YOUSEQ

THE ONE 16S NGS LIBRARY PREPARATION KIT USER GUIDE

CAT NO. YS-NGS-ONE16S-IL-96

96 samples (For Illumina Sequencers)

VERSION 3.0

For Research Use Only

YOUSEQ

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INTENDED USE

The ONE 16S Library Preparation Kit allows the user to generate an indexed library of the V3 and V4 regions of the bacterial 16S gene in a single-PCR. After purification the amplicons generated are ready to sequence. We also provide an online bioinformatic tool for automated analysis.

PRODUCT DESCRIPTION

YouSeq believes that the vast potential of NGS should be accessible to every researcher, regardless of specialism and experience.

The ONE 16S NGS library preparation kit provides the worlds simplest and most comprehensive NGS workflow, which is designed to take you from sample to analysis report with minimal hands-on time.

This protocol combines the 16S V3-V4 fragment amplification step with the indexing step in a 'single tube' reaction reducing the time, effort and chances of user error compared with a more 'traditional' library preparation protocol. The integrated bioinformatics pipeline allows you to easily determine the bacterial community composition of your samples, without the requirement for specialist bioinformatics training. Simply upload the fastq files from your Illumina instrument to our app and we will do the rest.

BACKGROUND INFORMATION

It is highly recommended that you use a DNA extraction kit that is compatible with your type of sample. High inhibitor carryover from the original sample to the qPCR mix may prevent the reaction from performing optimally.

It is also highly recommended that you quantify the amount of bacterial DNA present in your sample (via fluorometric assay) in addition to total DNA (using Qubit or equivalent). This will enable you to optimise the qPCR protocol much faster and will ensure the best results from the ONE kit.

To prevent contamination of the reactions and your workspace, good molecular practice should be maintained at all times. Clean your workspace and equipment with DNA Away or 7% bleach solution prior to commencing the protocol.

WORKFLOW

Step	Action	Hands-on time/ Hands-off time
Initial assessment of the template*	Determine the optimal PCR cycle number for preparing libraries by running an initial qPCR with indexed primers.	30 minutes/1.5hours
16S library preparation	Generate an indexed library in a single PCR reaction, ready for pooling.	30 minutes/1.5hours
Pooling and normalisation	Using the endpoint fluorescence measurement of your qPCR machine, calculate the volume of each sample to add to the final library pool	30 minutes
Bead purification	Purify the pooled libraries using magnetic beads	30 minutes
Preparation of library for sequencing	Quantify the pooled, purified library and prepare for loading onto your Illumina instrument	15 minutes

*Optional step

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KIT CONTENTS

	Volume/Rxns
16S Oligo Mix Primer Plate	96 x 15 µl
The ONE MasterMix	2 x 1ml
Magnetic Beads	0.5 ml
Wash Buffer	2 ml
Elution Buffer	0.5 ml

PROVIDED ONLINE

16S Metagenomic analysis NGS (youseq.com)

	Document
Plate Layout Template	Excel spreadsheet showing which samples have which index code
Pooling Calculations Template	Excel spreadsheet for calculating sample volume to add to the final library pool

REAGENT PREPARATION

Prepare the reagents as per the table below.

	Reagent	Volume to add
Wash buffer	100% Ethanol	1.6 ml

MATERIALS REQUIRED BUT NOT PROVIDED

- PCR hood
- 100% ethanol
- qPCR instrument
- Benchtop microcentrifuge
- Vortex mixer
- Adjustable micropipettes (2 or 10 ml, 200 ml and 1000 ml)
- Multichannel pipette (10 ml)
- Racks for 1.5 ml microcentrifuge tube
- 1.5 ml microcentrifuge tubes (DNase/RNase free)
- Aerosol barrier pipette tips with filters
- Disposable gloves
- 96 well PCR plate
- Optically clear plate seals
- Ice or chilled beads in container

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REAGENT STORAGE, SAFETY AND QUALITY CONTROL

Both The ONE MasterMix and the 16S Oligo mix primer plate should be stored at –20C and kept on ice while being used. The 16S Oligo mix primer plate should be kept level and upright at all times including during storage. The magnetic beads, wash buffer and elution buffer should be kept in the fridge at 4C while not is use.

While none of the kit components are classified as hazardous, good laboratory practice should be maintained while carrying out the protocol. A suitable lab coat and gloves should be worn while handling chemicals.

Each component of The ONE 16S kit is tested against a set of rigorous specifications to ensure consistency and the best quality product.

INPUT SAMPLE REQUIREMENTS

This kit has been optimised for a wide variety of different sample types, including traditionally 'difficult' inputs such as faeces and soil. This kit has been optimised for between 5-25 ng of bacterial DNA per reaction, we recommend you load DNA within this range. Less DNA can be used, however you will have to adjust the cycling protocol (see number 3 in the Library Preparation Protocol below)

16S LIBRARY PREPARATION PROTOCOL

Please note:

a. Each well of the provided 16S Oligo mix primer plate has a unique barcode ID. Therefore, you must make careful note which well from the 16S Oligo primer plate you can use for each sample. This well will become the 'sample ID' in downstream processes and will allow you to identify each sample after demultiplexing. Use the supplied plate layout template to assist you with assigning each sample to a well.

b. Do not exceed the volume of DNA stated in the formula below. Samples with a high DNA concentration (>5 ng/µL) should be diluted to within 1-5 ng/µL) with water or elution buffer. Samples should be diluted to a similar concentration to prevent unnecessary PCR cycles being used.

c. To prevent sample and index contamination between wells, ensure that you use a fresh tip per well when dispensing both the primers and DNA samples.

