Sequencing Nextera® Libraries on the HiSeq® System

User Guide

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Introduction

Sequencing Nextera® libraries on the HiSeq® system follows single-read or paired-end sequencing workflows with the exception that you must use sequencing primers provided in the TruSeq Dual Index Sequencing Primer Box in addition to indexing and paired-end reagents provided in the TruSeq Cluster Kit.

You can perform six types of runs with Nextera libraries:

- **Single-read runs**—non-indexed, single-indexed, and dual-indexed
- **Paired-end runs**—non-indexed, single-indexed, and dual-indexed

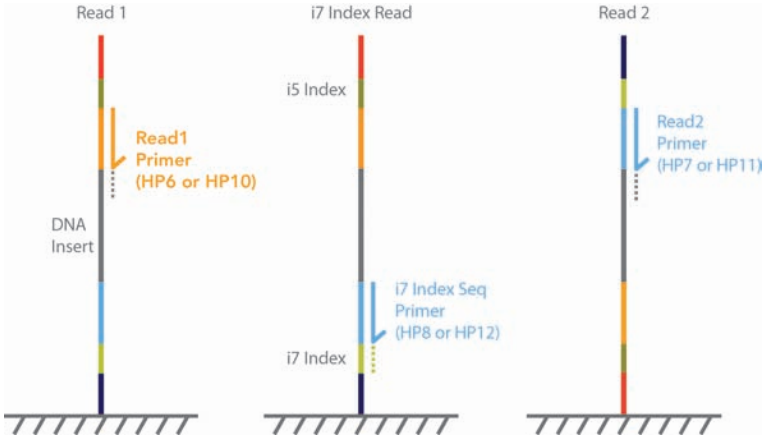
This guide explains the run setup steps for each type of run, including how to prepare and load required reagents.

For more information about instrument components and software, see the *HiSeq 2000 User Guide*, Part # 15011200, or the *HiSeq 1000 User Guide*, Part # 15023355. Additional documentation is available from the Illumina website (http://www.illumina.com).

Single-Indexed Sequencing Overview

Single-indexed sequencing includes one Index Read following Read 1.

Figure 1 Single-Indexed Sequencing



Read 1

Read 1 follows the standard Read 1 sequencing protocol using reagents provided in the TruSeq SBS Kit. The Read 1 sequencing primer is annealed to the template strand during the cluster generation process on the cBot.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP10**, which is used in place of HP6.

Index Read Preparation

The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand, producing the Index 1 (i7) Read.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP12**, which is used in place of HP8.

Index 1 (i7) Read

Following preparation of the Index 1 (i7) Read, the Index 1 (i7) Read is performed.

For most runs, the Index Read consists of seven cycles of sequencing. However, when sequencing Nextera libraries, the Index Read consists of eight cycles of sequencing.

Read 2 Resynthesis

The Index Read product is removed and the original template strand is used to regenerate the complementary strand. Then, the original template strand is removed to allow hybridization of the Read 2 sequencing primer.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP11**, which is used in place of HP7.

Read 2

Read 2 follows the standard paired-end sequencing protocol using reagents provided in the TruSeq SBS Kit.

Dual-Indexed Sequencing Overview

NOTE
Dual-indexed sequencing is supported for Nextera libraries only. You must use HCS v1.5 to perform dual-indexed sequencing.

Dual-indexed sequencing includes two Index Reads following Read 1.

Figure 2 Dual-Indexed Single-Read Sequencing

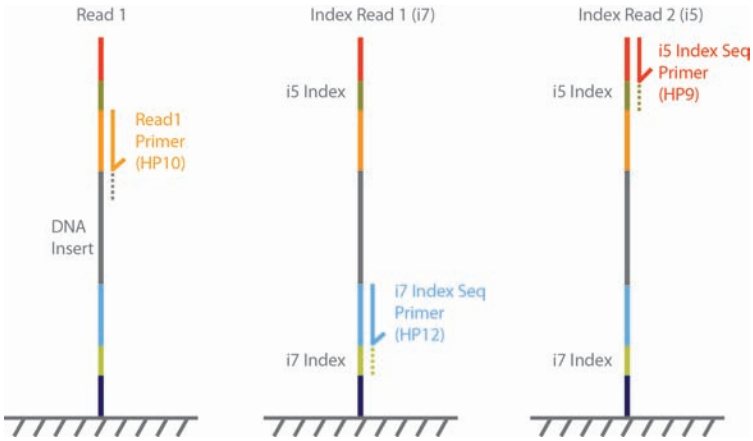
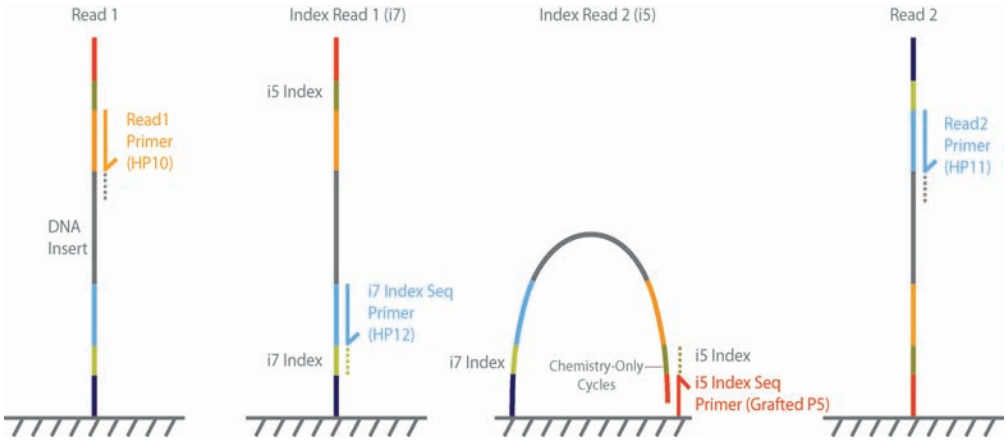


Figure 3 Dual-Indexed Paired-End Sequencing



Read 1

Read 1 follows the standard Read 1 sequencing protocol using reagents provided in the TruSeq SBS Kit. The Read 1 sequencing primer is annealed to the template strand during the cluster generation process on the cBot.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP10**, which is used in place of HP6.

