow of the kit User Manual for library preparation, with ¼ of the recommended reaction volume for denaturation and cDNA synthesis, and ½ for PCR, all steps done in 384-well plates.

NOTE: *the kit contains a Ribodepletion PCR-based module not used in this protocol, so PCR reagents are in excess and a higher volume for PCR can be used safely without depleting the kit of reagents unevenly.*

- The absence of an RNA fragmentation step coupled with more stringent cleanups (at 0.68x) generates libraries with longer insert size, therefor this protocol is only to be used on RNA samples of high quality, and unnecessary freeze-thaw of the RNA **MUST** be avoided. As the RNA extracted from plasma/swabs is below the detection level for QC, if in doubt about the quality of the RNA provided, proceed with library preparation and pooling, but check the size of the libraries before cleanup of the pool as the 0.68X beads ratio recommended in this protocol would remove most of the library if the starting RNA is of lower quality.
- To maximize RNA input and improve sensitivity of the assay, an RNA concentration step at the start of the procedure has been added, though not necessary to generate libraries.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

David Bonsall, Tanya Golubchik, **Mariateresa de Cesare**, Mohammed Limbada, Barry Kosloff, George MacIntyre-Cockett, Matthew Hall, Chris Wymant, Azim Ansari, Lucie Abeler-Dörner, Ab Schaap, Anthony Brown, Eleanor Barnes, Estelle Piwowar-Manning, Susan Eshleman, Ethan Wilson, Lynda Emel, Richard Hayes, Sarah Fidler, Helen Ayles, Rory Bowden, Christophe Fraser, and The HPTN 071 (PopART) team, **A comprehensive genomics solution for HIV surveillance and clinical monitoring in low income settings,** J Clin Mic (Accepted)

ATTACHMENTS

Sequencing_COVID-19_target_enrichment.pdf

GUIDELINES

Timeframe

The protocol can be completed in **2-3 days**, depending on the length of the hybridization time: if the capture is set up overnight on Day1, it can be completed on Day2.

Citation: Mariateresa De Cesare (04/02/2020). Probe-based target enrichment of SARS-CoV-2. https://dx.doi.org/10.17504/protocols.io.bd5di826

If there is not enough time to start the capture on Day1, set up the capture early on Day2 and hybridize for 4h: mind that once the hybridization is stopped, all following steps prior to LM-PCR must be completed!

***NOTE**: The time to dry with the SpeedVac the libraries pooled on Day1 is variable and dependent on the volume of the pool and could take >>1h*

Day1: Library prep with pooling, cleanup and QC; +/-set up for O/N capture
Day2: Capture; 10 nM, +/- qPCR

Day1	Step	Estimated time
	Preparation	*30 minutes*
	RNA concentration	30 minutes
	cDNA synthesis setup	10 minutes
	cDNA 1st strand synthesis	*1h 40 minutes*
	PCR setup	15 minutes
	PCR	35 minutes
	Pooling by volume	10 minutes
	Pool cleanup	30 minutes
	QC	15 minutes
Optional Day1 or Day2	*SpeedVac drying of pool*	*20 minutes – 1.5 hours*
	Capture setup	20 minutes
Day2	*Probe Hybridization*	*4 hours (or overnight)*
	Preparation of streptavidin beads and wash buffers	15 minutes
	Hybridization to streptavidin beads	*45 mins*
	Streptavidin washes and PCR setup	20 mins
	Post-Capture LM-PCR	*35 mins*
	Capture cleanup	30 minutes
	QC	15 mins
	qPCR setup	30 minutes
	qPCR	1.5 hours

References

Supplementary Documents:

- Library Preparation – Takara-Clontech, SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian
- Roche, SeqCap EZ Library SR

MATERIALS

NAME	CATALOG #	VENDOR
Qubit® dsDNA HS Assay Kit	Q32854	Thermo Fisher Scientific
High Sensitivity D5000 ScreenTape	5067-5592	Agilent Technologies
High Sensitivity D5000 Reagents	5067-5593	Agilent Technologies
High Sensitivity D1000 Reagents	5067-5585	Agilent Technologies
High Sensitivity D1000 ScreenTape	5067-5584	Agilent Technologies

