User Guide

YOUSEQ

SARS-COV-2

NGS LIBRARY PREP KIT

96 samples (For Illumina® Sequencers)

Product Code: YS-COVID-IL-96

Version 1.4

Kit contents

RT Pack

	Cap colour	Size
10X RT Buffer	○	200 µL
YouSeq Reverse Transcriptase	●	10 µL
100 mM DTT	○	200 µL
100 mM dNTPs	○	100 µL
Random Hexamers	○	100 µL

PCR1 & Cleaning Pack

	Cap colour	Size
SARS-CoV 2 primers Pool A	◌	1.5 mL
SARS-CoV 2 primers Pool B	◌	1.5 mL
YouSeq NGS MasterMix	◌	5 mL
YouSeq Cleaning Reagent	●	60 µL
YouSeq Cleaning Solution	◌	500 µL

Pouch 1 of 3
Store at -20°C

PCR2 Pack

	Cap colour	Size
YouSeq NGS MasterMix	◌	3 x 1 mL
YouSeq dual-labelled Index Primers	96 well Plate	(x96) 10 uL each well

Pouch 2 of 3
Store at -20°C

Bead clean pack

	Size
YouSeq clean up beads	40 ml
Wash buffer*	120 ml
Elution buffer	22 ml

*Add 96 mL of 100% Ethanol to the wash buffer to total 120 mL

Pouch 3 of 3
Store at 4°C

Other items to be supplied by the user

- **RNase-free water**
- 100% Ethanol
- Ice
- Illumina sequencing instrument
- (optional) PhiX control – Illumina catalogue no. FC-110-3001
- PCR machine
- 96-well plates suitable for your PCR machine
- Bench top vortex
- Magnetic rack
- 1.5 mL tubes
- Manual pipettes, filter tips and foils
- Qubit Fluorometer with hsDNA reagents
- Agilent Bioanalyzer (optional)

Workflow

Total hands on-time required approximately 3 hours.

Stage	Hands on Time	Hands off Time	Description
RNA/RT primer hybridization	10 mins	5 mins	Improve cDNA synthesis by pre-hybridization of random hexamers to RNA template
cDNA synthesis	5 mins	1.2 hours	cDNA synthesis
PCR 1	20 mins	2.5 hours	Multiplex PCR to amplify cDNA targets using two pools of primers
Bead clean 1	30 mins	45 mins	Purification with 1.2X YouSeq NGS beads
Multiplex cleaning	10 mins	20 mins	Removal of primer-dimer causing oligonucleotides and artefacts with YouSeq Cleaning reagent
Bead clean 2	30 mins	45 mins	Purification with 1.2X YouSeq NGS beads
PCR 2	20 mins	25 mins	Unique index added to sample, and library amplified in singleplex PCR reaction
Bead clean 3	30 mins	30 mins	Purification with 0.8X YouSeq NGS beads
Quantification and pooling	Depends on the method of choice		

Protocol

cDNA synthesis

i) Set a thermal cycler to incubate at 65°C, with heated lid at 105°C
ii) In a 0.2 mL PCR tube prepare the following <u>on ice</u>;

Hybridization of Random Hexamers to RNA template

1 µL Random Hexamers
4 µL Purified RNA template

iii) Add the PCR tube to the thermal block and incubate for 5 minutes to denature any secondary structure RNA.
iv) Dilute YouSeq Reverse Transcriptase 1:50 with RNase-free water.

Dilution of YouSeq Reverse Transcriptase

1 µL YouSeq Reverse Transcriptase
49 µL RNase-free water

v) Immediately transfer the product onto ice, and prepare the following in a separate PCR tube;

cDNA synthesis

For each sample prepare the following

2 µL 10X RT Buffer
2 µL 100 mM DTT
0.8 µL 100 mM dNTPs
1 µL YouSeq Reverse Transcriptase (1/50 dilution)
9.2 µL RNase-free water

vi) Add this 15 µL mix directly into the 5 µL of Random Hexamers/RNA template on ice.
vii) Run the following protocol;

Temp	Time
25°C	10 mins
45°C	1 hour
70°C	15 mins
4°C	Hold

PCR1 (Multiplex)

Each 20 µL cDNA synthesis reaction is divided into two. 10 µL of the reaction is used as the template in two discrete multiplex PCR reactions.

Load each sample well with the following:

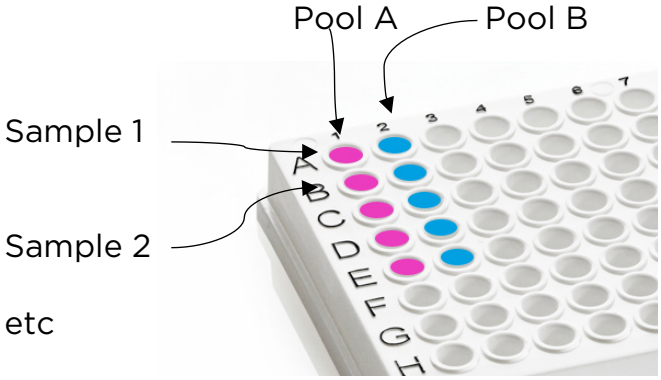
i) Vortex the NGS MasterMix well, with 5 pulse vortexes

Pool A
10 µL cDNA reaction
25 µL YouSeq One-Step NGS Mastermix
15 µL SARS-Cov 2 Pool A primers

Pool B
10 µL cDNA reaction
25 µL YouSeq One-Step NGS Mastermix
15 µL SARS-Cov 2 Pool B primers

ii) Mix well and spin down the plate prior to loading on the PCR machine.