Index Read Preparation

The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand, producing the Index 1 (i7) Read.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP12**, which is used in place of HP8.

Index 1 (i7) Read

Following preparation of the Index 1 (i7) Read, the Index 1 (i7) Read is performed.

For most runs, the Index Read consists of seven cycles. However, when sequencing Nextera libraries, the Index Read consists of eight cycles of sequencing.

Index 2 (i5) Read

The process for Index 2 (i5) Read is different for single-read runs and paired-end runs:

- **Single-Read Flow Cells**—The Index 1 (i7) Read product is removed and the Index 2 (i5) Sequencing Primer Mix, **HP9**, is annealed to the same template strand. The run proceeds through eight cycles of sequencing.
- **Paired-End Flow Cells**—The Index 1 (i7) Read product is removed. The Resynthesis Mix, **RMX**, deprotects the grafted P5 primer on the surface of the flow cell and the template anneals to the grafted P5 primer. The run proceeds through an additional seven chemistry-only cycles (no imaging occurs), followed by eight cycles of sequencing.

Read 2 Resynthesis

The Index Read product is removed and the original template strand is used to regenerate the complementary strand. Then, the original template strand is removed to allow hybridization of the Read 2 sequencing primer.

With Nextera libraries, you must use the appropriate sequencing primer provided in the TruSeq Dual Index Sequencing Primer Box, **HP11**, which is used in place of HP7.

Read 2

Read 2 follows the standard paired-end sequencing protocol using reagents provided in the TruSeq SBS Kit.

Sequencing Consumables

Illumina-supplied consumables are required for sequencing on the HiSeq. The kits that you need depend on the type of run you perform.

Required Consumables for Nextera Libraries

For dual-indexed paired-end sequencing runs of up to 209 total cycles, one 200-cycle SBS kit is sufficient. For runs where the total number of cycles exceeds 209, such as a 101-cycle dual-indexed paired-end run totaling 225 cycles, you can use four 50-cycle SBS kits. For more information, see *SBS Kits for Dual-Indexed Sequencing* on page 11.

Run Type	Single-Read Runs			Paired-End Runs			
Index Type	None	Single	Dual	None	Single	Dual ≤ 209	Dual > 209
TruSeq SBS Kit (200 Cycles)	1	1	1	1	1	1	
TruSeq SBS Kit (50 Cycles)							4
TruSeq SR Cluster Kit	1*	1	1				
TruSeq PE Cluster Kit				1	1	1	1
TruSeq Dual Index Sequencing Primer Box (SR)	1*	1	1				
TruSeq Dual Index Sequencing Primer Box (PE)				1	1	1	1

* Contains components required for cluster generation on the cBot. However, no additional reagents from this kit are required for a non-indexed single-read run on the HiSeq.

SBS Reagents

Sequencing reagents are provided in the TruSeq SBS Kit. One 200-cycle kit provides sufficient reagents for up to 209 cycles. One 50-cycle kit provides sufficient reagents for up to 58 cycles.

Kit	Catalog #
TruSeq SBS Kit v3 - HS (200 Cycles)	FC-401-3001
TruSeq SBS Kit v3 - HS (50 Cycles)	FC-401-3002

Indexing Reagents

Indexing reagents for single-indexed sequencing runs are provided in the TruSeq Cluster Kit. One kit provides sufficient reagents for sequencing one flow cell.

Kit	Catalog #
TruSeq SR Cluster Kit v3 (cBot - HS)	GD-401-3001
TruSeq PE Cluster Kit v3 (cBot - HS)	PE-401-3001

Paired-End Reagents

Paired-end reagents are provided in the TruSeq PE Cluster Kit. One kit provides sufficient reagents for sequencing one flow cell.

Kit	Catalog #
TruSeq PE Cluster Kit v3 (cBot - HS)	PE-401-3001

Dual Index Sequencing Primers

With Nextera libraries, you ***must*** use sequencing primers provided in the TruSeq Dual Index Sequencing Primer Box for any type of run (indexed or non-indexed) and any number of index reads (single-index or dual-index), in addition to reagents provided in the TruSeq Cluster Kit.

One kit provides sufficient reagents for sequencing one flow cell.

Kit	Catalog #
TruSeq Dual Index Sequencing Primer Box (Single Read)	FC-121-1003
TruSeq Dual Index Sequencing Primer Box (Paired End)	PE-121-1003

Dual Index Sequencing Primer Box Contents

The TruSeq Dual Indexing Sequencing Primer Box is available in a single-read kit and a paired-end kit, each with specific sequencing primers.

Kit Contents, Single-Read Kit

Reagent	Storage	Description	Replaces
HP9	-15° to -25°C	Index 2 (i5) Sequencing Primer Mix	--
HP10	-15° to -25°C	Read 1 Sequencing Primer Mix	HP6 (on the cBot)
HP12	-15° to -25°C	Index 1 (i7) Sequencing Primer Mix	HP8

Kit Contents, Paired-End Kit

Reagent	Storage	Description	Replaces
HP10	-15° to -25°C	Read 1 Sequencing Primer Mix	HP6 (on the cBot)
HP11	-15° to -25°C	Read 2 Sequencing Primer Mix	HP7
HP12	-15° to -25°C	Index 1 (i7) Sequencing Primer Mix	HP8

Sequencing Primers by Run Type

Sequencing primers for Nextera libraries are used throughout the sequencing process from cluster generation on the cBot, sequencing of the Index Reads, and sequencing of Read 2.

Run Type	Read 1 Primer (cBot)	Index 1 (i7) Primer	Index 2 (i5) Primer	Read 2 Primer
Single-Read, Non-Indexed	HP10	--	--	--
Single-Read, Single-Indexed	HP10	HP12	--	--
Single-Read, Dual-Indexed	HP10	HP12	HP9	--
Paired-End, Non-Indexed	HP10	--	--	HP11
Paired-End, Single-Indexed	HP10	HP12	--	HP11
Paired-End, Dual-Indexed	HP10	HP12	--	HP11

NOTE
The Index 2 (i5) Read of a dual-indexed paired-end run uses RMX, a paired-end reagent provided in the TruSeq PE Cluster Kit.

