

User Guide



YOUSEQ

The ONE™ 16S NGS Library preparation Kit

For use on Thermo Fisher Ion Torrent sequencers

Version 2.0

8, 24 & 96 Reactions

World's simplest NGS Library Preparation Workflow

qPCR

Pool


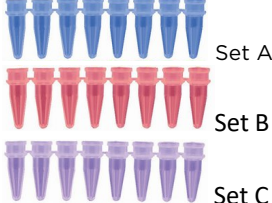


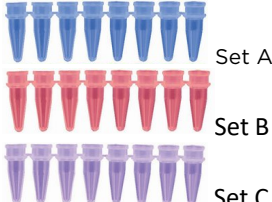




Clean

Validate




Sequence

Kit contents

PCR Pack

	Size (rxns)		
	8	24	96
16S Oligo Mix (v3)		 Set A Set B Set C	
	5 μ L each well		
16S Oligo Mix (v4)		 Set A Set B Set C	
	5 μ L each well		
The ONE™ MasterMix	 1 mL per vial	 1 mL per vial	 2 mL per vial

Bead clean pack

	Cap colour
YouSeq beads	
Wash buffer*	
Elution buffer	

*Add molecular biology grade ethanol to the supplied diluent as per label to make the wash buffer ready to use.

Other items to be supplied by the user

- ThermoFisher Ion Torrent NGS sequencing instrument, reagents and Sequencing chips
- Real-Time qPCR instrument
- Plasticware suitable for your qPCR instrument
- Magnetic rack
- Molecular Grade Ethanol
- Pipettes and general laboratory equipment
- Phix control - Illumina catalogue no. FC-110-3001

qPCR Protocol

The ONE™ Ion Torrent 16S kit has the ability to sequence either or both v3 and v4 region of the 16S rRNA gene. This kit is supplied with both v3 and v4 region primer sets and to achieve both, it would be required to run two separate reactions. YouSeq would recommend running both v3 and v4 region to achieve a more accurate microbial classification result.

***IF RUNNING BOTH V3 and V4 REACTIONS, YOU MUST RUN THE SAME INDEX WELL FOR EACH SAMPLE**

- i.e. Microbial sample 1 is run with v3 index 1 and v4 index 1*

1. Load each well of your 96-well PCR plate or PCR tubes with the following:
 - a. **ESSENTIAL** - Clearly note the location of each well used from oligo mix plate, this well position will become the samples ID further downstream
 - b. i.e. sample 1 used oligo mix from well A1
i.e. sample 2 used oligo mix from well A2
etc.....

V3 Region Reaction

10 µl - YouSeq NGS Master Mix

5 µl - v3 specific Oligo Mix

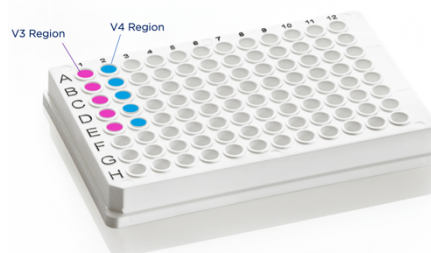
5 µl - Microbial Sample

V4 Region Reaction

10 µl - YouSeq NGS Master Mix

5 µl - v4 specific Oligo Mix

5 µl - Microbial Sample



2. Seal the plate/PCR tubes and load into your qPCR machine.

3. Run the following PCR protocol:

	Temp	Time	
25 cycles*	95°C	3 minutes	
	95°C	30 seconds	
	56°C	45 seconds	
	72°C	30 seconds	Acquire SYBR
	72°C	5 minutes	
	Melt Curve (65-95C)		Acquire SYBR
	4°C	HOLD	

*It is not desirable for all samples amplifications to reach plateau. Instead the ideal reaction will reach “take off” point thus triggering a threshold C_q value. This means the sample is within the exponential phase of amplification rather than reaching plateau.

If after 25 cycles, amplification has not begun in your samples as indicated by the beginnings of an amplification plot then add 4 cycles. Consult your instrument instructions for best method to add 4 more cycles.