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NAME	CATALOG #	VENDOR
SeqCap EZ Hybridization and Wash Kit	5634253001	Roche
SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian	634418	Takara
xGen® Universal Blockers—TS Mix 96 rxn	1075475	Integrated DNA Technologies
NG SeqCap EZ Accessory Kit V2	7145594001	Roche
RNAClean XP Kit	A66514	Beckman Coulter
AMPure XP	A63881	Beckman Coulter
High Sensitivity D5000 Ladder	5067-5594	Agilent Technologies
High Sensitivity D1000 Ladder	5067-5587	Agilent Technologies
Dynabeads™ M-270 Streptavidin	65305	Thermo Fisher Scientific

MATERIALS TEXT

Store
- SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian at **-20 °C**
- SMART TSO Mix v2 (from SMARTer® Stranded Total RNA-Seq Kit v2) at **-80 °C**
- NG SeqCap EZ Accessory Kits v2 at **-20 °C**
- SeqCap Hybridization and Wash Kit at **-20 °C**
- Dynabeads™ M-270 Streptavidin at **4 °C**

Required Content of the Kits:

Takara-Clontech, SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian
- SMART TSO Mix v2 (Cat#: ST1250)
- SMART Pico Oligos Mix v2 (Cat#: ST1262)
- 5X First-Strand Buffer (Cat#: ST1266)
- SMARTScribe RT (100 U/µl) (Cat#: ST1270)
- RNase Inhibitor (40 U/µl) (Cat#: ST1272)
- SeqAmp DNA Polymerase (Cat#: ST1280)
- SeqAmp CB PCR Buffer (2X) (Cat#: ST1282)
- Nuclease-Free Water
- Tris Buffer (5 mM) (Elution Buffer, EB)

SeqCap Hybridization and Wash Kit (Cat#: 5634253001)
- 10X SC Wash Buffer I (Vial 1)
- 10X SC Wash Buffer II (Vial 2)
- 10X SC Wash Buffer III (Vial 3)
- 10X Stringent Wash Buffer (Vial 4)
- 2x Hybridization Buffer (Vial 5)
- Hybridization Component A (Vial 6)
- 2.5X Bead Wash Buffer (Vial 7)

SeqCap EZ Accessory Kits v2 (Cat#: 07145594001)
- COT-1 Human DNA
- Water, DNA Grade

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- KAPA HiFi HotStart ReadyMix
- Post- LM-PCR Oligos 1 & 2

Additional Reagents

- IDT xGen® Lockdown Probes

Equipment

- Gilson Platemaster
  - Pipetman Diamond Tips DF30ST Tipack (Gilson, F171303)
  - Pipetman Diamond Tips DF200ST Tipack (Gilson, F171503)
- Invitrogen, DynaMag-2 Magnet (Cat#: 123-21D)
- 384 block Thermocycler
- DNA Vacuum Concentrator
- Heat block
- Single and multi-channel pipettes with tips, 2-1000 µl

- Qubit® 3.0 Fluorometer (Q33216)

- Tapestation 2200

- 1.5 ml LoBind tubes
- 0.2 ml PCR tubes

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING

Use RNase Zap to **decontaminate all work surfaces** prior to practical work, only work in a dedicated pre-PCR Area.

Preparations

1 Bring the **RNAClean XP** (A63987) to **Room temperature** .

2 Ensure a chilling block to accommodate 384-well plates is at **-20 °C** .

3 Defrost reagents (SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian and SMART TSO Mix v2) **On ice** .

4 Prepare the following program on a 384 thermal cycler:

Temperature	Time
72 °C	∞
72 °C	3 min
42 °C	∞
42 °C	90 min
70 °C	10 min
4 °C	∞

Thermal Cycler Program

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Concentration of the RNA using magnetic beads

5 Thaw the RNA samples **On ice** (up to 2 plates of 96 samples).

This protocol describes how process 2 plates of 96 samples in parallel, and uses a Gilson PlateMaster. If a PlateMaster or equivalent is not available, it is not recommended to process more than one 96-well plate.
For 96 or fewer samples, the protocol can be executed with multichannel pipettes.

6 

Add in **RNAClean XP beads** at **1.8 ratio** to all RNA samples.

NOTE: Precise measurement of the RNA volume of each sample is not crucial at this stage, as this “cleanup” has the only purpose of concentrating the RNA, and not removing unwanted oligos

7 

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

8 Place the plate on magnet: allow beads to separate for **00:08:00** .

9 

Remove supernatant and put aside in a new 96-well plate.