Compatibility of Sequencing Consumables

For best performance and run results, always use compatible versions of sequencing consumables and software.

Cluster Kit and Flow Cell	SBS Kit	HiSeq Control Software
TruSeq Cluster Kit v3 (cBot - HS) with Flow Cell v3	TruSeq SBS Kit v3 - HS	HCS v1.4 or later

Sequencing Primer Kit	SBS Kit	HiSeq Control Software
TruSeq Dual Index Sequencing Primer Box (SR or PE)	TruSeq SBS Kit v3 - HS	HCS v1.5 or later

NOTE

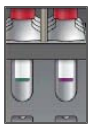
More information about version compatibility is available on the Illumina website at http://www.illumina.com/VersionCompatibility.

Sequencing Workflow

Prepare SBS reagents.

For indexed runs:
Prepare indexing reagents. Make sure that you prepare the appropriate sequencing primers for your library and type of run.

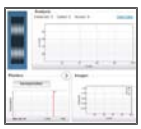
Using the HiSeq Control Software (HCS), enter parameters for your run.



When prompted, load SBS reagents.
For indexed runs:
Load indexing reagents.

With a used flow cell on the instrument, confirm proper flow as prompted by the HCS user interface.
Prime SBS reagents and measure priming waste.

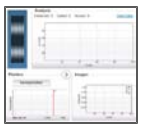
Load a flow cell that was previously clustered on the cBot. Confirm proper flow as prompted by the HCS user interface.



Start your sequencing run. After cycle 1, inspect the first base report and then continue Read 1.
For indexed runs:
Following Read 1, the run proceeds to the Index Read (single-indexed runs) or two Index Reads (dual-indexed runs).

For paired-end runs:
Prepare paired-end reagents for Read 2 Resynthesis.
Prepare fresh ICB for Read 2.

For paired-end runs:
Load paired-end reagents onto the paired-end rack.
Load fresh ICB in position 1.



For paired-end runs:
Continue the run. The software automatically primes paired-end reagents and performs Read 2 resynthesis and Read 2.

When the run is complete, unload and weigh reagents.
Perform a maintenance wash.

Preparing SBS Reagents

The first step in setting up a run is to prepare SBS reagents. Reagent preparation requires overnight thawing at 2° to 8°C, or about 90 minutes in a room temperature deionized water bath. When completely thawed, reagents are ready for use with the exception of ICB (Incorporation Mix), which requires preparation prior to use.

NOTE
TruSeq SBS v3 reagents enable an alternative workflow of loading all SBS reagents at the start of a paired-end sequencing run for both Read 1 and Read 2. Although this alternative SBS workflow provides acceptable sequencing performance in Read 2, Illumina recommends that you prepare and load fresh ICB at the beginning of Read 2 for optimal performance.

SBS Kits for Dual-Indexed Sequencing

One 200-cycle SBS Kit provides sufficient reagents for up to 209 cycles of sequencing. For runs greater than a total of 209 cycles, and to maximize reagent use for other read lengths, you can use multiple 50-cycle kits.

Run Type	Run Cycles	Total Cycles	SBS Kits (200-cycle)	SBS Kits (50-cycle)
Dual-Indexed Paired-End	101+8+(7)+8+101	225	--	4*
	93+8+(7)+8+93	209	1	4*
	76+8+(7)+8+76	175	1	3
	51+8+(7)+8+51	125	1	3
	36+8+(7)+8+36	95	1	2
Dual-Indexed Single-Read	101+8+8	117	1	2
	76+8+8	92	1	2
	51+8+8	67	1	2
	36+8+8	52	1	1

*Fill the container of SRE and SB3 to the top. No replenishing is required.

Thaw SBS Reagents

1 Remove SRE and CMR from -15° to -25°C storage and thaw according to the thawing method and time required listed in the following table:

Reagent Name	SBS Kit (200 Cycles)	SBS Kit (50 Cycles)
SRE	Thaw at 2° to 8°C for 16 hours or in a room temperature water bath for 90 minutes	Thaw in a room temperature water bath for one hour
CMR	Thaw at 2° to 8°C for 16 hours or in a *separate* room temperature water bath for 90 minutes	Thaw in a *separate* room temperature water bath for one hour

2 When thawed, set SRE and CMR aside on ice until you are ready to load reagents.

3 Remove LFN36 from -15° to -25°C storage and thaw in a beaker containing room temperature deionized water for 20 minutes.

Reagent Name	SBS Kit (200 Cycles)	SBS Kit (50 Cycles)
LFN	Thaw two tubes to prepare ICB for Read 1. Thaw the other two tubes when you prepare ICB for Read 2 or a subsequent run. Alternatively, thaw four tubes to prepare ICB for both Read 1 and Read 2.	Thaw one tube. One tube of LFN prepares ICB for 58 cycles of sequencing.

4 When thawed, set LFN36 aside on ice until you are ready to prepare ICB.

5 Leave EDP in -15° to -25°C storage until you are ready to prepare ICB.

6 Use ICB, SB1, SB2, and SB3 directly from 2° to 8°C storage.

7 Record the lot numbers of each reagent on the lab tracking form.

Preparing ICB

Prepare ICB according to your type of run and the size of kit you are using:

- For non-indexed or single-indexed runs using a 200-cycle kit, see *Prepare ICB for Read 1 (200-Cycle Kit)* on page 12 and *Prepare ICB for Read 2 (200 Cycle Kit)* on page 28.
- For non-indexed or single-indexed runs using a 50-cycle kit, see *Prepare ICB for 58 Cycles (50-Cycle Kit)* on page 12.
- For dual-indexed runs, see *Prepare ICB for Dual-Indexed Runs* on page 13.
- For non-indexed or single-indexed runs using the alternative workflow for SBS v3 reagents, see *Prepare ICB for Read 1 and Read 2 (200-Cycle Kit)* on page 13.

NOTE
When you prepare ICB, gently invert the bottle of ICB to mix. For best performance, make sure that the temperature of ICB remains below 8°C.

