Illumina Custom Protocol

Cholera-Haiti protocol

For Research Use Only. Not for use in diagnostic procedures.

Introduction

These instructions are based on selections made in the Custom Protocol Selector. The instructions are assembled from the comprehensive product documentation, listed under Related Documentation. Before using this custom protocol, make sure that you have read and understand the listed documents, and have all the necessary equipment and consumables they describe.

Supported Combination

Sequencing Instrument:	MiSeq
Library Preparation Kit:	Nextera XT DNA Sample Prep
Index Adapters:	Nextera XT Indexes
Number of Samples:	< 96
Container Option:	Plate Protocol
Indexing:	Dual Indexing
Reagent Kits:	MiSeq Reagent Kit v3
Analysis Software:	BaseSpace Sequence Hub
Analysis App:	Generate FASTQ

Related Documentation

This document is derived from the following documents:

Document Title	Document Number	Publication Date
Illumina Experiment Manager Software Guide	15031335 v08	January 2019
Nextera XT DNA Library Prep Kit Reference Guide	15031942 v05	June 2019
MiSeq Denature and Dilute Libraries Guide	15039740 v09	November 2018
MiSeq System User Guide	15027617 v06	January 2021
BaseSpace Sequence Hub Online Help	1000000009008 v03	November 2017

Legal

Generating a Sample Sheet

Create a Nextera Sample Plate

1. Open Illumina Experiment Manager.
2. From the main screen, select **Create Sample Plate**.
3. Select an index adapter based on your kit type, and then select **Next**.
4. In the Unique Plate Name field, enter a unique name for the sample plate.
5. For Index Reads, select **2** for dual-indexed libraries.
6. Select **Next**.
7. Select the **Table** or **Plate** tab, depending on your preferred view.
   - The Table tab lists each well of a 96-well plate with the sample ID, name, indexes, project, and description.
   - The Plate tab mimics the layout of a 96-well plate, with columns A–H and rows 1–12. Use the Currently Displaying menu to select which type of information is displayed for each sample: sample ID, name, indexes, project, or description.
8. [Optional] Use the fill and copy features to populate all wells quickly.
   - To generate sequential data, enter sequential data in two adjacent wells, and then select those cells and all other cells to populate. Right-click the selected area and select **Fill Down** or **Fill Right**.
   - To copy data, populate one cell, and then select that cell and all other cells you want to populate with the same value. Right-click the selected area and select **Fill Down** or **Fill Right**.
   - Paste content from an Excel table into the Sample ID column.
9. Enter a unique sample ID for each well that contains a sample.

   The sample ID is used to track a sample from preparation through sequencing and analysis. The sample ID is typically a barcode, but any value is acceptable.
10. If appropriate, select a well in the Index Well field for each well that contains a sample.
11. In the Index1 and Index2 fields, select the index adapter being used for each Index Read.
    - To autopopulate the indexes for all index reads for 96 wells, select **Apply Default Index Layout**. If desired, edit the autopopulated indexes.
    - To make an edited index layout the default index layout, select **Save As Default Index Layout**.
    - To restore the Illumina layout as the default index layout, select **Restore Illumina Default Index Layout**.

> **Note**
> Shaded wells contain invalid or missing values.

12. [Optional] Enter a sample name, project, and description for each well to record more detailed information about the plate.
13. [Optional] Select the Plate Graphic tab to view the plate with the sample ID and index adapter shown in each well.
    - To copy an image of the sample plate, select **Copy to Clipboard**. The image can be pasted into Paint, PowerPoint, Word, PhotoShop, or other graphics-enabled software.
    - To print an image of the sample plate, select **Print**.
14. Select **Finish**, and then save the sample plate file in the desired location.

Create a MiSeq Sample Sheet

1. From the main screen, choose whether to create or edit a sample sheet.
    - To create a sample sheet, select **Create Sample Sheet**.
    - To edit a sample sheet, select **Edit Sample Sheet**, navigate to the appropriate sample sheet, and proceed to step 4.
2. Select **MiSeq**, and then select **Next**.
3. Select the appropriate application, and then select **Next**.
4. In the Reagent Kit Barcode field, enter the reagent kit ID from the label of box 1 or box 2 of the SBS kit.
5. Select the appropriate Library Prep Workflow.
6. Select the appropriate Index Adapter.
7. For Index Reads, select **2** for dual-indexed libraries.
8. [Optional] Enter the Experiment Name, Investigator Name, Description, and Date.
9. Select the **Paired End** Read Type.
10. In the Cycles Read fields, enter one more than the number of cycles.

    For example, for a 150-cycle read, enter 151.

