YOUSEQ

THE ONE 16S NGS LIBRARY PREPARATION KIT USER GUIDE

With Library Quantification Kit

CAT NO. YS-NGS-ONE16SLQ-IL-96

96 samples (For Illumina Sequencers)

VERSION 6

For Research Use Only

YOUSEQ

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INTENDED USE

The ONE 16S NGS 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 and quantification using the included YouSeq Library Quantification kit, the libraries generated are ready to sequence on any Illumina instrumentation. After sequencing, YouSeq's online bioinformatic tool can be used for automated sequence 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 a simple and comprehensive NGS workflow, 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 chance of user error compared with a more 'traditional' library preparation protocol. YouSeq's companion application allows you to easily determine the bacterial community composition of your samples, without the requirement for specialist bioinformatics training.

The app can be found at https://youseq.basepairtech.com. Contact us at support@youseq.com to receive a coupon code to start uploading your sequence data for analysis.

This kit also includes a separate qPCR Library quantification kit that can be used, if you so choose, to precisely quantify your final library prior to sequencing.

WORKFLOW OVERVIEW

Throughout the protocol there are 'Safe Stop' points that allow flexibility in the workflow.

Step	Action	Hands on time	Hands off Time
16S library preparation	Generate an indexed library in a single PCR reaction, ready for pooling.	30 minutes	1.5 hours
Sample quantification and library pooling	Using the endpoint fluorescence measurement from the qPCR machine, calculate the volume of each sample to add to the final library pool	5 minutes	-
Bead purification	Purify the pooled libraries using magnetic beads	30 minutes	-
qPCR quantification of pooled library	Quantify the pooled, purified library	15 minutes	1.5 hours
Preparation of library for sequencing	Prepare library for loading onto the Illumina sequencing instrument	15 minutes	-

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KIT CONTENTS

Pouch 1: The ONE 16S reagents (store at -20°C)

	Lid Colour/Plate	Volume
16S Oligo Mix Primers, Indexes 1-96 (PCR Plate)		96 x 15 µL
The ONE MasterMix		1 x 1 mL

Pouch 2: Bead Clean-up Reagents (store at 4°C)

	Lid Colour/Bottle	Volume
Magnetic Beads		0.5 mL
Wash Buffer		0.4 mL *
Elution Buffer		10 mL

*Supplied concentrated and requires dilution before use. See Component Preparation step below for instructions.

Pouch 3: Library Quant Reagents (store at -20°C***)

	Lid Colour/Bottle	Volume
NGS Library Quantification Kit primers		110 µL
qPCR SYBR green MasterMix		1 mL
Standards 1-4		4 x 100 µL**
Dilution Buffer***		40 mL
DNase/RNase free water		1.5 mL

**Supplied lyophilised and requires resuspension before use. See Component Preparation step below for instructions
*** Can be stored at 2-8C after thawing for subsequent use.

ASSOCIATED DOCUMENTS

These helpful documents can be found on the associated kit product page at www.youseq.com, or can be obtained by contacting support@youseq.com

	Document
The ONE16S Index Sequences	Excel spreadsheet showing which Primer indexes are in which plate wells to allow association with samples
Pooling Calculations Template	Excel spreadsheet for calculating sample volume to add to the final library pool
Sample Sheet - 96	CSV file with index sequences for uploading to local run manager.

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COMPONENT PREPARATION

Prepare the designated kit components as per the table below. Spin or gently tap the vials to ensure all content is at the bottom before opening.

After adding the reagent, pulse vortex the tube to ensure it is mixed well.

