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Ion 16S™ Metagenomics Kit

Catalog Number A26216

Pub. No. MAN0010800 **Rev.** B.0

Component	Cap color	Storage conditions
2X Environmental Master Mix	Orange	Shipped at –20°C. After first use, store at 2–8°C if used frequently, otherwise store at –20°C. Protect the 2X Environmental Master Mix from light.
DNA Dilution Buffer	Clear	
16S Primer Set V2–4–8 (10X)	Green	Store at –15 to –25°C.
16S Primer Set V3–6, 7–9 (10X)	Blue	
Negative Control	White	
E. coli DNA control (30 µg/mL)	Red	Store at –15 to –25°C. Store separately from other reagents to prevent cross-contamination.

Note: For safety and biohazard guidelines, refer to the "Safety" appendix in the *Ion 16S™ Metagenomics Kit User Guide* (Pub. no. MAN0010799). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Methods

Prepare amplicons

1 Amplify the 16S hypervariable regions

See the *Ion 16S™ Metagenomics Kit User Guide* for guidelines on input amount and cycle number.

a. Thaw all Ion 16S™ Metagenomics Kit reagents and keep on ice.

b. For each sample, prepare two reactions (one for each of the 2 primer sets). Include one positive and negative control per PCR run. Before you pipet each reagent, vortex for 5 seconds and pulse-spin the reagent tube.

Component	Sample or positive control volume	Negative control volume
2X Environmental Master Mix	15 µL	15 µL
16S Primer Set (10X)[1]	3 µL	3 µL
DNA (sample or diluted *E. coli* DNA control)	2–12 µL sample[2] *or* 2 µL diluted control[3]	N/A
Negative Control (water)	to 30 µL	to 30 µL
Total	**30 µL**	**30 µL**

[1] V2-4-8 or V3-6, 7–9

[2] 2.5–5 ng pure microbial DNA or 1–2 µL DNA for samples with large amounts of non-microbial DNA.

[3] Dilute the *E. coli* DNA control stock 1:20 (1.5 ng/µL) with DNA Dilution Buffer. Use 2 µL of the diluted DNA control (3 ng DNA input) in the positive control reaction.

1 Amplify the 16S hypervariable regions *(continued)*

c. Place the tubes or plate in the thermal cycler and run the following program:

Stage	Temperature	Time
Hold	95°C	10 min
Cycle 18–25 cycles	95°C	30 sec
	58°C	30 sec
	72°C	20 sec
Hold	72°C	7 min
Hold	4°C	∞[1]

[1] Remove samples within 24 hours and continue to next step or store at –20°C for up to 2 weeks.

d. *(Optional)* If samples contain non-microbial DNA, confirm the presence of PCR products (use a Bioanalyzer® instrument or 2% agarose gel) before you continue to the purification step.

2 Prepare reagents for purification

a. Allow the Agencourt® AMPure® XP beads to come to room temperature (~30 minutes).
b. Prepare 70% ethanol. Store in a tightly closed container at room temperature when not in use.

IMPORTANT! Always use 70% ethanol for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol may cause sample loss.

Continue to "Purify the amplification products in the PCR plate" on page 2 or "Purify the amplification products in tubes" on page 2.

3 Purify the amplification products in the PCR plate

a. Pulse-spin the plate. Vortex the Agencourt® AMPure® XP Reagent to resuspend, add 54 µL (or 1.8 × *sample volume*) to each 30 µL sample, then pipet up and down 5 times to thoroughly mix.
b. Incubate the mixture for 5 minutes at room temperature.
c. Place the plate in a magnetic rack such as the DynaMag™-96 Bottom Magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the bead pellet.
d. Without removing the plate from the magnet, add 100 µL of 70% ethanol, then incubate for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.
e. Repeat step d for a second wash.
f. To remove residual ethanol, keep the plate on the magnetic rack and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
g. Keeping the plate on the magnetic rack, air-dry the beads at room temperature for 4 minutes.

IMPORTANT! Do not let the pellet dry out completely.

h. Remove the plate from the magnetic rack and add 15 µL of Nuclease-free Water directly to the pellet to disperse the beads. Mix thoroughly by pipetting the suspension up and down 5 times or more as needed to resuspend the beads.
i. Place the plate in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new PCR plate or tube without disturbing the pellet.