10 

Add **200 µl 80% ethanol** to wash the beads, incubate for **00:00:30** .

11 Remove **80% EtOH**.

12 

Repeat the ethanol wash (steps 10 – 11)

If two 96-well plates are being prepared together, do both ethanol washes on the 1st plate before moving to the 2nd plate, so that the same PlateMaster tips can be used for both washes on a plate.

WARNING: Keep to time with washes on the 2nd plate to avoid over drying beads on the 1st!

12.1 Add **200 µl 80% ethanol** to wash the beads, incubate for **00:00:30** .

12.2 Remove **80% EtOH**.

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13 Briefly spin the plate and remove additional ethanol with fresh tips. Ensure any visible quantities of ethanol are removed.

14 Remove the plate(s) from magnet and resuspend beads in **3 µl EB** .

15 

Leave the beads in EB for **00:05:00** at **Room temperature** .

16 

During the 5 mins incubation, prepare a 384-well plate containing **0.25 µl SMART Pico Oligos Mix v2** .

In our lab we use a Labcyte Echo 525 to dispense the Oligo Mix v2.
If accuracy of pipetting such small volume is a concern, consider scaling up the volume of the all protocol from 1/4 to 1/2 or more.

RNA denaturation

17 Pre-heat the thermocycler to **72 °C** .

18 Once the 5 mins elution time is passed, place the RNA plate on the magnet and allow beads to separate for **00:02:00** .

19 

Transfer **2 µl sample** into the plate containing **0.25 µl freshly-dispensed SMART Pico Oligos Mix v2** .

20 Mix with PlateMaster, quickly spin down the plate.

21 Final plate for denaturation should contain:

Reagent	Volume (µl)
SMART Pico Oligos Mix v2	0.25
RNA	2
Total	*2.25 µl / reaction*

22 

Place plate containing **RNA** and **SMART Pico Oligos Mix v2** onto the pre-set thermocycler and incubate at **72 °C** for **00:03:00** .

22.1 Take the chilling block out of the freezer during the incubation

23 Immediately move samples to the chilling block and leave for **00:02:00** .

24 Proceed **immediately** to First-strand cDNA synthesis

Citation: Mariateresa De Cesare (04/02/2020). Probe-based target enrichment of SARS-CoV-2. https://dx.doi.org/10.17504/protocols.io.bd5di826

First-strand cDNA synthesis

25 Pre-heat thermal cycler to **42 °C** .

26

Prepare First Strand reverse transcription mastermix by mixing the following reagents in the order shown (no. reactions + 10%):

Reagent	Volume per reaction (µl)
5X First-Strand Buffer	1
SMART TSO Mix v2	1.125
RNase Inhibitor	0.125
SMARTScribe Reverse Transcriptase	0.5
Total	*2.75 µl*

27

Add **2.75 µl** of MasterMix to each sample, mix by pipetting and then spin down the plate.

28

Place in the pre-heated thermocycler for first strand RT incubation:

42 °C for **01:30:00** → **70 °C** for **00:10:00** → **4 °C** hold.

29 Leave samples at **4 °C** until next step.

30

SAFE STOPPING POINT!

Overnight at **4 °C** or store at **-20 °C** .

PCR amplification of cDNA and library generation

31 Prepare thermocycler in a post-PCR area for second strand amplification.

32

Set up the PCR as follow:

Component		Volume per reaction (µl)
Sample	*First-strand cDNA*	*5*
EACH	Indexed i7 primer (6.25 µM)	1
	Indexed i5 primer (6.25µM)	1
Master Mix (MM)	Nuclease-free Water	5
	SeqAmp CB PCR Buffer (2X)	12.5
	SeqAmp DNA Polymerase	0.5
Total		5 samples + 2 primers + 18 MM = 25 µl / reaction

PCR Setup

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33

Mix by pipetting, spin down the plate and start PCR:

Step	Temperature	Time	No. cycles
Initial Denaturation	94 °C	60 seconds	1
Denaturation	*98 °C*	*15 seconds*	*12*
Annealing	*55 °C*	*15 seconds*	
Extension	*68 °C*	*30 seconds*	
Final Extension	68 °C	2 minutes	1
Hold	10 °C	∞	

34

SAFE STOPPING POINT!