Prepare ICB for Read 1 (200-Cycle Kit)

Use the following instructions to prepare ICB for up to a 2 x 101-cycle run or a 2 x 101 cycle single-indexed run. For dual-indexed runs, see *Prepare ICB for Dual-Indexed Runs* on page 13.

1 Aliquot 47 ml of ICB-200 into a spare 250 ml bottle to result in two bottles of ICB.

2 Store the spare bottle containing 47 ml of ICB at 2° to 8°C storage until you are ready to prepare and load reagents for Read 2.

3 Add the contents of two tubes of LFN to the original bottle of ICB-200.

4 Rinse each tube of LFN with ICB-200 to make sure that all LFN is transferred.

5 Aliquot 1.1 ml of EDP-200 into the ICB/LFN mix.

6 Return the unused portion of EDP-200 to -15° to -25°C storage.

7 Cap the bottle and *gently* invert several times to mix.

8 Set aside on ice until you are ready to load reagents onto the instrument.

Prepare ICB for 58 Cycles (50-Cycle Kit)

Use the following instructions to prepare ICB for up to 58 cycles of sequencing.

1 Add the contents of one tube of LFN to the bottle of ICB-50.

2 Rinse the tube of LFN with ICB-50 to make sure that all LFN is transferred.

3 Add the contents of one tube of EDP-50 into the ICB/LFN mix.

4 Rinse the tube of EDP-50 with ICB/LFN mix to make sure that all EDP is transferred.

5 Cap the bottle and *gently* invert several times to mix.

6 Set aside on ice until you are ready to load reagents onto the instrument.

Prepare ICB for Dual-Indexed Runs

Illumina recommends the following procedure to calculate the volume of ICB required for a dual-indexed run.

For more information about the number of SBS Kits required for a dual-indexed run based on the read length, see *SBS Kits for Dual-Indexed Sequencing* on page 11.

1 At the beginning of your run, aliquot ICB, LFN, and EDP using the following volumes for every ten cycles of sequencing to be performed. Do ***not*** mix ICB, LFN, and EDP.

Reagent	Volume per Ten Cycles	Storage
ICB	4.7 ml	2° to 8°C
LFN	0.7 ml	-15° to -25°C
EDP	0.11 ml	-15° to -25°C

2 Store each volume of ICB, LFN, and EDP at the proper storage, as needed. For paired-end runs, store the aliquots calculated for Read 2 until you are ready to prepare fresh ICB for Read 2.

3 Prepare ICB, LFN, and EDP as follows:
   a Add LFN to the bottle of ICB.
   b Rinse each tube of LFN with ICB to make sure that all LFN is transferred.
   c Add EDP to the ICB/LFN mix.
   d Rinse the tube of EDP with ICB/LFN mix to make sure that all EDP is transferred.
   e Cap the bottle and *gently* invert several times to mix.
   f Set aside on ice until you are ready to load reagents onto the instrument.

Prepare ICB for Read 1 and Read 2 (200-Cycle Kit)

If you plan to use the alternative workflow and load ICB for Read 1 and Read 2 at the start of your run, use the following instructions for runs up to 209 total cycles.

1 Add the contents of four tubes of LFN to the bottle of ICB-200.

2 Rinse each tube of LFN with ICB-200 to make sure that all LFN is transferred.

3 Add the contents of one tube of EDP-200 into the ICB/LFN mix.

4 Rinse the tube of EDP-200 with ICB/LFN mix to make sure that all EDP is transferred

5 Cap the bottle and *gently* invert several times to mix.

6 Set aside on ice until you are ready to load reagents onto the instrument.

Preparing Indexing Reagents

Indexing sequencing requires indexing reagents provided in the TruSeq Cluster Kit, and sequencing primers for Nextera libraries provided in the TruSeq Dual Index Sequencing Primer Box. Indexing reagents take less than 30 minutes to prepare.

NOTE
HP8, provided in the TruSeq PE Cluster Kit, is not compatible with Nextera libraries. You must use **HP12** from the TruSeq Dual Index Sequencing Primer Box.

Single-Indexed Sequencing, Single-Read or Paired-End

Reagents	Storage	Kit Name
HP3	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
HT2	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
HP12	-15° to -25°C	TruSeq Dual Index Sequencing Primer Box (SR or PE)

Dual-Indexed Single-Read Sequencing

Reagents	Storage	Kit Name
HP3	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
HT2	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
HP12	-15° to -25°C	TruSeq Dual Index Sequencing Primer Box (SR or PE)
HP9	-15° to -25°C	TruSeq Dual Index Sequencing Primer Box (SR)

Dual-Indexed Paired-End Sequencing

Reagents	Storage	Kit Name
HP3	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
HT2	-15° to -25°C	TruSeq Cluster Kit (SR or PE; Indexing Reagents Box)
RMX	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
HP12	-15° to -25°C	TruSeq Dual Index Sequencing Primer Box (SR or PE)

Thaw Reagents

1. Remove the following reagents from -15° to -25°C storage:
   - Single-indexed runs—HP3, HT2, and **HP12**
   - Single-read dual-indexed runs—HP3, HT2, **HP12**, and **HP9**
   - Paired-end dual-indexed runs—HP3, HT2, **HP12**, and RMX
2. Thaw reagents in a beaker filled with room temperature deionized water for about 20 minutes, or until reagents are fully thawed.

Prepare HT2

1. Invert the tube of HT2 five times to mix the reagent.
2. Label the tube of HT2 **Reagent #19**.
3. Centrifuge the reagent at 1,000 rpm for one minute.
4. Set aside at room temperature.

Prepare HP3 for Single-Indexed Runs

1 Invert the tube of HP3 five times to mix the reagent, and then pulse centrifuge.

2 Transfer 3,325 µl of PW1 into a 15 ml Sarstedt conical tube and add 175 µl of HP3.

3 Invert the tube five times to mix the reagent.

4 Label the tube of diluted HP3 **Reagent #18**.

5 Centrifuge at 1,000 rpm for one minute.

6 Set aside at room temperature.

Prepare HP3 for Dual-Indexed Runs

NOTE
For dual-indexed runs, the preparation instructions for HP3 differ from single-indexed runs, in order to provide sufficient HP3 for both Index Reads.