Relative Quantification and Library Pooling

Quantify

**YouSeq can provide an excel sheet with automatic calculator built in: hello@youseq.com **

Adding the same amount of library for each sample on to the sequencing run is best practise and something YouSeq would highly recommend. Our unique protocol allows for relative quantification of each sample without any additional steps or costs.

4. Set the threshold
 - a. YouSeq recommend 10% of the delta Rn - however, user can use their preferred method
 - b. Simply export the Ct values from your qPCR instrument to an excel spreadsheet.

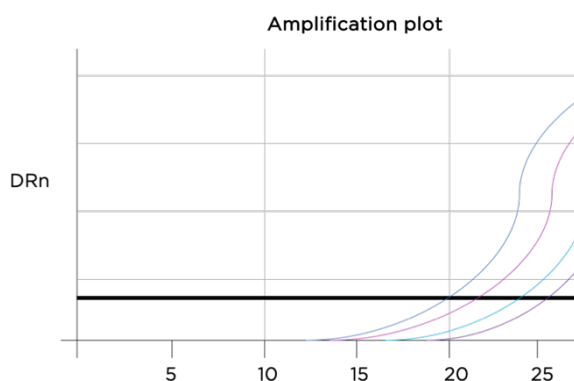


Figure 1, Example qPCR traces to represent a typical result. In this instance, purple trace with Ct value around 20 would be the highest Ct value of the set.

Pooling Calculations

5. Discount obvious outlying samples that did not produce good quality amplification plots.
6. From the remaining data set, find the highest Ct value (lowest concentration) from the results
7. Now calculate the relative amount of library for each of your samples based on the Ct value, relative to that of the sample with the highest Ct value. Follow the calculation below
 - a. *i.e. Relative Ratio = Sample Ct value / Highest Ct Value*

Calculate Sample volume for pool

8. Times the calculated ratio for each sample by 10. This value is now the volume of each sample you need to add to your pooled tube.
 - a. If the total volume < 100ul (because you are using only a small number of samples) then increase the volume of each pooled sample by 2x, 3x, 4x etc... as appropriate to make the total >100 μ L
 - b. Stated protocol requires 100 μ L for bead cleaning, if your total pooled mix is less than this, even after doubling up the volumes of each sample, simply times the total volume by 0.8 to give you the volume of beads to add for the following bead clean up step.

Table 1 Shows an example dataset and calculation values from the random 4 subset from figure 1.

Sample	Ct	Relative	Volume to pool
Swab_1	19.1	0.69	6.9
Swab_2	23.6	0.85	8.5
Swab_3	25.4	0.91	9.1
Swab_4	27.8	1.00	10.0

9. Add required volume of each sample to a new low-bind microcentrifuge tube.

Clean up protocol

Bring the beads to room temperature by removing from fridge 30 minutes before use

- Aliquot **100 μL** of pooled library into a new low-bind tube.

Bind

- Mix beads well until suspension is homogeneous
- Add **80 μL** YouSeq NGS Beads to the pooled product and mix well
- Incubate for 3 mins at room temp
- Place on magnet and wait until liquid is clear
- Remove and discard the supernatant

1st Bead Wash

- Leave your tube on the magnet

***N.B.** ensure the wash buffer has had correct volume of ethanol added*

- Add **200 μL** wash buffer
- Incubate for 30s
- Remove and discard the supernatant carefully

2nd Bead Wash

- Repeat as per 1st Bead Wash
- Ensure all remainder ethanol has been removed

Dry beads

- Leave until noticeably dry (cracking) on the magnet * (should be around 10 minutes)

***N.B.** if you notice any residual buffer on the sides of the tubes, carefully remove with pipette.

Elute

- Add **20 μL** Elution buffer
- Remove your tube from the magnet and mix well
- Incubate for **5 min** at RT
- Place on magnet and wait until liquid is clear

Your clean library is in the clear liquid. Carefully transfer to a fresh DNase/RNase free tube and proceed to the next step.