Overnight at **4 °C** or store at **-20 °C** .

Pooling, cleanup and QC

35

Pool in a 1.5 ml LoBind tube equal volume of each sample to be captured together

NOTE: Library prepared with this protocol would yield on average 2-5 ng/µl: pool enough of each library for a final pool with >> 500 ng (3 µl per sample for a 96-plex is usually plenty).

36 Accurately measured with a pipette the volume of the pool: this is crucial for correct size-selection of the libraries with Ampure XP beads.

37

Add **0.68x Ampure XP** to each pool and perform a cleanup

WARNING: The **0.68x ratio** is to be used only on libraries from high quality RNA! If that is not the case, use 0.8x of Ampure XP instead.

38 Mix thoroughly either by pipetting or vortexing.

39

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

40

Transfer tubes to magnet and incubate for **00:08:00** .

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41 

Remove and keep supernatant in a clean tube. Do not transfer any beads in the supernatant. If this is a risk, leave **<5µl supernatant** behind with the beads.

42 

Wash beads with **200 µl 80% ethanol** . Leave for **00:00:30** .

43 

Remove and discard the supernatant.

44 

Repeat wash with **ethanol**.

44.1 Wash beads with **200 µl 80% ethanol** . Leave for **00:00:30** .

44.2 Remove and discard the supernatant.

45 Centrifuging tube briefly to collect any residual ethanol at the bottom of the well and pipette this off with a P10 or P20 pipette.

46 

Ensure removal of all ethanol.

47 Air dry beads until the well looks dry and the beads are starting to crack.

48 Resuspend beads in a volume of **EB** equal to the starting pool volume or less.

NOTE: Eluting in less EB will speed the drying of the pool in the SpeedVac

49 Mix by pipetting or vortexing. If vortexing, leave for **00:02:00** before centrifuging.

50 Return tubes to magnet for **00:02:00** .

51 Transfer the clean eluant to a fresh 1.5 ml LoBind tube.

52 

Quantify the libraries pool using **Qubit® dsDNA HS Assay Kit** (Cat#: Q32854).

53 

Check the size of the libraries pool on an Agilent Tapestation with a **High Sensitivity D1000 Screen Tapes assay** (Cat#: 5067-5584).

54 Bring forward *500 ng* from each pool for capture (next section) and store the remaining uncaptured pool.

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Hybridize xGen Lockdown Probes to Target

55 Turn on a heat block that takes 1.5 ml tubes, and let it equilibrate to **95 °C** .

56 Defrost **xGen® Universal Blockers (IDT)**, **COT Human DNA** (vial 1, Roche, SeqCap EZ Accessory Kit, stored at -20°C).

57 Bring the **2X Hybridization buffer** (vial 5) and **Hybridization Component A** (vial 6) to **Room temperature** (Roche, SeqCap EZ Hybridization and Wash Kit, stored at -20°C).

58 Remove the **xGen Lockdown Probes (IDT)** from -20°C freezer and defrost **On ice** .

The xGen Lockdown Probes are custom biotinylated oligos designed to capture cDNA libraries derived from the viral RNA.
This protocol is based on our work with HIV, and has been validated for SARS-CoV-2 using a panel of probes designed by our lab.

59

In a 1.5 ml tube, add:

Component	Amount
Multiplex DNA Sample Library Pool	500 ng
COT Human DNA	5 µl
xGen® Universal Blockers - TS Mix	2 µl

60 Dry down the contents of the tube (libraries + COT Human DNA + Blocking Oligos) using a SpeedVac with high temperature.

61

Once the pool is completely dry, resuspend in:

Component	Amount
2X Hybridization buffer (vial 5)	7.5 µl
Hybridization Component A (vial 6)	3 µl

62 Vortex for **00:00:10** then spin down

63 Place the tube in the **95 °C** heat block for **00:10:00** to denature the DNA.

64 Spin down and transfer the content of the tube to a 0.2 ml PCR tube.

65

Add **4 µl xGen Lockdown Probes** and top up with water to a final volume of **15 µl**.

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66 

Mix by pipetting.