	Reagent	Volume to add
Wash buffer	100% Ethanol	1.6 mL
Standard Templates 1-4	Dilution buffer	100 µL

MATERIALS REQUIRED BUT NOT PROVIDED

- 100% ethanol
- PhiX
- Magnetic rack for 1.5 ml tubes
- qPCR instrument - This YouSeq kit will work with any qPCR instrument with a SYBR/FAM channel.
- Pipettes, microcentrifuge tubes and general laboratory equipment

INPUT SAMPLE REQUIREMENTS

This kit has been optimised for use with samples containing between 5-25 ng of bacterial DNA per reaction. Initial quantification of bacterial DNA within the sample is recommended. All samples should be used at a similar concentration to ensure an equal efficiency of the PCR reaction. Samples with a high DNA concentration (>5 ng/µL) should be diluted to within 1-5 ng/µL with RNase/DNase free water or elution buffer (supplied).

16S LIBRARY PREPARATION PROTOCOL

Please note:

Sample identification - Each well of the provided 16S Oligo Mix Primer plate has a unique Primer index ID. It is recommended to give all samples a sample ID 1-96 and match sample ID with index ID to allow identification of each sample after demultiplexing. The ONE16S Indexes sheet shows each index with an associated well position. A multichannel pipette should be used to dispense the primers to ensure they remain in the correct configuration.

Contamination avoidance - To prevent sample and Oligo Mix Primer contamination between wells, ensure that you use a fresh pipette tip per well when dispensing.

1. Thaw the One MasterMix and 16S Oligo Mix Primers. Once thawed completely, keep on ice and thoroughly vortex before use.

2. The library preparation will be performed in a new qPCR plate or PCR tubes and 16S Oligo mix primers will need to be moved from the supplied primer plate to the Library Preparation plate following the instructions below.

Component	Volume
The ONE MasterMix	10 µL
16S Oligo Mix Primers	5 µL
Sample DNA (1-5 ng/µL)	5 µL
Final Volume	20 µL

3. 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.

4. Perform a PCR using the following cycling conditions, with the lid temperature set to 105°C

	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: No fluorescence acquisition is required during cycles in stage 1.

**Phase 2: With the recommended amount of sample DNA, 10 cycles in phase 2 will enable amplification to enter the exponential phase. If no amplification is seen by cycle 7, then refer to the supplementary information section for guidance on adding cycles.

5. Review qPCR output to confirm amplification. If amplification is not apparent in the qPCR output, then refer to the supplementary information section of this guide.

6. If proceeding directly to the next step of the protocol, place the qPCR products on ice.

7. Return all components to the recommended storage temperature.

Safe Stop Point: qPCR products can be stored at -20°C for up to 1 week.

SAMPLE NORMALISATION AND LIBRARY POOLING

An equal amount of each of the qPCR products should be added to the library pool. This will prevent one sample 'crowding out' the others during sequencing.

1. Review qPCR output to rule out anomalous results. If anomalous results are suspected, refer to the Supplementary Information section of this guide.

2. Export the endpoint fluorescence values from your qPCR instrument to a spreadsheet.

3. Insert the endpoint fluorescence values into the correct cells of the available Pooling Calculations Template file available on the product page at www.youseq.com. The volume of each sample to use in the library pool will be automatically calculated.

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

Please note: Extreme care should be taken when opening the plate after the qPCR. It now contains millions of copies of the library and is a significant contamination risk. Always use good laboratory practice.

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BEAD PURIFICATION PROTOCOL

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

1. Bring the Magnetic Beads to room temperature by removing from the fridge 30 minutes before use.
2. Obtain the volume of bead mix to add to the pooled library from the Pooling Calculations Template spreadsheet. This volume is 0.8 X the volume of your pooled library.
3. Thoroughly vortex the magnetic bead solution until it appears homogeneous.
4. Add the required volume of magnetic beads to the tube containing the pooled library and mix well by pipetting up and down. Take care to expel all of the liquid out of the tip during the last mix.
5. Incubate at room temperature for 3 minutes.
6. Put the tube onto a magnetic rack and leave until the supernatant becomes clear and the beads have formed a tight pellet on the magnet. Your DNA library is now bound to the beads.
7. Leaving the tube on the magnetic rack, carefully remove and discard the supernatant using a suitably sized pipette tip without disturbing the bead pellet.
8. Make sure that 1.6 mL of 100% Ethanol has been added to the Wash Buffer tube. Leaving the tube on the magnetic rack, add 200 µL of the Wash Buffer to the beads and mix by pipetting. It does not matter if the pellet becomes dislodged during this process.
9. Wait until the supernatant is clear and then carefully remove and discard the supernatant using a suitably sized pipette tip without disturbing the bead pellet.
10. Remove the tube from the magnet, briefly spin the tube and return to the magnet. Remove any left-over supernatant carefully with a 10 µL pipette tip.
11. Leave the cap of the tube open and leave the bead pellet to dry at room temperature until all visible liquid has evaporated but beads remain dark brown and has lost it's shiny appearance. (approximately 2 minutes).