IMPORTANT! The supernatant contains your sample. **Do not discard.**

STOPPING POINT *(Optional)* Store the DNA at –30°C to –10°C for up to 2 weeks.

Continue to "Calculate DNA input for library preparation" on page 3.

4 Purify the amplification products in tubes

a. Combine the following in an Eppendorf® tube. Vortex the Agencourt® AMPure® XP Reagent to resuspend before taking aliquots.

Component	Volume	Example
Pooled amplification reaction[1]	X (equal volumes of V2-4-8 and V3-6, 7–9 reactions)	40 µL (20 µL each of V2-4-8 and V3-6, 7–9 reactions)
Agencourt® AMPure® XP Reagent	1.8X	72 µL

[1] Combine equal volumes of the 2 reactions. Alternatively, purify the 2 reactions separately, then combine equal volumes before you perform "Calculate DNA input for library preparation" on page 3.

b. Vortex the mixture briefly, pulse-spin, then incubate the mixture at room temperature for 5 minutes.
c. Follow the instructions in "Wash and elute" on page 3. Wash with 300 µL of 70% ethanol. Elute into 15 µL of Nuclease-free Water.

Continue to "Wash and elute" on page 3.

5 Wash and elute

a. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the bead pellet.
b. Without removing the tube from the magnetic rack, add the specified volume of 70% ethanol to each tube. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
c. Repeat step 5b for a second wash.
d. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
e. Keeping the tube on the magnetic rack, air-dry the beads at room temperature for 4 minutes.

IMPORTANT! Do not let the pellet dry out completely.

f. Remove the tube from the magnetic rack and add the specified volume of eluent directly to the pellet to disperse the beads. Vortex the tube for 5–10 seconds as needed to resuspend the beads.
g. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

IMPORTANT! The supernatant contains the eluted DNA. **Do not discard.**

STOPPING POINT *(Optional)* Store the DNA at –30°C to –10°C for up to 2 weeks.

Continue to "Calculate DNA input for library preparation" on page 3.

6 Calculate DNA input for library preparation

Calculate DNA input for library preparation using the the Agilent® 2100 Bioanalyzer® instrument and an Agilent® High Sensitivity DNA Kit, or one of the recommended Quant-iT™ kits.

Prepare the library

Use the Ion Plus Fragment Library Kit (Cat. no. 4471252) according to the following procedures. Refer to the *Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit User Bulletin* (Pub. no. MAN0006846) for details.

1 End repair and purify pooled amplicons

a. Combine the following in a 1.5-mL Eppendorf® LoBind® tube:

Component	Volume
Pooled short amplicons, 10–100 ng	79 µL
5X End Repair Buffer	20 µL
End Repair Enzyme	1 µL
Total	**100 µL**

b. Pipet up and down to thoroughly mix, then incubate at room temperature for 20 minutes.
c. Add 180 µL Agencourt® AMPure® XP Reagent (1.8 × *sample volume*) to each sample, vortex the mixture briefly, pulse-spin, then incubate for 5 minutes at room temperature.
d. Follow the instructions in "Wash and elute" on page 3. Wash with 500 µL of 70% ethanol. Elute into 25 µL of Low TE.

2 Ligate and nick-repair

a. In a 0.2-mL PCR tube, combine the reagents as indicated in the table, and mix well by pipetting up and down.

Component	Volume for Non-barcoded Libraries	Volume for Barcoded Libraries
DNA	~25 µL	~25 µL
10X Ligase Buffer	10 µL	10 µL
Adapters (non-barcoded libraries) or Ion P1 Adapter (barcoded libraries)	2 µL	2 µL
Ion Xpress™ Barcode X[1]	—	2 µL
dNTP Mix	2 µL	2 µL
Nuclease-free Water	51 µL	49 µL
DNA Ligase	2 µL	2 µL
Nick Repair Polymerase	8 µL	8 µL
Total	**100 µL**	**100 µL**

[1] X = barcode chosen.

IMPORTANT! When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.

b. Place the tube in a thermal cycler and run the following program.

Stage	Temperature	Time
Hold	25°C	15 min
Hold	72°C	5 min
Hold	4°C	∞[1]

[1] Not a stopping point; continue directly to the next steps.

c. Transfer the entire reaction mixture to a 1.5-mL Eppendorf LoBind® Tube for the next cleanup step.