Example plate:



iii) Seal the plate and load into your PCR machine.

iv) Run the following PCR protocol:

	Temp	Time
24 cycles	95°C	3 mins
	95°C	30 secs
	64°C	4 mins
Hold	4°C	Hold

Bead Clean Up 1

Pool

For each sample combine 45 µL of PCR product from Pool A with 45 µL of Pool B

Bring YouSeq Magnetic Beads to room temperature by removing from the fridge 30 minutes before use.

Bind

- Vortex beads well until suspension is homogeneous
- Add 108 µL YouSeq NGS Beads directly into each PCR1 pool and mix beads well until suspension is homogeneous
- Incubate for 5 mins at room temp
- Place on magnet and wait until liquid is clear (approx. 5 min)
- Remove supernatant

1st wash

- Leave your tube on the magnet
- Add 200 µL of Wash Buffer
- Mix by pipetting
- Remove supernatant carefully

2nd wash

- Repeat as per 1st wash

Dry beads

- Leave to dry at RT **(do not take off the magnet)**
- Wait until the pellet of beads is completely dry and has taken on a cracked appearance.

Elute

- **Keeping the sample plate on the magnetic rack**, add 35 µL Elution buffer
- Remove your tube from the magnet and mix well by pipetting.
- Incubate for 5 min at RT. Place on magnet and wait until supernatant is clear.
- Remove 31 µL of the supernatant to a new PCR plate, ready to multiplex cleaning.

Safe Stop Point: 24 hours at 2-8°C or 7 days at -20°C

Multiplex cleaning

Prepare enough YouSeq Cleaning mix to remove primer-dimer causing artefacts and oligonucleotides from each reaction.

For individual reactions, the setup is as follows

Multiplex Cleaning Mix

31 µL Eluted PCR1 Product
3.5 µL YouSeq Cleaning Solution
0.5 µL YouSeq Cleaning Reagent

For multiple samples, in a 1.5 mL tube prepare the following. Mix well by pipetting. Do not vortex.

Number of columns	Volume YouSeq Cleaning reagent (µL)	Volume YouSeq Cleaning solution (µL)
1	4.5	31.5
2	9.0	63.0
3	13.5	94.5
4	18.0	126.0
5	22.5	157.5
6	27.0	189.0
7	31.5	220.5
8	36.0	252.0
9	40.5	283.5
10	45.0	315.0
11	49.5	346.5
12	54.0	378.0

Return the eluted PCR Product to the magnetic Rack

1. When the beads have pelleted, remove 31 µL of eluted product to a new 96-well PCR plate.

2. To each well, add 4 µL of YouSeq Cleaning mix and mix well by pipetting.

3. Run the following program on a thermal cycler

Temp	Time
37°C	15 minutes
80°C	5 minutes
4°C	hold

Bead Clean Up 2

Bring YouSeq Magnetic Beads to room temperature by removing from the fridge 30 minutes before use.

Bind

- Vortex beads well until suspension is homogeneous
- Add 42 µL YouSeq NGS Beads directly in to each PCR1 pool and Mix beads well until suspension is homogeneous
- Incubate for 5 mins at room temp
- Place on magnet and wait until liquid is clear (approx. 5 min)
- Remove supernatant

1st wash

- Leave your tube on the magnet
- Add 200 µL of Wash Buffer
- Mix by pipetting
- Remove supernatant carefully

2nd wash

- Repeat as per 1st wash

Dry beads

- Leave to dry at RT **(do not take off the magnet)**
- Wait until the pellet of beads is completely dry and has taken on a cracked appearance.

Elute

- **Keeping the sample plate on the magnetic rack**, add 20 µL Elution buffer
- Remove your tube from the magnet and mix well by pipetting.
- Incubate for 5 min at RT. Place on magnet and wait until supernatant is clear.
- Remove 15 µL of the supernatant to a new PCR plate, ready to PCR2

PCR 2 (Indexing)

Take a fresh 96-well plate and according to your experimental plan, load each well with the following:

i) Vortex the One-step Master Mix well, with 5 pulse vortexes

15 µl Clean PCR1 product
10 µl YouSeq index primer
25 µl YouSeq NGS MasterMix

ii) Mix well and spin down the plate prior to loading on the PCR machine.

Seal the plate and load into your PCR machine.