1 Invert the tube of HP3 five times to mix the reagent, and then pulse centrifuge.

2 Transfer 3,800 µl of PW1 into a 15 ml Sarstedt conical tube and add 200 µl of HP3.

3 Invert the tube five times to mix the reagent.

4 Label the tube of diluted HP3 **Reagent #18**.

5 Centrifuge at 1,000 rpm for one minute.

6 Set aside at room temperature.

Prepare HP12

Prepare HP12 for sequencing Nextera libraries.

1 Invert the tube of HP12 five times to mix the reagent.

2 Briefly pulse centrifuge to collect droplets.

3 Label the tube of HP12 **Reagent #17**.

4 Set aside at room temperature.

Prepare HP9

Prepare HP9 for dual-indexed single-read sequencing of Nextera libraries.

1 Invert the tube of HP9 five times to mix the reagent.

2 Briefly pulse centrifuge to collect droplets.

3 Label the tube of HP9 **Reagent #16**.

4 Set aside at room temperature.

Prepare RMX

NOTE
The Index 2 (i5) Read of a dual-indexed paired-end run uses RMX, a paired-end reagent provided in the TruSeq PE Cluster Kit.

Prepare RMX for dual-indexed paired-end sequencing of Nextera libraries.

1. Invert the tube of RMX five times to mix the reagent.
2. Label the tube of RMX **Reagent #10**.
3. Briefly centrifuge the reagent at 1,000 rpm for one minute or less. Alternatively, gently tap or flick the tube to make sure that reagent droplets collect at the bottom of the tube.

   NOTE
   Do not vortex RMX.

4. Set aside on ice.

Entering Run Parameters

The HiSeq Control Software (HCS) guides you through the run setup steps. From the HCS Start screen, select **Sequence** | **New Run**. The Scan screen opens.

Scan Screen

NOTE
It is critical that you scan the flow cell barcode or accurately enter the flow cell ID when you set up your run. The software uses the flow cell ID to determine flow cell type and reagent compatibility.

1. Scan the flow cell barcode or enter the flow cell ID of the flow cell to be sequenced.
2. Enter your experiment name and user name.
3. Confirm the flow cell type, which is automatically selected based on the flow cell ID.
4. Select the lane containing the control, if applicable. Otherwise, select **None**.
5. Enter or browse to the path of your run folder.
6. Select **Advanced** to expand the following additional settings:
   a. Select the **Confirm First Base** checkbox to generate a first base report confirmation.
   b. Select **Keep Intensity Files** for later reanalysis or custom processing (optional).
   c. Select a setting from the **Save Images** drop-down list.
      — Select **Save All Thumbnails** to save tile level and full thumbnails.
      — Select **Save Tile Thumbnails** to save tile level thumbnails only.
   d. Select the **Use Existing Recipe** checkbox only if you want to use a custom recipe. Otherwise, allow the software to create your recipe based on information you provide on the run setup screens.
7. Select **Next**. The Recipe screen opens.

Recipe Screen

1. Select one of the following indexing options:
   - **No Index**—Performs a non-indexed single-read or paired-end run.
   - **Single Index**—Performs a single-read or paired-end run with one Index Read (Index 1 (i7) Read).
   - **Dual Index**—Performs a single-read or paired-end run with two Index Reads (Index 1 (i7) Read and Index 2 (i5) Read).
   - **Custom**—Performs a single-read or paired-end run with a custom number of cycles for the Index Reads.
2. Enter the number of cycles you plan to sequence for Read 1 and Read 2, if applicable.
   If you selected the **Custom** indexing option, enter the number of cycles you plan to sequence for the Index Reads.
3. Select a flow cell format, either **Single Read** or **Paired End**.

4 Confirm the chemistry selections for your run:

a **SBS chemistry**—This setting is determined by the flow cell ID.

NOTE
The software defaults to the compatible kit versions based on the flow cell ID. For example, the ID for Flow Cell v3 defaults to the TruSeq SBS Kit v3.

b **Index chemistry**—This setting is determined by the indexing option selected. However, you must specify the kit name for single-indexed runs to make sure that the correct number of indexing cycles are applied to your run.

— For Nextera libraries, select **TruSeq Dual Index Sequencing Primer Box**. This sets up your run to perform an eight-cycle Index Read.

c **PE turnaround chemistry**—This setting is determined by the flow cell ID.

5 Select **Next**.

If you are performing an indexed run, the Sample Sheet screen opens.

If you are performing a non-indexed run, the Reagents screen opens.

Sample Sheet Screen

1 Enter the path or browse to a valid sample sheet for your run. For indexed runs, the sample sheet is required if you want indexes to be reported.

2 Select **Next**. The Reagents screen opens.

Reagents Screen

1 Scan or enter the SBS reagent kit ID. You can enter the kit ID from either box 1 or box 2.

2 Select the SBS reagent kit you are using for your run:

- Select **200 Cycles** for a 200 cycle kit.
- Select **50 Cycles** for a 50 cycle kit.
- Select **Used Kit** for a partial kit, and then enter the number of SBS cycles remaining, or the number of cycles that reagents are expected to last.
  For dual-indexed runs, selecting **Used Kit** defaults to 225 cycles, which you can change according to the number of cycles in your run.

3 For indexed runs, scan or enter the Indexing Reagent Kit ID.

4 For paired-end runs, scan or enter the PE Reagent Kit ID.

5 Select **Next**. The review run setup screen opens.

Review Screen

Review your run information. If it is correct, select **Next**.

Select **Back** if you need to change any entries.

Loading and Priming Reagents

The next step is to load sequencing reagents followed by the reagent priming step. The software interface guides you through the process.

Load SBS Reagents

Make sure that SBS reagents are thoroughly thawed and ready to load onto the instrument.

1 Record the weight of each reagent on the lab tracking form.

2 Open the reagent compartment door.

3 Raise the sippers for the sequencing reagent rack.

4 Slide the reagent rack out of the reagent compartment using the rack handle.

5 Place each reagent bottle onto the rack in the associated numbered position, making sure that the conical end of the bottle rests in the indentation on the base of the rack.