    For a paired-end run, enter the same number in Cycles Read 1 and Cycles Read 2.
11. See MiSeq Workflow-Specific Settings for information on workflow-specific settings. Make the appropriate selections.
12. Select **Next** to continue to Select Samples for a MiSeq Sample Sheet.

Select Samples for a MiSeq Sample Sheet

1. Select samples by creating a sample plate, or using an existing plate.
    - To create a sample plate, select **New Plate**.

- To use an existing plate, select **Select Plate**, and then navigate to the appropriate sample plate.

2) Choose wells to include in the sequencing run.
   - To include all wells, select **Select All**.
   - To specify which wells to include, select only the applicable wells.
3) Select **Add Selected Samples**.

> **Tip**
> Select the **Maximize** checkbox to hide the sample plate panel and view the sample sheet in full screen.

4) [Optional] Select **Add Blank Row** to add rows and manually enter sample information.
5) [Optional] To remove rows, select at least one field, and then select **Remove Selected Rows**.
6) For each sample, enter a sample name, reference, project, and description, if applicable.

Complete the Sample Sheet

1) Select **Finish**, and then save the sample sheet file (*.csv) in the desired location.
2) Select **Yes** to review the sample sheet in Excel, or **No** to exit the sample sheet wizard.

MiSeq Workflow-Specific Settings

Setting	Applications	Description
BWA-Backtrack	Enrichment, Library QC, PCR Amplicon, Small Genome Resequencing	Allows selection of v0.6.1 of the BWA aligner. This setting only applies to MiSeq Reporter v2.6 and later workflows, which use a newer version of the BWA aligner (BWA-MEM, v0.7.9a).
Custom Primer for Read 1	All except TruSeq Amplicon	Use a custom primer for Read 1.
Custom Primer for Index	All except TruSeq Amplicon	Use a custom primer for Index 1 and Index 2.
Custom Primer for Read 2	All except TruSeq Amplicon	Use a custom primer for Read 2.
Use Somatic Variant Caller	TruSeq Amplicon, PCR Amplicon, Enrichment, Resequencing	Select this checkbox if you are using the somatic variant caller. Somatic variant caller is an Illumina variant calling algorithm for TruSeq Amplicon – Cancer Panel and TruSight Myeloid Sequencing Panel. It detects low frequency mutations (even below 5%) in a mixed cell population. For more information, see the Somatic Variant Caller Technical Note (Pub. No. 970-2012-014) on the Illumina website.
Indel Realignment GATK	Resequencing, Enrichment	Locally realign reads around indels to minimize mismatches.
Flag PCR Duplicates	Resequencing, PCR Amplicon, Enrichment, Library QC	Flag apparent PCR duplicates in the BAM files and omit them from variant calling. PCR duplicates are two clusters from a paired-end run that have the same alignment positions for each read.
Variant Quality Filter	TruSeq Amplicon, PCR Amplicon, Resequencing, Enrichment	A cutoff parameter with a default setting of 30. For more information, see the MiSeq Sample Sheet Quick Reference Guide (document # 15028392) on the Illumina website.

Setting	Applications	Description
Use Adapter Trimming	All except TruSeq Amplicon	The software masks the adapter sequence, which improves the accuracy and speed of analysis. This setting is recommended when sequencing libraries prepared with the Nextera, Nextera XT, Nextera Rapid Capture Enrichment, or TruSight Enrichment kits. The default adapter sequence is the adapter present in all Nextera libraries. If a different adapter sequence is used, edit the sequence that is displayed in the final sample sheet. For TruSight HLA, do not change the default.
Use Adapter Trimming Read 2	Plasmids, Assembly, RNA-Seq, Library QC, FASTQ Only, ChIP-Seq	Set the AdapterRead2 setting in the sample sheet to trim a different adapter sequence in Read 2.
Run Picard HsMetrics	Enrichment	Perform Picard hybrid selection (HS) analysis of the BAM file.
Reverse Complement	Resequencing, Library QC, FASTQ Only, Assembly	Convert Nextera Mate Pair libraries from a mate pair to a paired-end orientation as required by BWA and Velvet.
K-mer size	Assembly	Set the k-mer size used for assembly. The range is 2–255. Larger k-mer sizes require more memory and can impact the stability and performance of the analysis. K-mer optimization is suggested for optimal assemblies.
Genome	Small RNA	Provide the relative or absolute path to the following reference sequence folders. The typical settings for human runs are shown in parentheses: • **Contaminants** (HumanRNAContaminants) • **RNA** (HumanRNA) • **miRNA** (HumanRNAMature)
Export to gVCF	PCR Amplicon, TruSeq Amplicon, Enrichment	Enable output of gVCF files to the run folder.