Set up the following reaction mixture **on ice** in either a white qPCR plate or PCR tubes suitable for your qPCR instrument, depending on the number of samples to be run. Use a multichannel pipette to dispense the primers. Make sure you check each tip has the correct volume of liquid in.

Component	Volume
The ONE MasterMix	10 µl
16S Oligo mix primers	5 µl
Sample DNA	5 µl
Final Volume	20 µl

Please note: Seal the plate carefully and spin it briefly in a plate centrifuge to ensure that all the reagents are at the bottom of the well.

This YouSeq kit will work with any qPCR instrument capable of detecting the required fluorophores. Use the following cycling conditions:

	Temperature	Time	
	95°C	3 minutes	
*Phase 1 10 cycles	95°C	30 seconds	
	56°C	45 seconds	
	72°C	30 seconds	
*Phase 2 10 cycles	95°C	30 seconds	
	56°C	45 seconds	
	72°C	30 seconds	Acquire through SYBR/FAM channel
	72°C	5 minutes	
	4°C	HOLD	

*Phase 1: We suggest that you **do not** record the SYBR output for the first 10 cycles of the protocol.

*Phase 2: This is the recommended number of cycles to achieve successful entry to exponential phase when loading the suggested amount of bacterial DNA (see ESSENTIAL NOTES 1, above). If you are loading less than the recommended amount, you may have to optimise the cycle number. If after 10 cycles amplification has not begun, then increase the number of cycles to 15. It is not recommended to run the qPCR for more than 15 cycles at Phase 2 as this may introduce errors to the sequence (refer to Supplementary Information Table 1 for advice on cycle optimisation)

Please note:

a. The kit contains enough **The ONE MasterMix** and **16S Oligo Mix Primers** to run two 96 well plates. If after the first qPCR run you do not achieve satisfactory curves, then we suggest you optimise the cycling protocol, set up and run a new plate – ensuring you have followed all the instructions correctly

b. If you encounter any strange artifacts in your curves, refer to the Troubleshooting section of this document for advice

c. If proceeding directly to the next step of the protocol, then place your qPCR products on ice. If you are not proceeding directly to the next step, then the qPCR products can be stored at -20°C for up to a week.

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SAMPLE QUANTIFICATION AND LIBRARY POOLING

It is highly recommended that you add equimolar amounts of your qPCR products to the library pool to prevent one sample 'crowding out' the others during sequencing.

1. First check that all the amplification plots have behaved as expected. Discard any anomalous results. See supplementary information Figure 1 for an example of typical traces.

2. Export the endpoint fluorescence values from your qPCR instrument to an excel document.

3. Insert the endpoint values into the correct cells of the supplied Pooling Calculations Template spreadsheet. The spreadsheet will automatically calculate the volume of each sample that you will need to add to the final library pool using the following calculations:

Volume to add () = (Highest Sample Endpoint Value/Sample Endpoint Value) x 2*

*This multiplication factor can be changed – see Pooling calculations template for guidance.

4. Add the required volume of each sample to a single low-bind 1.5ml microcentrifuge tube.

Please note: Be very careful when opening your plate after the qPCR. It now contains millions of copies of your library which can easily contaminate other samples. Use good laboratory practice at all times.

BEAD CLEAN UP PROTOCOL

1. Obtain the volume of bead mix to add to your pooled library from the Pooling calculations template worksheet.

2. Thoroughly mix the bead solution until it becomes homogeneous and then add the required amount to the pooled library.

3. Flick the tube to mix and spin it down briefly before incubating at room temperature for 3 minutes.

4. Put the tube onto a magnetic rack and leave it there until the solution becomes clear and the beads have formed a tight pellet on the magnet. Your DNA library is now bound to the beads.

5. Leaving the tube on the magnetic rack, carefully remove and discard the supernatant.

6. Leaving the tube on the magnetic rack, add 200 ml of the wash buffer to the beads and wait for 30 seconds. Ensure that the solution is clear and then remove and discard the wash buffer.

7. Remove the tube from the magnet, briefly spin the tube and return to the magnet. Remove any excess wash buffer with 10 µl tip carefully

8. Leave the cap of the tube open and leave the bead pellet to dry for 1 minute until it has lost its glossy sheen and becomes dull. Do not over dry.

9. Add 100 ml of elution buffer directly to the bead pellet. Remove the tube from the magnet and vortex briefly to mix until homogenous. Incubate for 5 minutes at room temperature. Your DNA library is now in solution.

