ELISA protocol:

1. Coat microtiter plate wells with 100 µl of the antigen/analyte solution, at a concentration of between 1-10 µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.
2. Add 150 µl of blocking solution to each well. Incubate for 1 hour at 37°C. Wash 4 times in wash buffer.
3. Add 100 µl of unconjugated detection antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.
4. Add 100 µl enzyme-conjugated secondary antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.
5. Add 100 µl of the appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.
6. Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes
7. All procedures will be replicated three times.

16S or 18S Metagenomic Sequencing Library Preparation using iSeq 100 protocol:

1. Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached.

Consumables needed:

Items	Quantity	Storage
Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5)	2.5 µl per sample	-15° to -25°C
Amplicon PCR Reverse Primer (1 µM)	5 µl per sample	-15° to -25°C
Amplicon PCR Forward Primer (1 µM)	5 µl per sample	-15° to -25°C
2x KAPA HiFi HotStart ReadyMix	12.5 µl per sample	-15° to -25°C
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	
[Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504)		

Procedures:

a. Set up the following reaction of DNA, 2x KAPA HiFi HotStart Ready Mix, and primers:

Item	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
2x KAPA HiFi HotStart ReadyMix	12.5 µl
TOTAL	**25 l**

b. Seal plate and perform PCR in a thermal cycler using the following program:
   1) 95°C for 3 minutes
   2) 25 cycles of:
      i. 95°C for 30 seconds
      ii. 55°C for 30 seconds
      iii. 72°C for 30 seconds
   3) 72°C for 5 minutes
   4) Hold at 4°C

c. Run 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace after the Amplicon PCR step is ~550 bp. Other option, using gel electrophoresis to verify the size is also viable. Mix 2 µl of the PCR product with 3 µl Blue Juice. Run the sample in 1% agarose gel in 1x TAE Buffer with the configuration of 110 V, 15 minutes. With 1kb DNA ladder as the control, the band should be produced near ~550 bp.

2. PCR Clean Up I

This step uses AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species.

Consumables needed:

Items	**Quantity**	**Storage**
10 mM Tris pH 8.5	52.5 µl per sample	-15° to -25°C
AMPure XP beads	20 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Preparation:

- Bring AMPure XP beads to room temperature

Procedure:

a. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
b. [Optional - for use with shaker for mixing] Using a multichannel pipette set to 25 µl, transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples.
   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
c. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.
d. Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.
e. Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
f. Incubate at room temperature without shaking for 5 minutes.
g. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
h. With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
i. With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   1) Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   2) Incubate the plate on the magnetic stand for 30 seconds
   3) Carefully remove and discard the supernatant.
j. With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:
   1) Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   2) Incubate the plate on the magnetic stand for 30 seconds.
   3) Carefully remove and discard the supernatant.
   4) Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.
k. With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

l. Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.
m. Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.
n. Incubate at room temperature for 2 minutes.
o. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
p. Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

3. Index PCR

This step attaches dual indices and Illumina sequencing adapters using the Nextera UD Indexes Kit.

Consumables Needed:

Items	**Quantity**	**Storage**
2x KAPA HiFi HotStart ReadyMix	25 µl per sample	-15° to -25°C
Nextera™ DNA UD Indexes Set A	10 µl per sample	-15° to -25°C
PCR Grade Water	10 µl per sample	
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	

Note: The Nextera™ DNA UD Indexes is already premixed, packaged in 96-well plate, and ready to use in single pipetting. Pierce the aluminum foil attached to the 96-well plate with a pipette tips, discard after piercing. Pipette the needed amount for reaction with new pipette tips.

Procedure:

a. Set up the following reaction of DNA, Index Primers, 2x KAPA HiFi HotStart ReadyMix, and PCR Grade Water:

Item	**Volume**
DNA from PCR Clean Up I	5 µl
Nextera™ DNA UD Indexes Set A	10 µl
2x KAPA HiFi HotStart ReadyMix	25 µl
PCR Grade Water	10 µl
TOTAL	**50 µl**

b. Transfer 5 µl the product of PCR Clean-Up I from each well to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored for other uses.
c. Gently resuspend the mixture for 10 seconds to mix.
d. Cover the plate with Microseal 'A'.
e. Centrifuge the plate at 1000 x g at 20°C for 1 minute.
f. Perform PCR on thermal cycler using the following program:
    1) 95°C for 3 minutes
    2) 8 cycles of:
        i. 95°C for 30 seconds
        ii. 55°C for 30 seconds
        iii. 72°C for 30 seconds
    3) 72°C for 5 minutes
    4) Hold at 4°C.