Setting	Applications	Description
Genus-Level Classification	Metagenomics	Enable genus-level classification, which overrides the species-level taxonomic classification default.

Creating Nextera XT DNA Libraries

Tagment Genomic DNA

Consumables

- Amplicon Tagment Mix (ATM)
- Tagment DNA Buffer (TD)
- Neutralize Tagment Buffer (NT)
- 96-well PCR plate
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
ATM	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
TD	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
NT	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

Procedure

1. Add the following volumes *in the order listed* to each well of a new 96-well PCR plate.
   - TD (10 µl)
   - 1 ng DNA (0.2 ng/µl per sample) (5 µl)
2. Pipette to mix.
3. Add 5 µl ATM to each well.

4. Pipette 10 times to mix, and then seal the plate.
5. Centrifuge at 280 × g at 20°C for 1 minute.
6. Place on the preprogrammed thermal cycler and run the TAG program. When the program reaches 10°C, *immediately* proceed to step 7 because the transposome is still active.
7. Add 5 µl NT to each well.
8. Pipette 10 times to mix, and then seal the plate.
9. Centrifuge at 280 × g at 20°C for 1 minute.
10. Incubate at room temperature for 5 minutes.

Amplify Libraries

Consumables

- Nextera PCR Master Mix (NPM)
- Index adapters (tubes or plates)
- Microseal 'A' adhesive film

About Reagents

- Index adapter plates
  - A well may contain >10 µl of index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.
- Index adapter tubes
  - Open only one index adapter tube at a time to prevent misplacing caps. Alternatively, use fresh caps after opening each tube.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
Index adapters	-25°C to -15°C	Thaw at room temperature . [Tubes] Vortex to mix, and then centrifuge briefly. [Plates] Spin briefly before use.
NPM	-25°C to -15°C	Thaw on ice for 20 minutes.

2. Save the following NXT PCR program on a thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl

- 72°C for 3 minutes
- 95°C for 30 seconds
- 12 cycles of:
  - 95°C for 10 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Procedure

1. Add the following index adapter volumes per sample according to your index adapter kit type.

Index Adapter Kit Type	Volume of Index Adapter per Sample
Index Adapter Tubes	5 µl i7 adapter 5 µl i5 adapter
Index Adapter Plate	10 µl pre-paired i7 and i5 index adapters

2. Add 15 µl NPM to each well.
3. Pipette 10 times to mix, and then seal the plate.
4. Centrifuge at 280 × g at 20°C for 1 minute.
5. Place on the preprogrammed thermal cycler and run the NXT PCR program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Libraries

Consumables

- Purification Beads (PB)
- Freshly prepared 80% ethanol (EtOH)
- Resuspension Buffer (RSB)
- 96-well 0.8 ml polypropylene deepwelll storage plate (midi plate) (2)
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' foil seals
- Nuclease-free water

About Reagents

- Purification Beads
  - Must be at room temperature before use.
  - Vortex before each use.
  - Vortex frequently to make sure that beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
PB	2°C to 8°C	Let stand on the benchtop for 30 minutes to bring to room temperature. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw and bring to room temperature. Vortex to mix. RSB can be stored at 2°C to 8°C after the initial thaw.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge at 280 x g at 20°C for 1 minute to collect contents at the bottom of the well.
2. Transfer 50 µl supernatant from each well of the PCR plate to corresponding wells of a new midi plate.

> **Note**
> The ratio of supernatant to volume of PB is 3:2. If you transfer less than 50 µl supernatant, adjust the volume of PB accordingly.

3. If you are using standard DNA input, add 30 µl PB to each well containing supernatant.
4. If you are using small PCR amplicon sample input, add the PB volume according to your input size in the table below.

Input Size (bp)	AMPure XP Recommendation	AMPure XP Volume (µl)
300–500	1.8x AMPure XP	90
> 500	0.6x AMPure XP (0.5x AMPure XP for ≥ 2 x 250 cycles)	30 (25 µl for ≥ 2 x 250 cycles)

5. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
8. Without disturbing the beads, r emove and discard all supernatant.
9. Wash two times as follows.
    a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
    b. Incubate for 30 seconds.
    c. Without disturbing the beads, remove and discard all supernatant.
10. Use a 20 µl pipette to remove and discard residual EtOH.
11. Air-dry on the magnetic stand for 15 minutes.
12. Remove from the magnetic stand.
13. Add 52.5 µl RSB to the beads.
14. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
15. Incubate at room temperature for 2 minutes.
16. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
17. Transfer 50 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

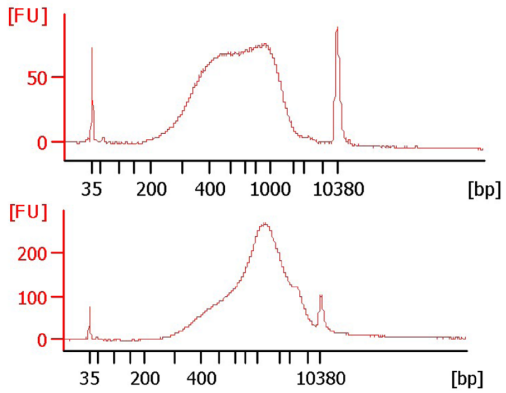
If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal and store at -25°C to -15°C for up to 7 days.