6 Add 25 ml of PW1 or laboratory-grade water to the bottle in position 2.

7 Remove the cap from each bottle and replace it with a funnel cap. Replace the cap on the bottle of CMR last, and then replace your gloves.

CAUTION
After handling the bottle of CMR, discard your gloves and replace them with a new pair.

8 Slide the reagent rack into the reagent compartment, aligning the rack with the raised guide on the floor of the compartment.

9 Lower the sippers into the sequencing reagent bottles.

10 Select the checkbox labeled **PW1 (25mL) loaded in Position 2**.

11 Do one of the following:
- If you are performing an indexed run, select **Next**. The software prompts you to load indexing reagents.
- If you are performing a non-indexed run, close the reagent compartment door and select **Next**. The software proceeds to priming reagents.

SBS Reagent Positions

Table 1 SBS Reagent Positions

Position	Reagent	Description
1	ICB	Incorporation Mix
2	PW1 (25 ml)	Wash Buffer
3	SRE	Scan Mix Reagent
4	SBS Buffer 1 (SB1)	High Salt Buffer
5	SBS Buffer 2 (SB2)	Incorporation Wash Buffer
6	SBS Buffer 2 (SB2)	Incorporation Wash Buffer
7	CMR	Cleavage Mix Reagent
8	SBS Buffer 3 (SB3)	Cleavage Buffer

Load Indexing Reagents

If you are performing an indexed run, load indexing reagents onto the instrument before starting the run.

1 Before beginning, record the weight of each reagent on the lab tracking form.

2 Raise the sippers for the paired-end reagent rack and remove the rack.

3 Place each reagent tube onto the rack in the associated numbered position indicated according to your type of run.

4 Remove the caps from each reagent tube.

5 Slide the reagent rack into the reagent compartment, aligning the rack with the raised guide on the floor of the compartment.

6 Lower the sippers into the paired-end reagent tubes.

7 Close the reagent compartment door.

8 Select **Next** to proceed to *Priming Reagents* on page 20.

Reagents for Nextera Libraries

Table 2 Indexing Reagent Positions for a Single-Indexed Run (Single-Read or Paired-End)

Position	Reagent	Description
17	HP12	Index 1 (i7) Sequencing Primer Mix
18	HP3	Denaturation Solution
19	HT2	Wash Buffer

Table 3 Indexing Reagent Positions for a Dual-Indexed Single-Read Run

Position	Reagent	Description
16	HP9	Index 2 (i5) SR Sequencing Primer Mix
17	HP12	Index 1 (i7) Sequencing Primer Mix
18	HP3	Denaturation Solution
19	HT2	Wash Buffer

Table 4 Indexing Reagent Positions for a Dual-Indexed Paired-End Run

Position	Reagent	Description
10	RMX	Resynthesis Mix
17	HP12	Index 1 (i7) Sequencing Primer Mix
18	HP3	Denaturation Solution
19	HT2	Wash Buffer

Priming Reagents

To prime reagents, a used flow cell must be loaded on the instrument. If you do not have a used flow cell, contact your Illumina Field Applications Scientist (FAS) or Field Service Engineer (FSE).

Use the following instructions to clean the flow cell holder, load a used flow cell, and confirm proper flow before priming reagents.

Clean the Flow Cell Holder

1 Open the flow cell compartment door.

2 Make sure that the flow cell lever is in the OFF position.

3 Put on a new pair of powder-free latex gloves.

4 Using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol, carefully wipe the surface of the flow cell holder until it is completely clean.

CAUTION
Do not allow alcohol to drip into the vacuum holes or around the manifolds. Use a low-lint lab tissue to dry the stage, if necessary.

5 Visually inspect the flow cell holder to make sure that it is free of lint, and that the vacuum holes are free of obstructions.

Load the Used Flow Cell

You can reuse existing gaskets when loading a used flow cell for instrument washes or reagent priming.

1 Remove the used flow cell from storage buffer and rinse the flow cell with laboratory-grade water. Dry it with lens cleaning tissue or lint-free tissue.

2 Clean the flow cell using alcohol wipes and lens cleaning tissue.

3 Place the used flow cell on the flow cell holder with the inlet and outlet ports facing *down* and the barcode on the right. The arrow on the left edge of the flow cell, which indicates flow direction, should point towards the instrument.

4 Gently slide the flow cell towards the top and right guide pins until it stops.

5 Slowly, move the flow cell lever to position 1. This engages the vacuum and secures the flow cell into position. When the flow cell lever is green, the vacuum is engaged. If the flow cell lever remains orange, the vacuum seal is not secure. Repeat the cleaning steps and reload the flow cell.

6 Wait for about five seconds, and then slowly move the flow cell lever to position 2 (far-right). When the flow cell lever is solid green, the manifolds are in position and the flow cell is ready for use.
If the flow cell lever remains orange, the manifolds did not engage or the vacuum seal has been lost. Remove the flow cell, and repeat the cleaning steps, and then reload the flow cell.

7 Make sure that the **Vacuum Engaged** checkbox is selected on the load prime flow cell screen.

8 Select **Next** to proceed to the fluidics check.

Confirm Proper Flow

After the used flow cell is loaded, you are ready to check for proper flow using the fluidics check screen. Checking for proper flow confirms that the flow cell and gaskets are properly installed and the manifold is engaged.

1 Select solution 2 (laboratory-grade water) from the drop-down list.

CAUTION
You can use water to confirm proper flow on a used flow cell only. Never use water to confirm proper flow on a clustered flow cell.

2 Enter the following default values:
- Volume: **250**
- Aspirate Rate: **250**
- Dispense Rate: **2000**

3 Select **Pump**.

4 Visually inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
If you see excessive bubbles, check the gaskets for obstructions, reduce the aspirate rate to 100, and pump another 250 µl of water to the flow cell. If problems persist, remove the flow cell, change the gaskets, repeat the cleaning steps, and reload the flow cell. If this does not resolve the issue, go to the HiSeq support page on the Illumina website.