67 The tube should contain the following:

Component	Amount
Multiplex DNA Sample Library Pool	500 ng*
Cot-1 DNA	5 µg*
xGen® Universal Blockers - TS Mix	2 µl*
2X Hybridization Buffer (vial 5)	7.5µl
Hybridization Component A (vial 6)	3 µl
xGen Lockdown Probes	4 µl
Nuclease-Free Water	0.5 µl
Total	*15 µl*

*Dried in the SpeedVac

68 

Incubate hybridization reaction at **47 °C** in a thermocycler (lid heated at **57 °C**) for **04:00:00** (or **Overnight**).

69 **If proceeding after a 4 h hybridization**, change the heat block's temperature used in step 63 to **47 °C** .

70 Allow time to equilibrate to the set temperature.

Prepare wash buffers

71 **If proceeding after an overnight hybridization**, turn on a heat block that takes 1.5 ml tubes to **47 °C** , and let it equilibrate to the set temperature.

72 

2 h before the end of the hybridization, dilute **10X Wash Buffers** (I, II, III and Stringent) and **2.5X Bead Wash Buffer** (Roche, SeqCap EZ Hybridization and Wash Kit, stored at -20°C) to create **1X working solutions**.

	Buffer (µl)	*Water (µl)*	*Final volume (µl)*
10X Wash Buffer I (vial 1)	***33***	***297***	***330***
10X Wash Buffer II (vial 2)	22	198	220
10X Wash Buffer III (vial 3)	22	198	220
10X Stringent Wash Buffer (vial 4)	44	396	440
2.5X Bead Wash Buffer (vial 7)	220	330	550

73 For each capture reaction, preheat the following wash buffers to **47 °C** in the heat block:

- 1X Stringent Wash Buffer (all)
- **110 µl 1X Wash Buffer I**

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74 Equilibrate buffers at **47 °C** for at least **02:00:00** before starting wash steps of the captured DNA (section "Wash streptavidin beads to remove unbound DNA" below).

Prepare the Streptavidin Dynabeads

75 Allow **Dynabeads M-270 Streptavidin** (stored at 4°C) to equilibrate to **Room temperature** for **00:30:00** before use (**~30minutes before the end of the hybridization**).

76 Mix the beads thoroughly by vortexing for **00:00:15** .

77 

Aliquot **100 µl streptavidin beads** per capture into a single 1.5 ml tube (i.e., for 1 capture use 100 µl beads, for 2 captures use 200 µl beads, etc.).

78 Place the tube in a magnetic separation rack. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.

79 

Add **200 µl 1X Bead Wash Buffer** per 100 µl beads. Vortex for **00:00:10** .

80 Place the tube back in the magnetic rack to bind the beads. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.

81 

Repeat wash with **1X Bead Wash Buffer**.

81.1 Add **200 µl 1X Bead Wash Buffer** per 100 µl beads. Vortex for **00:00:10** .

81.2 Place the tube back in the magnetic rack to bind the beads. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.

82 

After removing the buffer following the second wash, add 1X the original volume of beads of **1X Bead Wash Buffer** (i.e., for 100 µl beads, use 100 µl buffer) and resuspend by vortexing.

83 

Transfer **100 µl** of the resuspended beads into a new 0.2 ml tube for each capture reaction.

84 Place the tube in a magnetic rack to bind the beads. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.

Bind hybridized target to the streptavidin beads

85 Take the samples pool from **go to step #68** out of the thermocycler (**do not stop the program!**) quickly spin down and transfer to the tube containing prepared streptavidin beads.

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86 

Mix thoroughly by pipetting up and down *10 times*.

87 Place the tube back into the thermal cycler set to **47 °C** and incubate for **00:45:00** (set heated lid at **57 °C**) to bind the DNA to the beads.

88 Vortex the tube for **00:00:03** **every 15 min** to ensure that the beads remain in suspension.

Wash streptavidin beads to remove unbound DNA

89 

Take the samples out of the thermal cycler and add **100 µl pre-heated 1X Wash Buffer I** to the tube and vortex for **00:00:10** to mix.

90 

Spin down and transfer the mixture to a fresh low-bind 1.5 ml tube.

91 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

92 

Add **200 µl preheated 1X Stringent Wash Buffer** and pipette up and down 10 times to mix.

Incubate on the heat block at **47 °C** for **00:05:00** .

93 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

94 

Repeat wash with preheated **1X Stringent Wash Buffer**.