4. PCR Clean Up II

This step uses AMPure XP beads to clean up the final library before quantification.

Consumables:

Items	Quantity	Storage
10 mM Tris pH 8.5	27.5 µl per sample	-15° to -25°C
AMPure XP beads	56 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Procedures:

a. Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
b. [Optional - for use with shaker for mixing] Using a multichannel pipette set to 50 µl, transfer the entire Index PCR product from the PCR plate to the MIDI plate. Change tips between samples.
   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
c. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.
d. Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.
e. Gently pipette mix up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
f. Incubate at room temperature without shaking for 5 minutes.
g. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
h. With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
i. With the Index PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    1) Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    2) Incubate the plate on the magnetic stand for 30 seconds
    3) Carefully remove and discard the supernatant.
j. With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:
    1) Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    2) Incubate the plate on the magnetic stand for 30 seconds.
    3) Carefully remove and discard the supernatant.
    4) Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.
k. With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
l. Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of 10 mM Tris pH 8.5 to each well of the Index PCR plate.
m. If using a 96-well PCR plate, gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column. If using a MIDI plate, seal plate and shake at 1800 rpm for 2 minutes.

n. Incubate at room temperature for 2 minutes.
o. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
p. Using a multichannel pipette, carefully transfer 25 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross contamination.
q. (OPTIONAL) Run 1 µl of a 1:50 dilution of the final library on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace of the final library is ~630 bp. Other option, using gel electrophoresis to verify the size is also viable. Mix 2 µl of the PCR product with 3 µl Blue Juice. Run the sample in 1% agarose gel in 1x TAE Buffer with the configuration of 110 V, 15 minutes. With 1kb DNA ladder as the control, the band should be produced near ~630 bp.

5. Library Quantification, Normalization, and Pooling

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes, such as Qubit 3.0. Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by Qubit 3.0 Fluorometer using the given formula:

$$\frac{(concentration\ in\ \frac{ng}{\mu l})}{(\frac{660g}{mol}x\ average\ library\ size)}\ x\ 10^6 = concentration\ in\ nM.$$

For example, in this metagenomic sequencing:

$$\frac{(15\ \frac{ng}{\mu l})}{(\frac{660g}{mol}x\ 630)}\ x\ 10^6 = 36\ nM.$$

Dilute concentrated each sample final library using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 or Nuclease-Free Water to a concentration 4 nM.

6. Final Library Preparation

Preparation:

a. Thaw the bagged cartridge that was stored in -25°C to -15°C storage. If the cartridge is boxed, remove it from the box but **do not open the white foil bag**. Thaw the bagged cartridge using one of the following methods. Use **immediately** after thawing, without refreezing or otherwise storing.

Method	Thaw Time	Instruction
20°C to 25°C water bath	6 hours, not exceeding 18 hours	Use 6 L (1.5 gal) water per cartridge. • Set a temperature-controlled water bath to 25°C ***or*** mix hot and cold water to achieve 20°C to 25°C. • Face the bag label up, submerge the cartridge completely, and apply ~2 kg (4.5 lbs) weight to prevent floating. • Do not stack cartridges in the water bath unless it is temperature-controlled.
2°C to 8°C refrigerator	36 hours, not exceeding 72 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.
Room-temperature air (20°C to 25°C)	9 hours, not exceeding 18 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.

b. While thawing the bagged cartridge, remove the flow cell from 2-8oC storage and set aside the unopened package at room temperature for 10-15 minutes to prevent condensation.

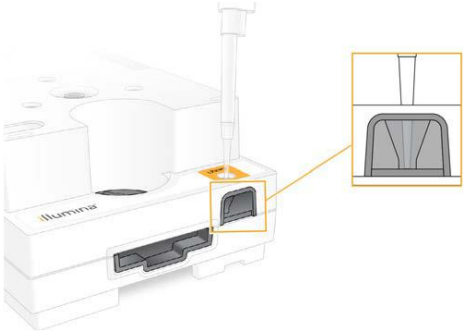
Final Library Preparation:

