USER GUIDE

YOUSEQ

SARS-CoV-2 WHOLE GENOME NGS LIBRARY PREP KIT

CAT NO: YS-NGS-nCoV19-96-IL

96 samples
(For Illumina® Sequencers)

Version 1.9

For Research Use Only

YOUSEQ

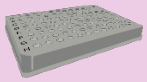
CONTENTS

KIT CONTENTS

cDNA SYNTHESIS/TARGET ENRICHMENT PACK

Component	Cap Colour	Size
C9 Buffer		1.2 mL
cDNA Synthesis Reagent		100 µL
100 mM DTT		200 µL
SARS-CoV 2 Primer Pool A v3		1.5 mL
SARS-CoV 2 Primer Pool B v3		1.5 mL
YouSeq NGS MasterMix		(5x) 1 mL
Cleaning Reagent		60 µL
Cleaning Solution		500 µL

INDEXING PACK

Component	Lid Colour	Size
YouSeq NGS MasterMix		(3x) 1 mL
YouSeq Index Adapter Set A		96 x 10 µL

BEAD CLEAN PACK

Component	Bottle	Size
Magnetic Beads		40 mL
Wash Buffer *Add 96 mL of 100% Ethanol to the wash buffer to total 120 mL*		120 mL
Elution Buffer		22 mL

MATERIALS REQUIRED BUT NOT PROVIDED

- RNase-free water
- 100% Ethanol
- Ice or frozen block
- Illumina sequencing instrument and sequencing reagents (300 cycle)
- (optional) PhiX control – Illumina catalogue no. FC-110-3001
- PCR machine
- (Optional) – qPCR machine
- 96-well plates suitable for your PCR machine
- Bench top vortex
- Magnetic rack for 96 well plates
- 1.5 mL tubes
- Manual pipettes with compatible filter tips
- Multichannel pipettes with compatible filter tips
- Foil seals
- Qubit Fluorometer with hsDNA reagents
- Agilent Bioanalyzer (optional)

WORKFLOW OVERVIEW

Total hands on-time required approximately 3 hours.

Stage	Hands on Time	Hands off Time	Description
cDNA synthesis	5 minutes	1.2 hours	cDNA synthesis
Target enrichment	20 minutes	2.5 hours	Multiplex PCR to amplify cDNA targets using two pools of primers
Bead clean 1	30 minutes	45 minutes	Purification with 1.0X Magnetic Beads
Cleaning reaction	10 minutes	20 minutes	Removal of primer-dimer causing oligonucleotides and artefacts with Cleaning Reagent
Bead clean 2	30 minutes	45 minutes	Purification with 1.0X Magnetic Beads
Indexing	20 minutes	25 minutes	Unique index added to sample, and library amplified in singleplex PCR reaction
Library purification	30 minutes	30 minutes	Purification with 0.8X Magnetic Beads
Quantification and pooling	Depends on the method of choice		

WORK AREAS

It is critical to use separate work areas with dedicated equipment for each stage of the library preparation process. If this is not possible, then make sure all work areas and equipment are cleaned with a DNA-destroying reagent such as DNA Away or a 0.1% Bleach solution, followed by isopropyl alcohol/isopropanol.

If contamination occurs, non-specific products of PCR reactions in downstream stages will be amplified in later stages and can cause the failure of library preparation due to an abundance of primer dimers or PCR artefacts, and a loss of library yield.

All reactions should be sealed in plates before being carried into the dedicated work area for thermal cycling.

Stage	Dedicated work area for setting up reaction	Dedicated work area for thermal cycling
cDNA synthesis	RNase free/Pre-PCR	RNase free/Pre-PCR
Target enrichment	RNase free/Pre-PCR	Target enrichment
Bead clean 1	Target enrichment	
Cleaning reaction	Target enrichment	Target enrichment
Bead clean 2	Target enrichment	
Indexing	Target enrichment	Indexing/Post-PCR
Library purification	Indexing/Post-PCR	
Quantification and Pooling	Indexing/Post-PCR	
Sequencing	Indexing/Post-PCR	

cDNA SYNTHESIS

cDNA Synthesis Reagent, DTT, and a buffer containing random oligonucleotides are used to generate cDNA from the swab RNA template.