Caution: Do not over-dry the beads as this may result in lower recovery of DNA. The beads turn lighter brown and start to crack if they become too dry.

12. Leaving the tube on the magnetic rack, add 100 µL of Elution Buffer directly to the bead pellet.
13. Remove the tube from the magnetic rack and resuspend the pellet by pipetting unitl homogenous.
14. Incubate for at least 2 minutes at room temperature. The DNA library is now in solution.
15. Return the tube to the magnetic rack and wait until the supernatant is clear.
16. Carefully remove the supernatant using a suitably sized pipette tip and place into a fresh low-bind DNase/RNase free tube. This tube contains the purified, pooled DNA library.
17. Return all components to the recommended storage temperature if pausing protocol at this point.

Safe Stop Point: Purified DNA Library can be stored for 24 hours at 2-8°C or 2 weeks at -20°C. Longer term storage is at user's risk. Repeat quantification before any new sequencing run.

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qPCR QUANTIFICATION OF POOLED LIBRARY

The purified library pool can be quantified before loading onto the sequencing system to provide a more accurate loading concentration. This step is optional but recommended.

qPCR REACTION SET UP

Each reaction requires the component volumes as listed in the table below. It is recommended to make a reaction mix to cover all reactions and dispense this into all appropriate wells.

Pooled libraries should be run in triplicate reactions. All 4 standards should be included, in triplicate reactions, and no-template controls should be included, in triplicate. Add an extra reaction to the reaction mix to account for overage.

1. Calculate the total number of reactions required:

   Total number of reactions required = (number of pooled libraries X 3) + 12 standard reactions + 3 no-template control reactions + 1 reaction for overage

An example for the preparation of reaction mix when 1 pooled library is being quantified in triplicate – a total of 19 reactions.

2. Set up the reaction on ice. Follow the table below to create a reaction mix for your samples and standards and controls using the calculated total number of reactison required.

Component	Volume / reaction	Volume required	E.g., 1 pooled library, standards, no-template controls and overage
YouSeq qPCR MasterMix	10 µL	10 µL X Total number of reactions	190 µL
Library specific primers	1 µL	1 µL X Total number of reactions	19 µL
DNase/RNase free water	4 µL	4 µL X Total number of reactions	76 µL
Total volume	15 µL	15 µL X Total number of reactions	285 µL

LIBRARY DILUTIONS

Perform a serial dilution of your pooled library to create a library product at the optimum concentration.

1. Add 99 µL of Dilution Buffer into 2 microcentrifuge tubes and label them ‘tube 2’ and ‘tube 3’.
2. Add 90 µL of Dilution Buffer into a microcentrifuge tube and label it ‘tube 4’.
3. Pipette 1 µL of the purified library pool into tube 2
4. Mix by pipetting up and down 5 times
5. Change pipette tip and pipette 1 µL of tube 2 into tube 3
6. Mix by pipetting up and down 5 times
7. Change pipette tip and pipette 10 µL of tube 3 into tube 4
8. Mix by pipetting up and down 5 times

Tube No.	Dilution factor
4	1 : 100,000

9. Pipette 5 µL of tube 4 to the designated wells on your qPCR plate

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POSITIVE CONTROL

Pipette 5 µL of each positive control standard into your designated triplicate wells to create a positive control standard curve. For no-template control wells add 5 µL of RNase/DNase free water instead of positive control

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. Load the plate into the qPCR instrument.