Run the following PCR protocol:

	Temp	Time
6 cycles	95°C	3 min
	95°C	15 sec
	60°C	30 sec
	72°C	5 min
	4°C	HOLD

Safe Stop Point: 24 hours at 2-8°C or 7 days at -20°C

Bead Clean Up 3

Bring YouSeq Magnetic Beads to room temperature by removing from the fridge 30 minutes before use.

Bind

- Vortex beads well until suspension is homogeneous
- Add 40 µL YouSeq NGS Beads directly in to each PCR1 pool and Mix beads well until suspension is homogeneous
- Incubate for 5 mins at room temp
- Place on magnet and wait until liquid is clear (approx. 5 min)
- Remove supernatant

1st wash

- Leave your tube on the magnet
- Add 200µL of wash Buffer
- Mix by pipetting
- Remove supernatant carefully

2nd wash

- Repeat as per 1st wash

Dry beads

- Leave to dry at RT **(do not take off the magnet)**
- Wait until the pellet of beads is completely dry and has taken on a cracked appearance.

Elute

- **Keeping the sample plate on the magnetic rack**, add 20 µL Elution buffer
- Remove your tube from the magnet and mix well by pipetting.
- Incubate for 5 min at RT. Place on magnet and wait until supernatant is clear.
- Your library is in the supernatant and is ready to be quantified and normalised.

Quantification

Quantification can be done via multiple methods; we'd recommend qPCR via our library quantification kit. However, using Qubit or PicoGreen quantification methods are acceptable.

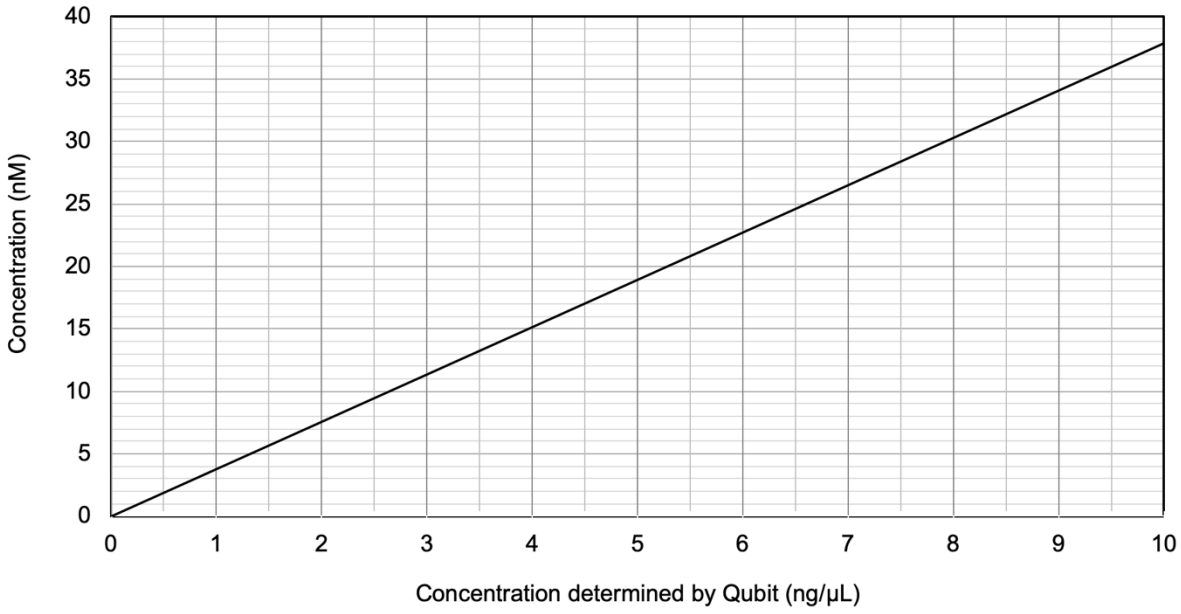
If using the qubit:

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

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

$$\frac{\text{ng/µL}}{(660 \times 410)} \times 1{,}000{,}000$$

Guide to check conversion for 410 bp Coronavirus library.



Pooling and library dilution

Dilute each library to 4 nM. Combine 5 µL of each 4 nM libraries to a single 1.5 mL tube.
Optional - Check size of pooled library on Bioanalyzer/TapeStation hsDNA chip

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.

Tips and tricks to succeed

a. Work areas

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

b. Separation of Lab Equipment

i. It is critical to separate all lab equipment used for the bead-based purification of PCR 2 products, final library pooling and quantification, from any equipment used to set up PCR 1 or multiplex cleaning.

c. Specific reduction of contamination risk

i. Once the library has been eluted after Bead Clean 3, any beads remaining should not be left to dry off the magnet. Once the libraries have been eluted, the plate should be sealed with foil and disposed of.
ii. 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.

d. RNA templates

i. 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.
ii. Efficiency of reverse transcription from the viral template can be improved by removing human ribosomal RNA from sample RNA. YouSeq recommend the use of NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) (E6350).