a. In the **RNase free/Pre-PCR** work area, prepare enough MasterMix for your templates and keep on ice.

cDNA Synthesis MasterMix components	Lid Colour	Volume per sample
C9 Buffer		12 µL
100 mM DTT		2 µL
cDNA Synthesis Reagent		1 µL

b. Add 15 µL of MasterMix to each well of a 96-well plate kept on ice, or in an open PCR block set to 4°C

c. Add 5 µL of RNA template to the plate and mix gently by pipetting.

d. Seal plate, and run the following protocol

Temperature	Time
25°C	10 minutes
45°C	1 hour
70°C	15 minutes
4°C	Hold

TARGET ENRICHMENT

The neat cDNA reaction is divided into two discrete multiplex PCR reactions. Each reaction contains a specific primer set to generate alternate amplicons across the entire SARS-CoV-2 genome. The two reactions will be combined before indexing to allow for full, tiled coverage of the genome.

a. In the RNase free/Pre-PCR work area thaw the NGS MasterMix, SARS-CoV-2 Pool A Primers, and SARS-CoV-2 Pool B Primers. Once thawed, vortex the NGS MasterMix well and keep on ice.

b. For each sample of cDNA synthesis in the previous reaction, prepare two reactions on 2 x 96 well plates

Reaction A	Lid Colour	Each sample
cDNA reaction		8 µL
NGS MasterMix		23 µL
SARS-CoV-2 Primer Pool A v3		15 µL

Reaction B	Lid Colour	Each sample
cDNA reaction		8 µL
NGS MasterMix		23 µL
SARS-CoV-2 Primer Pool B v3		15 µL

c. Seal and spin down the plates.

d. Move the plates into the **Target enrichment** work area and load onto the thermal cycler

e. Run the following protocol

	Temperature	Time
Initial denaturation	95°C	3 minutes
24 cycles	95°C	15 seconds
	65°C	4 minutes
Hold	4°C	Hold

BEAD CLEAN 1

Magnetic beads are supplied in a DNA binding buffer. When added to the sample, the multiplex PCR amplicons bind to the magnetic beads. The sample is washed, and the purified DNA is eluted.

a. For each sample combine 40 µL of PCR product from Pool A with 40 µL of Pool B in a plate

b. Bring YouSeq Magnetic Beads to room temperature by removing from the fridge 30 minutes before use. Vortex beads well until suspension is homogeneous

c. Add 80 µL YouSeq Magnetic Beads directly into each PCR1 pool and mix beads well until suspension is homogeneous

d. Incubate for 5 minutes at room temp

e. Place on magnet and wait until liquid is clear (approximately 5 minutes) Remove and discard supernatant

f. Wash samples with 200 µL of Wash buffer.

g. Remove as much of the Wash Buffer as possible.

h. Spin down the plate and remove as much residual Wash Buffer as possible.

i. Leave to dry at RT **(do not take off the magnet)**. Wait until the pellet of beads are dry (approximately 2 minutes) but do not take on a 'cracked' appearance

j. Keeping the sample plate on the magnetic rack, add 35 µL Elution Buffer

k. Remove your tube from the magnet and mix well by pipetting.

l. Incubate for 2 minutes at ambient temperature. Place on magnet and wait until supernatant is clear.

m. Remove 31 µL of the supernatant to a new PCR plate, ready for cleaning.

Safe Stop Point: 24 hours at 2-8°C or 1 month at -20°C

CLEANING REACTION

A reagent mix is used to remove unincorporated primers, PCR artefacts, and other non-specific products of the target enrichment multiplex PCR

a. Thaw the Cleaning Solution at ambient temperature.

b. Prepare sufficient mastermix for your samples, vortex well and keep on ice.