STANDARD CURVE SET UP

Program a standard curve into the qPCR instrument software with the input concentrations as in the table below:

Standard no.	Concentration
1	10 pM
2	0.5 pM
3	0.025 pM
4	0.00125 pM

qPCR AMPLIFICATION PROTOCOL

Perform a PCR using the following cycling conditions:

	Temperature	Time
Hot Start	95°C	3 minutes
40 cycles	95°C	10 seconds
	60°C*	60 seconds

*Make sure to collect fluorogenic data through the FAM/SYBR (Green) channel during these steps

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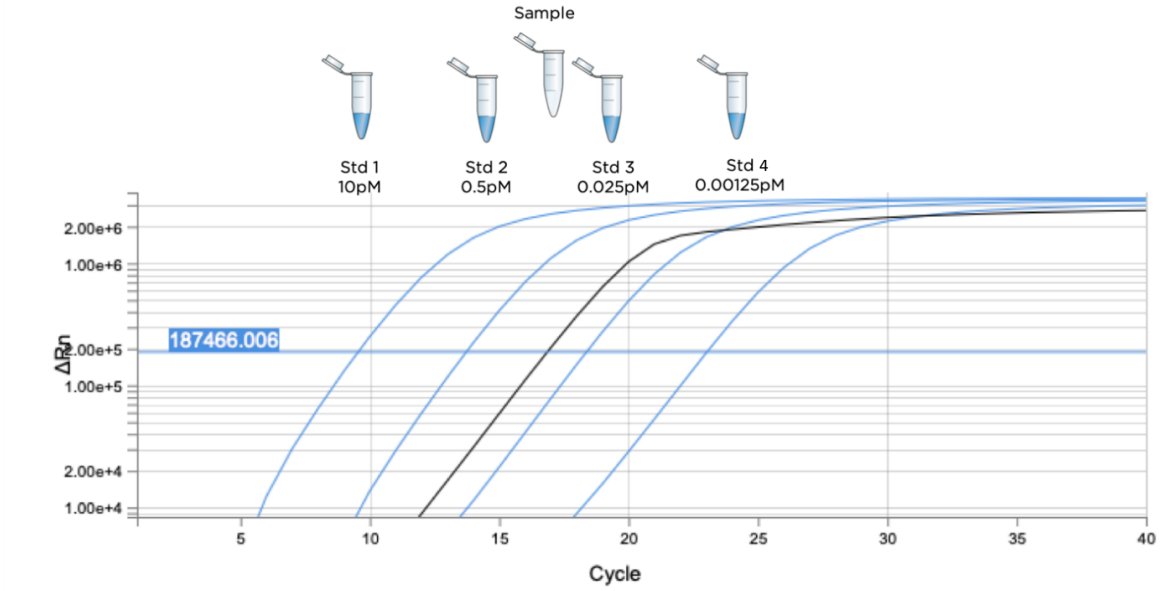
DATA INTERPRETATION



QUANTIFICATION OF LIBRARIES

The qPCR instrument software will automatically compare the Cq values obtained from the samples to those from the positive control standards in the kit. This calculation will deliver a 'calculated concentration' in pM of each of the diluted Libraries.

To determine the library concentration for the non-diluted library, perform a calculation for each library using the formula below. The calculation includes and adjustment for the library fragment size, ~600 bp, and a normalisation factor to adjust for the standards fragment size, 252bp.

Worked Example:

A library with an average fragment length of 600 bp was diluted 1:100,000 before qPCR and returned a calculated concentration of 0.6 pM.

Adjusted concentration = 0.6 x (252 / 600) x 100,000

Adjusted concentration = 25200 pM or 25.2 nM

PREPARATION OF LIBRARIES FOR SEQUENCING

Quality Check - Optional

Run a sample of the pooled library on an automated analyser such as a Bioanalyser or TapeStation. A prominent peak between 580-600bp indicates a good quality library. Peaks at other sizes indicate a potential problem, refer to the Supplementary Information section of this guide.