a. Dilute Library to 1 nM in a low-binding tube. Dilute library using Resuspension Buffer (RSB) or 10 mM Tris-HCl, pH 8.5 Buffer, or Nuclease-Free Water (NFW).
b. After dilution, pool all the diluted 1 nM library in one low-binding tube. (OPTIONAL) Reconfirm its 1 nM concentration using Qubit 3.0 Fluorometer.
c. Dilute the pooled 1 nM library to 50 pM concentration (5 µl 1 nM library in 95 µl RSB). Vortex briefly, then spin down briefly. Set aside diluted library on ice until sequencing.
d. Remove 10 nM Phi-X stock from -25°C to -15°C storage. Phi-X is needed only for an optional spike-in or a Phi-X only run.
e. Thaw Phi-X at room temperature for ten minutes.
f. Using RSB/10 mM Tris-HCl pH 8.5 Buffer/NFW, do a two-step dilution of Phi-X from 10 nM to 1 nM, and then to 50 pM (5 µl 1 nM Phi-X in 95 µl RSB).
g. Combine 19 µl of diluted 50 pM library and 1 µl of diluted 50 pM Phi-X in one tube. Vortex briefly and spin down briefly. This mixture is ready for injection to the cartridge.

7. Loading Consumables into the Cartridge

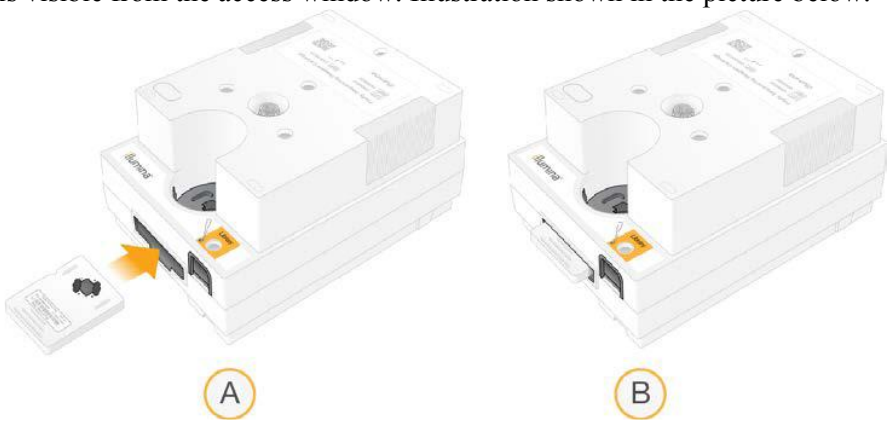
a. Open the cartridge bag from the notches.
b. While avoiding the access window on top of the cartridge, remove the cartridge from the bag. Discard the bag.
c. Invert the cartridge five times to mix reagents. Internal components can rattle during inversion, which is normal.
d. Tap the cartridge (label facing up) on the bench or other hard surface five times to ensure reagent aspiration.
e. Using a new pipette tip, pierce the Library reservoir and push the foil to the edges to enlarge the hole, as shown in the picture below.

f. Discard the pipette tip to avoid contamination.
g. Add 20 µl mixture of diluted library + Phi-X to the ***bottom*** of the reservoir. Avoid touching the foil. Illustration shown in the picture below.



h. Open the flow cell's white foil package from the notches. Use within 24 hours of opening.
i. Pull the flow cell out of the package. Touch only the plastic when handling the flow cell. Avoid touching the electrical interface, CMOS sensor, glass, and gaskets on either side of the glass. Illustration is shown in the picture below.

j. Hold the flow cell by the grip points with the label facing up.
k. Insert the flow cell into the slot on the front of the cartridge. An audible click indicates that the flow cell is in place. When properly loaded, the grip protrudes from the cartridge and the glass is visible from the access window. Illustration shown in the picture below.



A. Loading the flow cellB. Loaded flow cell

l. Make sure that the cartridge is thawed and contains the flow cell and diluted library.
m. Place the cartridge onto the tray so that the access window faces up and the flow cell is inside the instrument. Do not push the cartridge or tray into the instrument. Illustration shown in the picture below.

n. Select Close Door to retract the cartridge and close the door. A panel appears on the left side of the screen to show information from the scanned consumables.
o. Select and edit the run parameters:
    1) To change Read Type, select Single Read or Paired End.

2) To change Read Cycle, enter 26–151 cycles each for Read 1 and Read 2. Add one cycle to the desired number of cycles.
3) To change the output folder for the current run, enter the path to the location or select Browse and navigate to it.
4) Select Save, which updates the run in both the control software and Local Run Manager.

p. Select Start Run to initiate the pre-run check. Wait for about 15 minutes for pre-run check to complete. The run starts automatically after successful completion. Unless the system is muted, a chime sound indicates that the run has started. Monitoring run progress and metrics are available as they appear on the Sequencing screen after the cycle of 26.