	Lid Colour	Each sample
Cleaning Solution		3.5 µL
Cleaning Reagent		0.5 µL

c. Add 4 µL of this MasterMix to the 31 µL of purified amplicons from the target enrichment step.

d. Run the following program on a thermal cycler

Temperature	Time
37°C	15 minutes
80°C	5 minutes
4°C	Hold

BEAD CLEAN 2

The cleaned multiplex PCR amplicons bind to the magnetic beads. The sample is washed, and the cleaned amplicons are eluted ready for indexing.

a. Bring Magnetic Beads to room temperature by removing from the fridge 30 minutes before use. Vortex beads well until suspension is homogeneous

b. Add 35 µL Magnetic Beads directly into each sample and mix beads well until suspension is homogeneous

c. Incubate for 5 minutes at room temp

d. Place on magnet and wait until liquid is clear (approximately 5 minutes) Remove and discard supernatant

e. Add 200 µL of Wash Buffer to the beads and mix by pipetting.

f. Remove as much supernatant as possible. Spin down the plate and remove as much residual Wash Buffer as possible.

g. Leave to dry at RT **(do not take off the magnet)**. Wait until the pellet of beads are dry (approximately 2 minutes) but do not take on a 'cracked' appearance

h. Keeping the sample plate on the magnetic rack, add 20 µL Elution Buffer

i. Remove your tube from the magnet and mix well by pipetting.

j. Incubate for 2 minutes at ambient temperature. Place on magnet and wait until supernatant is clear.

k. Remove 15 µL of the supernatant to a new PCR plate, ready for indexing.

INDEXING

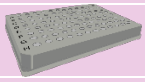
Indexed adapters are added to the ends of the amplicons which allow them to bind to the flow cell.

10 µL of each index is provided in a fully skirted PCR plate with a foil cover. To avoid cross contamination of indexes between samples, pierce the foil for each index required. A spare foil is provided to cover the opened wells.

a. In the **Target enrichment** work area, thaw the blue PCR plate containing the indexes at ambient temperature. Spin the plate down well, and make sure that there is no liquid on the wall of the well, or near the seal.

b. Thaw enough NGS MasterMix for your samples. Once thawed, vortex well and keep on ice.

c. For each sample, use a multi-channel pipette to prepare the following

	Lid colour	Each sample
Cleaned amplicons		15 µL
Index		10 µL
NGS Master Mix		25 µL

d. Mix well by pipetting, seal and spin down the plate.

e. Move the plate to the **Indexing/Post-PCR** work area and load onto the thermal cycler

f. Run the following PCR protocol:

	Temperature	Time
Initial denaturation	95°C	3 minutes
8 cycles	95°C	15 seconds
	60°C	30 seconds
Hold	4°C	Hold

Safe Stop Point: 24 hours at 2-8°C or 1 month at -20°C

LIBRARY PURIFICATION

The final libraries are purified ready for quantification and normalization. 0.8X volume of beads are added to the library.

a. Bring Magnetic Beads to room temperature by removing from the fridge 30 minutes before use. Vortex beads well until suspension is homogeneous

b. Add 40 µL Magnetic Beads directly into each sample and mix beads well until suspension is homogeneous

c. Incubate for 5 minutes at room temp

d. Place on magnet and wait until liquid is clear (approximately 5 minutes) Remove and discard supernatant

e. Add 200 µL of Wash Buffer to the beads and mix by pipetting.

f. Remove as much supernatant as possible. Spin down the plate and remove as much residual Wash Buffer as possible.

g. Leave to dry at RT **(do not take off the magnet)**. Wait until the pellet of beads are dry (approximately 2 minutes) but do not take on a 'cracked' appearance

h. Keeping the sample plate on the magnetic rack, add 20 µL Elution Buffer

   Remove your tube from the magnet and mix well by pipetting.

i. Incubate for 2 minutes at ambient temperature. Place on magnet and wait until supernatant is clear.

j. The final, purified library is in the supernatant. Remove 17 µL to a clean PCR plate for quantification and storage.

Safe Stop Point: 24 hours at 2-8°C or 1 month at -20°C

LIBRARY QUANTIFICATION

Quantification can be done via multiple methods; we'd recommend qPCR via our library quantification kit (YS-NGS-LQC-IL-96). Qubit or PicoGreen quantification methods can also be used.