3 Purify the adapter-ligated and nick-repaired DNA

IMPORTANT! Always use 70% ethanol for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol may cause sample loss.

a. Add 140 µL (1.4 × *sample volume*) of Agencourt® AMPure® XP Reagent to the sample, vortex the mixture briefly, pulse-spin, then incubate the mixture for 5 minutes at room temperature.
b. Follow the instructions in "Wash and elute" on page 3. Wash with 500 µL of 70% ethanol. Elute into 20 µL of Low TE.

Determine library concentration

Use qPCR or the Agilent® 2100 Bioanalyzer® instrument to determine the library concentration.

Determine library concentration using qPCR

Use the Ion Universal Library Quantitation Kit (Cat. no. A26217) and the following procedure to quantify libraries prepared using Ion 16S™ Metagenomics Kit amplicons.

1 Prepare serial dilutions of the *E. coli* DH10B Control Library

a. Thaw the *E. coli* DH10B Ion Control Library on ice. Vortex and briefly spin down before taking aliquots.
b. Prepare four sequential 10-fold dilutions from the *E. coli* DH10B Ion Control Library (68 pM; included in the Ion Universal Library Quantitation Kit) as shown in the table. Vortex and briefly spin down each standard before taking aliquots for the next dilution. Label the standards and store on ice.

Standard	Control Library	Nuclease-free Water[1]	Dilution factor	Concentration
1	5 µL undiluted Control Library	45 µL	1:10	6.8 pM
2	5 µL Std 1	45 µL	1:100	0.68 pM
3	5 µL Std 2	45 µL	1:1000	0.068 pM
4	5 µL Std 3	45 µL	1:10000	0.0068 pM

[1] Not DEPC-treated

2 Dilute the sample library

Prepare serial dilutions of the sample library as shown in the table. Label the dilutions and store on ice.

Dilution	Library input	Nuclease-free Water[1]
1:10	2 µL of sample library stock	18 µL
1:100	5 µL of 1:10	45 µL
1:1000[2]	5 µL of 1:100	45 µL
1:10,000[2]	5 µL of 1:1000	45 µL

[1] Not DEPC-treated

[2] Dilutions to be assayed.

3 Set up the PCR reactions

a. Thaw frozen components on ice. Gently but thoroughly mix each thawed component, then briefly centrifuge to bring the contents to the bottom of the tube. Do not vortex the TaqMan® Fast Universal PCR Master Mix.

b. Prepare a reaction mix as shown in the table. Store on ice.

Component	Volume per 20-µL reaction
TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase® UNG	10 µL
Ion Library TaqMan® Quantitation Assay, 20X	1.0 µL
Nuclease-free Water	4.0 µL

c. Vortex the reaction mix for 5 seconds, pulse-spin, then pipette 15 µL into the appropriate number of wells in the PCR plate.

d. Add 5 µL of the diluted (1:1000 or 1:10000) sample library or 5 µL of Standard 1–Standard 4 to each well (three wells per sample or standard), for a total reaction volume of 20 µL.

IMPORTANT! Use equivalent volumes of sample library and standards in PCR reactions.

e. Seal the plate, and centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

4 Run the real-time PCR reactions

a. Program your real-time instrument according to the manufacturer's instructions using the following settings.

- Enter the concentrations of the control library standards (see "Prepare serial dilutions of the E. coli DH10B Control Library" on page 4)
- Passive reference dye: **ROX™ Reference Dye**
- TaqMan® probe reporter/quencher: **FAM™ dye/MGB**
- Reaction volume: **20 µL**
- Enter the appropriate cycling program shown in the table

Real-time PCR System	Sample block and reaction plate	Run mode	Stage	Temp	Time
7900 HT Fast StepOnePlus™ ViiA™ 7	96-well Fast	Fast	Hold	95°C	20 sec
			Cycle (40 cycles)	95°C	1 sec
StepOne™	48-well Fast			60°C	20 sec
7500 Fast	96-well Fast	Fast	Hold	95°C	20 sec
			Cycle (40 cycles)	95°C	3 sec
				60°C	30 sec
7900 HT Fast ViiA™ 7 7900 HT	96-well standard 384-well	Standard	Hold	95°C	2 min
			Cycle (40 cycles)	95°C	15 sec
7500 7300	96-well standard			60°C	1 min

b. Place the plate in the real-time PCR instrument, run the reactions, and collect the real-time data.