Prime Reagents

Prepare the waste tubes for collection of priming waste as follows:

1 Loosen and remove the eight lines of waste tubing for the appropriate flow cell from the waste container. Make sure that you do not include the eight lines for the opposite flow cell or the lines for the condensation pump or the paired-end priming pumps.

2 Place each waste tubing into an empty 15 ml tube, one line per tube. Priming waste is collected and measured after the priming step.

3 Select **Next**. The priming screen opens and the priming step starts automatically. You can monitor the progress of the priming step from the priming screen.

4 When the priming step is complete, measure the collected priming waste and confirm that the volume is 2 ml from each reagent position, or a total of 14 ml. (Only seven of the eight reagent positions are used during reagent priming: positions 1, 3, 4, 5, 6, 7, and 8. Position 2, PW1, is the only reagent not primed during this step.)
Record the results on the lab tracking form.
If the measured delivery volume is less than 95% of the expected delivery volume, then repeat the priming step or troubleshoot the delivery issues.
For troubleshooting information, see the HiSeq User Guide.

NOTE
The color of priming waste might be brown in appearance when using SBS Kit v3. This is normal. The change in waste color is not toxic and does not impact run performance.

5 Return the waste tubing to the waste container before proceeding.

6 Select **Next**. You are ready to load the clustered flow cell.

Loading a Clustered Flow Cell

After priming is complete, the software prompts you to load the clustered flow cell for sequencing.

Remove the Used Flow Cell

1. Open the flow cell compartment door.
2. Slowly move the flow cell lever to position 1 to disengage the manifolds.
3. Slowly move the flow cell lever to the OFF position to disengage the vacuum seal and release the flow cell.
4. Lift the used flow cell from the flow cell holder.
5. Remove the used gaskets and discard them.

Clean the Flow Cell Holder

1. Put on a new pair of powder-free latex gloves.
2. Using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol, carefully wipe the surface of the flow cell holder until it is completely clean.

   CAUTION
   Do not allow alcohol to drip into the vacuum holes or around the manifolds. Use a low-lint lab tissue to dry the stage, if necessary.

3. Visually inspect the flow cell holder to make sure that it is free of lint, and that the vacuum holes are free of obstructions.
4. Let the surface air dry before installing new gaskets.

   NOTE
   Always install new manifold gaskets before loading the clustered flow cell.

5. Position two new gaskets in the slots on the front end and back end of the flow cell holder.

Clean the Flow Cell

1. Rinse the flow cell with laboratory-grade water and dry it with a lens cleaning tissue.
2. Fold an alcohol wipe to approximately the size of the flow cell.
3. Hold the edges of the clustered flow cell with two fingers. Make sure that the inlet and outlet ports are facing *up*.
4. Wipe off each side of the flow cell with a single sweeping motion. Repeat, refolding the alcohol wipe with each pass, until the flow cell is completely clean.
5. Dry the flow cell using a dry lens cleaning tissue.

   CAUTION
   If you clean the flow cell while it is lying on the bench top, you could easily apply too much pressure and break the flow cell. Illumina recommends cleaning the flow cell while holding the edges between your fingers.

6. Protect the flow cell from dust until you are ready to load it onto the instrument.

Load the Clustered Flow Cell

1. Place the flow cell on the flow cell holder with the inlet and outlet ports facing *down* and the barcode on the right. The arrow on the left edge of the flow cell, which indicates flow direction, should point towards the instrument.

   NOTE
   Flow Cell v3 has a mechanically keyed corner, which provides a visual orientation for loading the flow cell. Install Flow Cell v3 so that the keyed corner is on the output end of the flow cell facing towards the instrument, and on the left side of the flow cell by lane 1.

2. Gently slide the flow cell towards the top and right guide pins until it stops.
3. Slowly, move the flow cell lever to position 1. This engages the vacuum and secures the flow cell into position. When the flow cell lever is green, the vacuum is engaged.
   If the flow cell lever remains orange, the vacuum seal is not secure. Remove the flow cell, and inspect the gaskets and vacuum holes. Repeat the cleaning steps, if necessary, and reload the flow cell.
4. Wait for about five seconds, and then slowly move the flow cell lever to position 2. When the flow cell lever is solid green, the manifolds are in position and the flow cell is ready for use.
5. Make sure that the **Vacuum Engaged** checkbox is selected on the load sequencing flow cell screen.

Confirm Proper Flow

After loading the clustered flow cell, check for proper flow using the fluidics check screen. Checking for proper flow confirms that the flow cell and gaskets are properly installed and the manifold is engaged.

1. Select solution 5 (SB2) from the drop-down list.
2. Enter the following default values:
   - Volume: **250**
   - Aspirate Rate: **250**
   - Dispense Rate: **2000**
3. Select **Pump**.
4. Visually inspect the flow cell for bubbles passing through the lanes and leaks near the manifolds.
   If you see excessive bubbles, check the gaskets for obstructions, reduce the aspirate rate to 100, and pump another 250 µl of SB2 to the flow cell. If problems persist, remove the flow cell, change the gaskets, repeat the cleaning steps, and reload the flow cell.
5. After you have confirmed proper flow, select **Next** to proceed. Make sure that the flow cell lever is green and close the flow cell compartment door.
6. Confirm that the **Vacuum Engaged** and **Door Closed** checkboxes are selected, and then select **Next**.
7. Select **Start** to start the sequencing run.

Monitoring the Run

Use the run overview screen to monitor on-instrument analysis, fluidics, and imaging.

- **Progress Bar**—Use the progress bar to monitor how many cycles have been completed.
- **Flow Cell Image**—Use the flow cell image to monitor which lanes have been imaged for that cycle.
- **Analysis Graph**—Use the analysis graph to monitor quality scores by cycle.
- **Images Graph**—Use the images graph to monitor intensities by cycle.
- **Fluidics Graph**—Use the arrow button to expand the fluidics section and monitor chemistry steps in the protocol.

First Base Report

The first base report confirmation dialog box opens automatically after cycle one is complete, if you selected **Confirm First Base** on the scan screen during run setup. The run will pause at this step.