If using the Qubit:

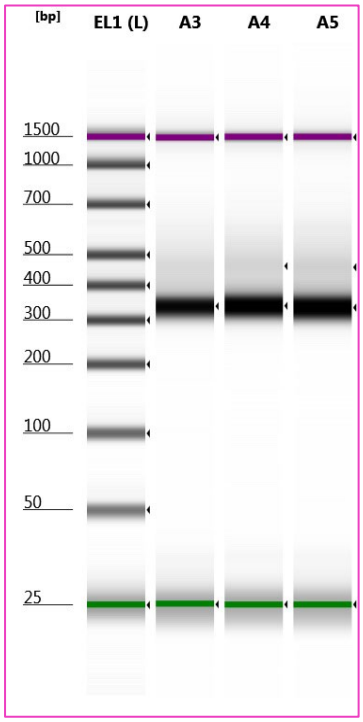
a. Prepare Qubit hsDNA reagent according to manufacturer's instructions and quantify each library.

b. Using the concentration of each library in ng/µL, convert to nM using the following formula.

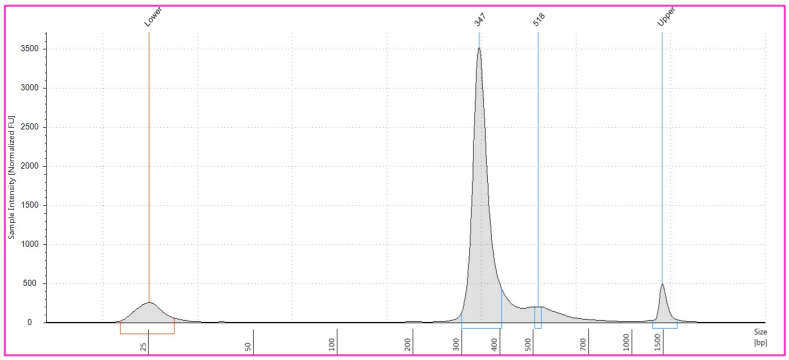
$$\frac{\text{ng/µL}}{(660 \times 360)} \times 1{,}000{,}000$$

If using the TapeStation System:

a. Prepare the Library and TapeStation according to manufacturer's instructions
b. The library should be a dark band between 310-360 bp band on the gel. An example Gel band and Electropherogram report is shown below.



TapeStation Gel, showing SARS-Cov-2 NGS library. The library has a weight of 347 bp. Acceptable range for the library size is between 310-360 bp



POOL LIBRARIES FOR SEQUENCING

a. Dilute each library to 1 nM.

b. Combine 5 µL of each 1 nM library to a fresh 1.5 mL tube

c. **Optional** - Check size of pooled library on Bioanalyzer hsDNA chip /TapeStation HS D1000 tape

Follow loading instructions relevant to your Illumina instrument according to manufacturer's instructions.

Optional - addition of PhiX is optional as the library is complex, however, for run performance characteristics, we'd recommend 5% spike of PhiX.

APPENDIX

Working with RNA

When working with RNA, it is essential to avoid contamination with RNases.

i. Wash all work areas, pipettes and equipment with RNaseZAP or equivalent.
ii. Ensure all water and plasticware used is RNase-free.
iii. Avoid multiple freeze-thaw cycles of stock RNA. Aliquot stock RNA into small expendable volumes and store at -80°C. Only thaw when needed.
iv. Thaw RNA on ice, do not leave at room temperature.
v. Prior to PCR1, an initial clean of pipettes and work areas with 5% bleach solution, followed by either 70% ethanol or isopropanol is recommended.
vi. Products such as DNA Away can also be used, to avoid handling concentrated bleach solution.

Separation of Lab Equipment

To help prevent contamination, it is critical to separate all PCR1 equipment from all other future steps

Specific Reduction of Contamination Risk

High background levels of PCR 2 products in the lab during library preparation can have a detrimental effect on the quality and integrity of your sequencing data.

If lab equipment has been used for PCR 2 and is required for either PCR 1 or multiplex cleaning, then it should be washed thoroughly with 5% bleach and 70% ethanol or isopropanol.

RNA Templates

YouSeq recommend the use of either QIAGEN RNeasy Mini kit (74104) or any other high-quality RNA extraction kit. Store all eluted RNA at -80°C in small aliquots until needed.

Bead Clean

After the removal of the wash buffer, make sure the pellets of magnetic beads do not take on a dry ‘cracked’ appearance as this may reduce the quality of DNA amplicons.