Check Library Quality

1. Run 1 µl undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit.

    Typical libraries show a broad size distribution of ~250–1000 bp, as shown in the top panel. Various libraries can be sequenced with average fragment sizes as small as 250 bp or as large as 1500 bp.

Figure 1: Example Bioanalyzer Trace



Normalize Libraries

- If the final library yield is < 10 nM.
- If your sequencing system uses onboard denaturation.

Consumables

- Library Normalization Additives 1 (LNA1)
- Library Normalization Beads 1 (LNB1)
- Library Normalization Wash 1 (LNW1)
- Library Normalization Storage Buffer 1 (LNS1)
- 0.1 N NaOH (fewer than 7 days old) (3 ml per 96 samples)
- 96-well 0.8 ml polypropylene deep-well storage plate (midi plate)
- 96-well PCR plate
- 15 ml conical tube
- Microseal 'B' adhesive seals

About Reagents

- Vortex LNA1 vigorously to make sure that all precipitates have dissolved. Inspect in front of a light.
- Vortex LNB1 vigorously, with intermittent inversion (at least 1 minute). Repeat until all beads are resuspended and no beads are present at the bottom of the tube when it is inverted.
- Always use a wide-bore pipette tip for LNA1.
- Mix only the required amounts of LNA1 and LNB1 for the current experiment. Store the remaining LNA1 and LNB1 separately at the recommended temperatures.
- Aspirate and dispense beads slowly due to the viscosity of the solution.

Warning

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
LNA1	-25°C to -15°C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNB1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNW1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNS1	Room temperature	Bring to room temperature.

Procedure

1. Transfer 20 µl supernatant from each well of the PCR plate to the corresponding well of a new midi plate.
2. Combine the following volumes in a 15 mL conical tube to prepare the LN master mix. Multiply each volume by the number of samples being processed.
   - LNA1 (46 µl)
   - LNA2 (8 µl)

   Reagent overage is included in the volume to ensure accurate pipetting.
3. Pipette 10 times to mix.
4. Pour the LN master mix into a trough.
5. Use a 200 µl multichannel pipette to transfer 45 µl LN master mix to each well.
6. Seal the plate, and then use a plate shaker at 1800 rpm for 30 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
8. Without disturbing the beads, remove and discard all supernatant.
9. Wash two times as follows.
   a. Add 45 µl LNW1 to each well.
   b. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.
   c. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
   d. Without disturbing the beads, remove and discard all supernatant.
10. Add 30 µl 0.1 N NaOH to each well.
11. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.
12. Add 30 µl LNS1 to each well of a new 96-well PCR plate labeled SGP.
13. After the 5 minute elution completes, make sure that all samples in the midi plate are resuspended. If they are not, resuspend as follows.
    a. Pipette 10 times to mix or lightly tap the sample plate on the bench.

b Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.

14 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

15 Transfer 30 µl supernatant from each well of the midi plate to the corresponding well of the SGP plate.

16 Seal the sample plate, and then centrifuge at 1000 × g for 1 minute.

> **Note**
> At this point, the libraries are single-stranded DNA, which resolves poorly on an agarose gel or Bioanalyzer chip. For quality control, use the double-stranded DNA saved from step 17 of the cleanup procedure.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal and store at -25°C to -15°C for up to 7 days.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends the read lengths indicated in the table below. If you would like additional overlapped reads, raw coverage, or adjusted the PB recommendations for ≥ 2 x 250 cycles, you can sequence up to 2 x 250 or 2 x 300, but it is not required.

Table 1: Recommended Read Length on Illumina Systems

Sequencing System	Read Length
MiSeq	2 x 151

*Assumes use of the 200 cycle kit.

1. Calculate the molarity value of the library or pooled libraries using the following formula.
   - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
   - For all other qualification methods, use 600 bp as the average library size.

$$\frac{ng/\mu l \times 10^6}{660\frac{g}{mol} \times average\ library\ size\ (bp)} = Molarity\ (nM)$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.
3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a multiplexed library pool.