10. Put the tube back onto the magnet and wait until the solution is clear. Carefully remove the supernatant and place it into a fresh low-bind DNase/RNase free tube. This tube contains your purified, pooled DNA library.

FINAL STEPS

Quality Check (Optional – highly recommended)

Run a sample of your pooled library on a bio-analyser or TapeStation (Agilent). Look for a prominent peak between 580-600bp to indicate a good quality library. Peaks at other sizes indicate a potential problem and you should troubleshoot this before proceeding. Refer to Supplementary Information Figure 2 for a typical Tape Station electropherogram.

Library Quantification

Check the concentration of your library pool. You can use the value from the TapeStation or use another method (Qubit or qPCR e.g. YouSeq qPCR Library Quantification Kit, Catalogue Number YS-NGS-LQC-IL-96).

Dilution

Use the results from the previous step to dilute the library pool to a suitable loading concentration for yout sequencing system. YouSeq recommends 80 pmol loading concentrations.

PhiX control

PhiX must be added to low complexity libraries (such as 16S libraries) for optimal sequencing performance. YouSeq advises adding 20% PhiX when using an iSeq100.

Your library is now ready to sequence. Follow the manufactures guidelines on the concentration and volume of library to load onto the instrument you are using,

All the required worksheets and barcode metadata are available from YouSeq Customer Services team.

SEQUENCING

DATA ANALYSIS & SUPPORT

We recommend use of the YouSeq 16S online application 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.

For customer support, please contact us for details on how to access and use this platform:

Email: support@youseq.com

Phone: +44 (0)333 577 6697

TROUBLESHOOTING

1. **No amplification curves seen after 10 cycles of PCR in Phase 2 of the protocol:**

   a. Increase the number of cycles in Phase 2 and 15

   b. If the amplification is still not satisfactory after 15 cycles, you may have a high amount of inhibitors, poor quality DNA or insufficient quantity of DNA in your sample. You may need to consider a purification and/or concentration step prior to qPCR. Ensure that you use an extraction kit suitable for your sample type.

2. **Bunching of amplification plots, strange fluorescence readings, inappropriate automatic baseline correction**

   a. This may be due to your qPCR cycler having trouble defining a baseline.

   b. Do not discard the qPCR plate. Instead run the protocol below on the same qPCR instrument you used to generate the curves. This will produce a fresh "end point" fluorescence reading that can be used for pooling caluculations.

Temperature	Time	
72°C	2 minutes	Acquire SYBR/FAM Channel

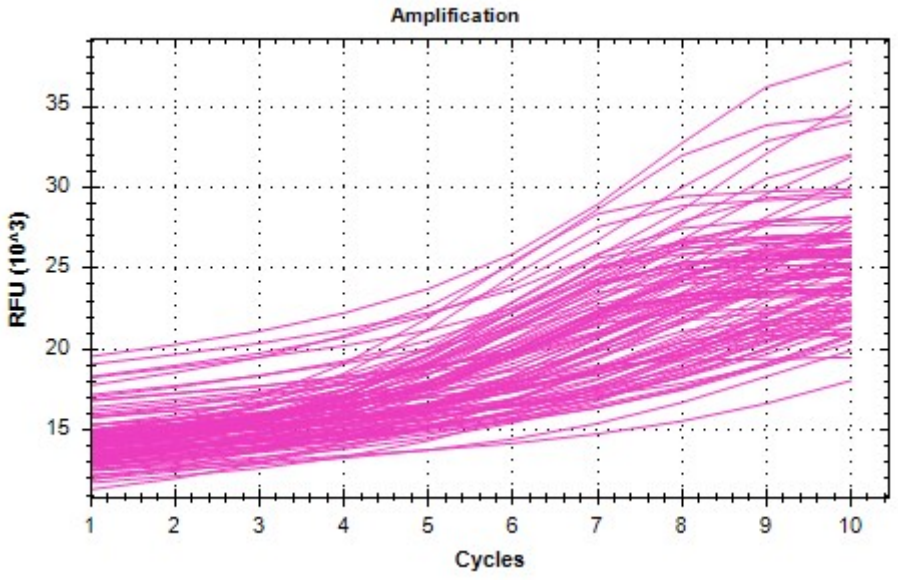
   c. Export and examine the fluorescence values. They should be more similar across the whole range of wells when compared to the endpoint values generated from curves similar to those displayed in Figure 1.

SUPPLEMENTARY INFORMATION

SI Table 1: Cycle optimisation matrix detailing how many cycles you should use in Phase 2for respective amounts of starting DNA and the Ct value you can expect that starting quantity to appear at:

Starting DNA amount	Ct	Recommended Phase 2 cycle
5 ng	15.5	10
2.5 ng	17.6	10
1 ng	17.8	10
0.5 ng	19.3	15
0.1 ng	21.6	15

SI Figure 1: Typical qPCR amplification plot (generated during Phase 2 of the qPCR, using 10 cycles) from all 96 index pairs, using a DNA template with a starting quantity of 5 ng.



Electropherogram: Obtained from a TapeStation (Agilent) for the pooled and cleaned 16S library. Strong peak at ~600bp