Finalise

Quality check *Optional*

If this protocol is new or is not routine

- Recommended - to check the quality of your DNA library with a Bioanalyzer or TapeStation (Agilent). Look for a prominent peak at around 600bp to indicate a good quality library.
- Significant peaks at other size points indicate a potential problem and you should troubleshoot before proceeding.

Quantify

- Check the concentration of the library pool. There are several methods to achieve this, YouSeq would recommend using qPCR for the most accurate measurement (e.g. YouSeq qPCR library quantification kit (Catalogue number: YS-LQK-IT-96))
- However, other methods are available such as a Fluorometer such as a Qubit (Thermo Fisher)

Dilute

- According to your results, dilute the pooled sample in YouSeq elution buffer to 4nM.

Your Library is now ready to sequence

Loading library

Depending on the intended Ion Torrent Sequencer model, follow the manufacturers guidelines on method and concentration of loading the library.

Samples sheets

Sample sheets containing all of the necessary info around indexing etc are available for download from The ONE 16S product page of www.youseq.com

Data Analysis

We recommend the use of the YouSeq 16S online App for data analysis found at:

<https://youseq.basepairtech.com>

This is a fully automated data analysis tool that delivers an easy to use choice of PDF, csv or html output.

If you have any questions or suggestions for our App we would love to hear from you.

If you can't get access to our web solution, please transfer your fastq files to YouSeq. We will process the data for you and return the reports within 24 hours.

Because the fastq file is large we recommend using a free data transfer service such as WeTransfer:

www.wetransfer.com

data@youseq.com

Tips and tricks to make life easy

Input DNA Amount

- While not a necessity and if you are new to this you may want to use a consistent amount of DNA in your reactions for more reliable results.
- We'd suggest an ideal input amount 10 ng in your PCR reaction. However, the kit is designed to work in the range of 5-40ng DNA. Use of qPCR method is ideal but Qubit Fluorometer to quantify your DNA is possible.
- It is good practice to set up your qPCR plate on ice or on a cold rack. This can improve your results.
- Be extremely careful when opening your plate after qPCR. The plate contains millions of copies of your library and can easily contaminate other samples if good laboratory practice is not followed.

Library Quantification

- Visual examination of your qPCR amplification plot will allow you to see which samples haven't amplified efficiently, any values with a negative number will need to be excluded as this will bias quantification.
- Inefficient or "dirty" starting DNA template may result in no amplification, so it is recommended you check your DNA quantity and quality post extraction and prior to qPCR.

Bead clean up

- Remove as much ethanol as you can (without disturbing the clump of beads) before the bead drying step. It is sometimes useful to centrifuge your tube, return it to the magnet, and then remove the final few microlitres.
- The ideal, precise bead drying time, will depend on the temperature and humidity in your lab. Inspect the pellet of beads by eye.
- If you over-dry the clump it is difficult to resuspend the pellet in the next step. You'll will need to increase the elution time as the bead has become too dry and ensure visually no clumping remains

Library Quality check

- A single peak close to 600 bp should be seen, this can be slightly broad, especially in a complex microbiome sample

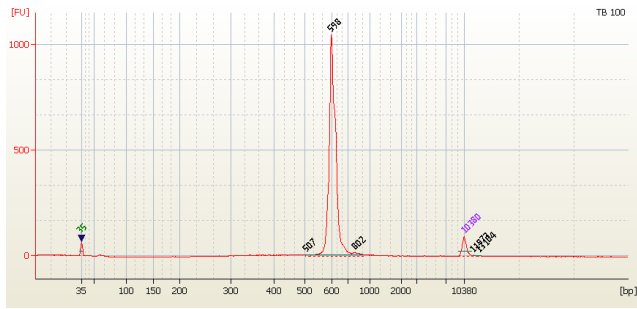


Figure 2, Example Bioanalyzer traces to represent a typical result. In this instance, this is a library formed from a single TB colony so very sharp peak around 600 bp.

Contact our technical support team if your library looks like it has unincorporated primers, adapters or non-specific PCR artefacts.