94.1 Add **200 µl preheated 1X Stringent Wash Buffer** and pipette up and down 10 times to mix.

Incubate at **47 °C** for **00:05:00** .

94.2 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

95 

Add **200 µl room temperature 1X Wash Buffer I** and vortex for **00:02:00** to mix.

96 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

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97

Add **200 µl room temperature 1X Wash Buffer II** and vortex for **00:01:00** to mix.

98 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

99

Add **200 µl room temperature 1X Wash Buffer III** and vortex for **00:00:30** to mix.

100 Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

101

Remove the tube from the magnetic rack and add **25 µl Nuclease-Free Water** to resuspend the beads. Mix thoroughly by pipetting up and down 10 times.

Do not pellet or remove the beads: The post-capture PCR is done on the beads

102

SAFE STOPPING POINT!

Overnight at **4 °C** or store at **-20 °C** .

Post-capture PCR

103 Thaw the **KAPA HiFi HotStart ReadyMix** and the **Post-LM-PCR Oligos** from the SeqCap EZ Accessory Kits v2 (stored at -20°C).

104 Set up the PCR as follow:

	Component	Volume
EACH	Captured library on beads	25
MASTER MIX	KAPA HiFi HotStart ReadyMix (Roche)	50
	Post-LM-PCR Oligos 1&2, 5µM (Roche)	5
	Nuclease free water	20
	Total	*75 +25 library =100 µl / reaction*

PCR Setup

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105

PCR incubation:

Step	Temperature	Time	No. cycles
Initial Denaturation	98 °C	45 seconds	1
Denaturation	*98 °C*	*15 seconds*	*12*
Annealing	*60 °C*	*30 seconds*	
Extension	*72 °C*	*30 seconds*	
Final Extension	72 °C	1 minute	1
Hold	10 °C	∞	

106

SAFE STOPPING POINT!

Store at **-20 °C** or **4 °C** .

107

Perform **Ampure XP** clean-up with **0.68x ratio** and elute into **20 µl** .

WARNING: The **0.68x ratio** is to be used only on libraries from high quality RNA! If that is not the case, use 0.8x of Ampure XP instead

108

SAFE STOPPING POINT!

Store at **-20 °C** or **4 °C** .

Captured Pool QC and 10nM-ing

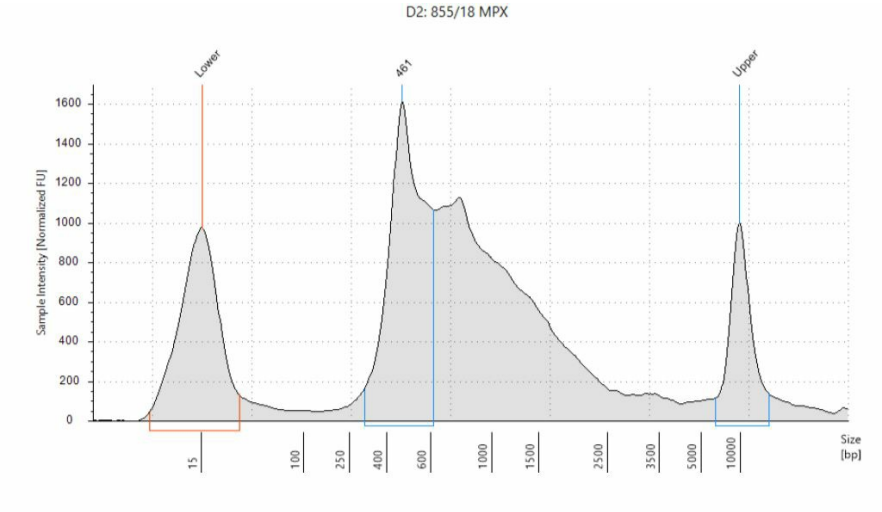
109

Quality check the captured pool using Qubit and Tapestation.

Pools concentrations is highly variable and dependent on samples viral load (<1 ng/µl is NOT to be considered a fail!)

Citation: Mariateresa De Cesare (04/02/2020). Probe-based target enrichment of SARS-CoV-2. https://dx.doi.org/10.17504/protocols.io.bd5di826

110 Tapestation profile should have a long tail, but it's dependent of the quality of the starting RNA. For high quality RNA, after two 0.68x Ampure XP cleanups, one pre- and one post-capture, the profile should look like this:



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