Dilution

Determine the correct loading concentration of the pooled library to add to the sequencing instrument. Illumina protocols will specify the optimal concentration of library to load on the chip/cartridge being used. Use the results from the Library Quantification qPCR to dilute the library pool to the correct loading concentration using the Elution Buffer supplied.

PhiX control

PhiX must be added to low complexity libraries (such as 16S libraries) for optimal sequencing performance. YouSeq advises adding PhiX to your library at a concentration of 20% in the final pool.

All the required worksheets and barcode metadata are available from www.youseq.com and/or the YouSeq Customer Services team.

SEQUENCING DATA ANALYSIS

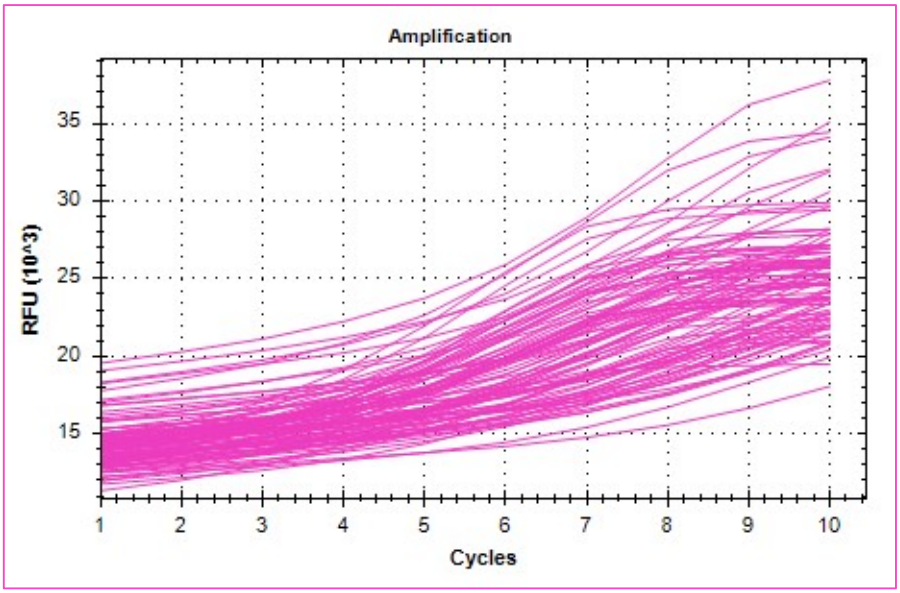
Use the Generate FASTQ Analysis Module on the Illumina sequencing instrument when inputting the sample indexes. Data generated is split into FASTQ files for each sample. Ready for analysis. Upload the FASTQ files to the companion application at https://youseq.basepairtech.com for analysis. This is a fully automated data analysis tool that delivers an easy-to-use choice of PDF, csv or html output.

SUPPLEMENTARY INFORMATION

Library Preparation qPCR output

When the recommended amount of input bacterial DNA is used in the qPCR reaction, amplification should be seen during the 10 cycles of phase 2. Amplification should look similar to the image below:

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



If no amplification is seen after 10 cycles of PCR in Phase 2 of the Library Preparation qPCR protocol:

a. Increase the number of cycles in Phase 2 to 15. It is not recommended to run the qPCR for more than 15 cycles at Phase 2 as this may introduce errors.

b. If the amplification is still not evident after 15 cycles, there may be a high level of inhibitors present, poor-quality DNA or insufficient quantity of DNA in the sample. It may be necessary to include a purification and/or concentration step prior to qPCR. Ensure that the extraction kit used is suitable for the sample type.

