User Guide

YOUSEQ

SARS-COV-2

NGS LIBRARY PREP KIT

96 samples (For Illumina® Sequencers)

Product Code: YS-nCoV19-IL-96

Version 1.6

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 v2.1	◌	1.5 mL
SARS-CoV 2 primers Pool B v2.1	◌	1.5 mL
YouSeq NGS MasterMix	◌	(5x) 1 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	◌	(3x) 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 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
- 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
1. RNA/RT primer hybridization	10 mins	5 mins	Improve cDNA synthesis by pre-hybridization of random hexamers to RNA template
2. cDNA synthesis	5 mins	1.2 hours	cDNA synthesis
3. PCR 1	20 mins	2.5 hours	Multiplex PCR to amplify cDNA targets using two pools of primers
4. Bead clean 1	30 mins	45 mins	Purification with 1.0X YouSeq NGS beads
5. Multiplex cleaning	10 mins	20 mins	Removal of primer-dimer causing oligonucleotides and artefacts with YouSeq Cleaning reagent
6. Bead clean 2	30 mins	45 mins	Purification with 1.0X YouSeq NGS beads
7. PCR 2	20 mins	25 mins	Unique index added to sample, and library amplified in singleplex PCR reaction
8. Bead clean 3	30 mins	30 mins	Purification with 0.8X YouSeq NGS beads
9. Quantification and pooling	Depends on the method of choice		

Protocol

1. RNA/ RT Primer Hybridization

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.

2. cDNA Synthesis

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) Seal the plate

viii) Run the following protocol;

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

3. 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:

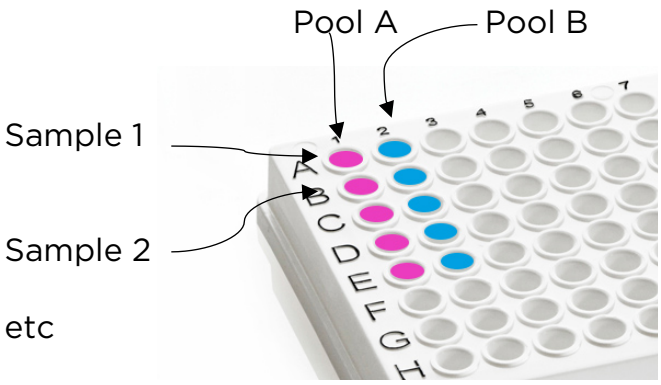
i) Vortex the NGS MasterMix well, with 5 pulse vortexes

Pool A
7 µL cDNA reaction
25 µL YouSeq NGS Mastermix
15 µL SARS-CoV 2 **Pool A** primers
3 µL RNase-free water

Pool B
7 µL cDNA reaction
25 µL YouSeq NGS Mastermix
15 µL SARS-CoV 2 **Pool B** primers
3 µL RNase-free water

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	15 secs
	65°C	4 mins
Hold	4°C	Hold

*Ensure you set the PCR reaction, so it has 50 µL reaction volume

4. Bead Clean 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 **90 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

5. Enzyme 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

Enzyme 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

6. Bead Clean 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 **35 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

7. 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.

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

Run the following PCR protocol:

	Temp	Time
	95°C	3 min
8 cycles	95°C	15 sec
	60°C	30 sec
	4°C	HOLD

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

8. Bead Clean 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 **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.
- Your library is in the supernatant and is ready to be quantified and normalised.

9. 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.

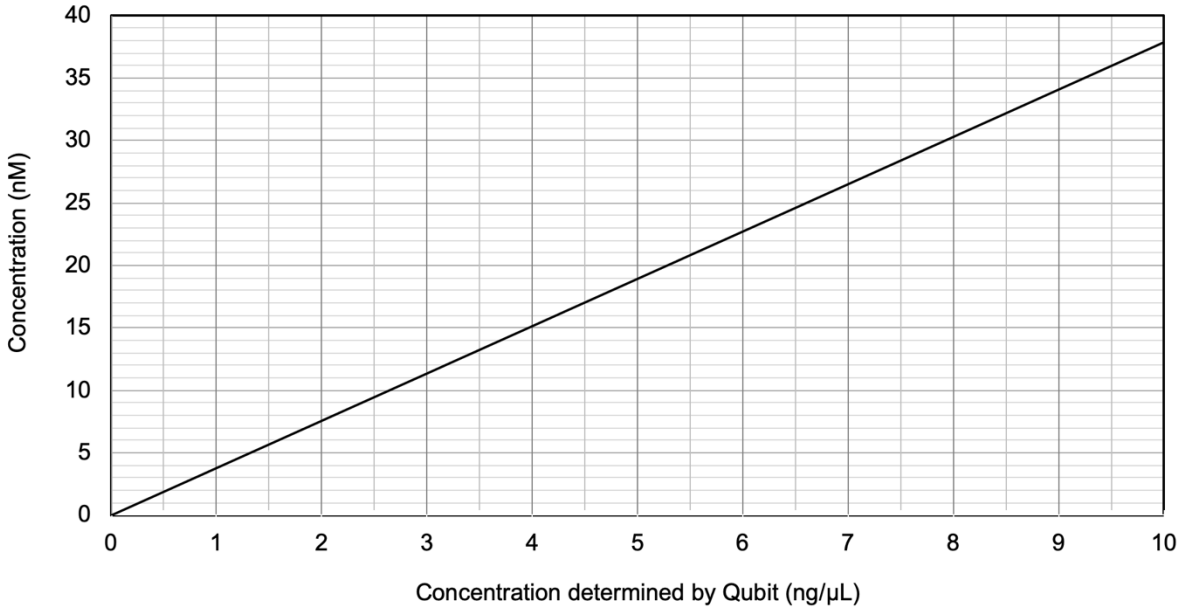
If using the qubit:

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

i) Dilute each library to **1 nM**.

ii) Combine **5 µL** of each **1 nM libraries** to a fresh 1.5 mL tube

iii) **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. 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.

b. Separation of Lab Equipment

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

c. Specific reduction of contamination risk

i. 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.