See the *Ion 16S™ Metagenomics Kit User Guide* for details on calculating the appropriate library dilution for template preparation.

1 Amplify the library

Use the components provided with the Ion Plus Fragment Library Kit for this step.

a. Add 5 µL of Low TE to the ~20 µL of purified, adapter-ligated library.

b. Combine the following reagents in an appropriately sized tube and mix by pipetting up and down.

Component	Volume
Platinum™ PCR SuperMix High Fidelity	100 µL
Library Amplification Primer Mix	5 µL
Unamplified library	25 µL
Total	**130 µL**

c. Split the 130-µL reaction into two 0.2-mL PCR tubes, each containing about 65 µL.

d. Place the tubes into a thermal cycler and run the following PCR cycling program.

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
5–7 cycles[1]	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	—	4°C	Hold for up to 1 hour

[1] 5 cycles for 50 ng of input, 7 cycles for 20 ng of input.

e. Combine previously split PCRs in a new 1.5-mL Eppendorf LoBind® Tube.

2 Purify the library

IMPORTANT! Always use 70% ethanol for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol may cause sample loss.

a. Add 195 µL of Agencourt® AMPure® XP Reagent (1.5 × *sample volume*) to each sample, vortex the mixture briefly, pulse-spin, then incubate the mixture for 5 minutes at room temperature.

b. Follow the instructions in "Wash and elute" on page 3. Wash with 500 µL of 70% ethanol. Elute into 20 µL of Low TE.

3 Assess the quality of the library

Analyze a 1-µL aliquot of the amplified library, diluted 1:10, on the Bioanalyzer® instrument with an Agilent® High Sensitivity DNA Kit.

See the *Ion 16S™ Metagenomics Kit User Guide* for details on calculating the appropriate library dilution for template preparation.

Proceed to template preparation

Proceed to template preparation, using the Ion OneTouch™ 2 System and the Ion PGM™ Hi-Q™ OT2 Kit. Follow the instructions in the *Ion PGM™ Hi-Q™ OT2 Kit User Guide* (Pub. no. MAN0010902), *with these modifications*:

1. Before you begin template preparation, dilute an aliquot of each library to the recommended concentration according to the calculations in the *Ion 16S™ Metagenomics Kit User Guide*.

Quantitation method	Recommended library concentration for template preparation
qPCR	10 pM
Agilent® 2100 Bioanalyzer® instrument	26 pM

Note: If you plan to sequence more than one library on the same chip, dilute each library to 10 pM (if you quantified the library using qPCR), or 26 pM (if you quantified the library using a Bioanalyzer® instrument), then pool the diluted libraries to obtain a final volume ≥25 µL. You can:

- Pool equal volumes of diluted library

  or

- Pool volumes in a ratio equivalent to the percentage of wells that you want to use to sequence each library

  For example, if you want to use 45% of the wells for library 1, 45% for library 2, and 10% for library 3, then pool volumes in a 45:45:10 ratio.

Note: Store diluted libraries at 2°C to 8°C and use within 48 hours. Store undiluted libraries at –30°C to –10°C.

2. Follow the instructions for template preparation as described in Chapter 3 of the *Ion PGM™ Hi-Q™ OT2 Kit User Guide* (Pub. no. MAN0010902) *but do not further dilute the library*. Add 20 µL of diluted library (prepared in step 1) and 5 µL of water to the amplification solution.
3. Remove the samples ≤ 16 hours after starting the run on the Ion OneTouch™ 2 Instrument.

Note: Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com**. Follow the links under **Protocols ▸ Prepare Template ▸ Prepare Template User Guides and Quick Reference**.

Sequence the library

Use the Ion Personal Genome Machine™ (PGM™) System and the Ion PGM™ Hi-Q™ Sequencing Kit. Follow the protocol in the *Ion PGM™ Hi-Q™ Sequencing Kit User Guide* (Pub. no. MAN0009816).

Use an Ion 314™ Chip v2, Ion 316™ Chip v2, or Ion 318™ Chip v2 depending on the number of barcoded libraries pooled for run, initial sample complexity and/or desired sequencing depth.

Limited product warranty

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