If amplification shows bunching of plots, strange fluorescence readings or inappropriate automatic baseline correction:

a. This may be due to the qPCR cycler having trouble defining a baseline. Set the baseline manually on the software and examine the fluorescence values. If the issue has now been corrected, export the fluorescence values and continue with the quantification analysis according to the Sample Quantification section of this User Guide. If this does not successfully correct the issue, attempt step b below.

b. Do not discard the qPCR plate. Instead run the protocol below on the same qPCR instrument that was used to generate the curves. This will generate new end point fluorescence values which can then be examined. If the issue has now been corrected, export the fluorescence values and continue with the quantification analysis according to the Sample Quantification section of this User Guide.

Temperature	Time	
72°C	2 minutes	Acquire SYBR

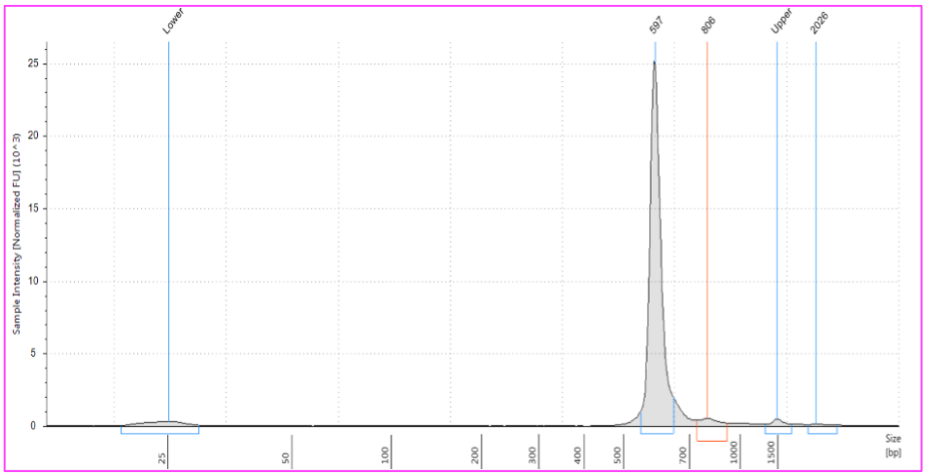
Sample normalisation

The required volume of qPCR product to add to Library pool is calculated automatically in the Pooling Calculations Template file available on the product page at www.youseq.com. The method uses using the following calculation:

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

Library Quality Check

Example electropherogram obtained from a TapeStation (Agilent) for the pooled and cleaned 16S library. Strong peak at 580-600bp indicates a good quality library.



PRODUCT SPECIFICATIONS

Storing your kit

Storage at -20°C

All components from the ONE 16S Reagents Pack and all components from the Library Quant Reagents Pack, except the Dilution Buffer, should be stored at –20°C 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.

Storage at 4°C

All components from the Bead Clean-up Reagents Pack and the Dilution Buffer from the Library Quant Reagents Pack should be kept in the fridge at 4°C while not is use.

Use good quality DNA

This kit has been optimised for a wide variety of different sample types, including traditionally 'difficult' inputs such as faeces and soil. High inhibitor carryover from the original sample to the qPCR mix may prevent the reaction from performing optimally. Therefore, it is highly recommended that a DNA extraction kit that is compatible with the sample type is used.

Regulatory status

This product has been developed for Research Use Only and is not intended for diagnostic use. It should not be used for diagnosis of disease unless specifically approved by the regulatory authorities in the country of use.

Laboratory Practices

Contamination control: It is essential to prevent contamination throughout the library preparation process. To achieve this, each step should be carried out in a dedicated work area with dedicated equipment. Clean workspaces and equipment with DNA Away or 7% bleach solution followed by isopropyl alcohol/isopropanol. All reactions should be sealed in plates before being transferred between work areas.

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.

Quality Control

In accordance with the YouSeq Ltd ISO EN 13485-certified Quality Management System, each lot of The ONE 16S NGS Library Preparation kit is tested against predetermined specifications to ensure consistent product quality.

Technical Assistance

For customer support, please contact us:

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