1. Review the first base report from the first base report confirmation dialog box or by opening First_Base_Report.htm from the root level of the run folder.
2. If the results are satisfactory, select **Continue** to continue your run.

Sequencing Analysis Viewer

If you are using HCS v1.3 or later, or installed the Sequencing Analysis Viewer (SAV), you can monitor the status page generated by real time analysis (RTA). The status page provides analysis metrics in plots, graphs, and tables. For more information, see the *Sequencing Analysis Viewer User Guide*.

The Sequencing Analysis Viewer launches automatically after image analysis begins, and opens to the status page. Select **View Data** at any time during the run to view the status page.

Preparing Reagents for Read 2

Prior to the completion of Read 1 and any Index Reads, prepare paired-end reagents for Read 2 resynthesis and fresh ICB for Read 2. Reagents take less than 30 minutes to prepare.

NOTE
For optimal performance, Illumina recommends that you prepare fresh ICB (Incorporation Mix) for Read 2 of a paired-end run.

Prepare Paired-End Reagents

WARNING
This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact.

Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit on www.illumina.com.

Reagents for Nextera Libraries

NOTE
HP7, provided in the TruSeq PE Cluster Kit, is not compatible with Nextera libraries. You must use **HP11** from the TruSeq Dual Index Sequencing Primer Box (PE).

Reagents	Storage	Kit Name
RMX**	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
LMX2	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
BMX	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
AMX2	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
APM2	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
AT2	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
HP3	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
HT2	-15° to -25°C	TruSeq PE Cluster Kit (PE Reagents Box)
HP11	-15° to -25°C	TruSeq Dual Index Sequencing Primer Box (PE)

** You do not need to prepare RMX for dual-indexed paired-end runs. RMX, used during the Index 2 (i5) Read, is not used again during Read 2 resynthesis.

Thaw Reagents

1 Remove the following reagents from -15° to -25°C storage:
   - Non-indexed or single-indexed runs—RMX, LMX2, BMX, AMX2, APM2, AT2, **HP11**, HP3, and HT2
   - Dual-indexed runs—LMX2, BMX, AMX2, APM2, AT2, **HP11**, HP3, and HT2

2 Thaw reagents in a beaker filled with room temperature deionized water for about 20 minutes, or until reagents are fully thawed.

3 After reagents have completely thawed, place RMX, LMX2, BMX, and AMX2 on ice.

Prepare RMX

Prepare RMX for non-indexed or single-indexed paired-end runs.

For dual-indexed runs, you do not need to prepare RMX. It was used during the Index 2 (i5) Read and is not required again for Read 2 resynthesis.

1 Invert the tube of RMX five times to mix the reagent.

2 Label the tube of RMX **Reagent #10**.

3 Briefly centrifuge the reagent at 1,000 rpm for one minute or less. Alternatively, gently tap or flick the tube to make sure that reagent droplets collect at the bottom of the tube.

NOTE
Do not vortex RMX.

4 Set aside on ice.

Prepare LMX2

1 Invert the tube of LMX2 five times to mix the reagent.

2 Label the tube of LMX2 **Reagent #11**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside on ice.

Prepare BMX

1 Invert the tube of BMX five times to mix the reagent.

2 Label the tube of BMX **Reagent #12**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside on ice.

Prepare AMX2

1 Invert the tube of AMX2 five times to mix the reagent.

2 Label the tube of AMX2 **Reagent #13**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside on ice.

Prepare APM2

1 Invert the tube of APM2 five times to mix the reagent.

2 Label the tube of APM2 **Reagent #14**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside at room temperature.

Prepare AT2

1 Invert the tube of AT2 five times to mix the reagent.

2 Label the tube of AT2 **Reagent #15**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside at room temperature.

Prepare HP11

Prepare HP11 for sequencing Nextera libraries.

1 Invert the tube of HP11 five times to mix the reagent.

2 Label the tube of HP11 **Reagent #16**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside at room temperature.

Prepare HP3

1 Invert the tube of HP3 five times to mix the reagent, and then pulse centrifuge.

2 Transfer 2.85 ml of PW1 into a 15 ml Sarstedt conical tube and add 150 µl of HP3.

3 Invert the tube five times to mix the reagent.

4 Label the conical tube of diluted HP3 **Reagent #18**.

5 Centrifuge at 1,000 rpm for one minute.

6 Set aside at room temperature.

Prepare HT2

1 Invert the tube of HT2 five times to mix the reagent.

2 Label the tube of HT2 **Reagent #19**.

3 Centrifuge the reagent at 1,000 rpm for one minute.

4 Set aside at room temperature.

Prepare ICB for Read 2 (200 Cycle Kit)

Use the following instructions for non-indexed or single-indexed runs. For dual indexed runs, prepare ICB using the volumes that you set aside for Read 2. For more information, see *Prepare ICB for Dual-Indexed Runs* on page 13.

1 Add the contents of two tubes of LFN to the bottle containing 47 ml of ICB-200.

2 Rinse each tube of LFN with ICB-200 to make sure that all LFN is transferred.

3 Aliquot 1.1 ml of EDP-200 into the ICB/LFN mix.

4 Return any unused portion of EDP-200 to -15° to -25°C storage.

5 Cap the bottle and invert several times to mix.

6 Set aside on ice until you are ready to load reagents onto the instrument.

Loading Reagents for Read 2

After completion of Read 1 and any Index Reads, the software prompts you to load paired-end reagents for Read 2 resynthesis and freshly-prepared ICB for sequencing Read 2.

Load Paired-End Reagents

When prompted by HCS, load paired-end reagents onto the paired-end reagent rack.

1 Record the weight of each reagent on the lab tracking form.

2 Make sure that the paired-end rack is not in use for Read 2 resynthesis, Index 1 (i7) Read preparation, or Index 2 (i5) Read preparation on the opposite flow cell.

3 Raise the sippers for the paired-end reagent rack and remove the rack.

WARNING

This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact.

For more information, see the MSDS for this kit, at http://www.illumina.com/msds.

4 Place each of the reagent tubes onto the rack in the associated numbered positions indicated in one of the following tables according to run type.

5 Remove the caps from each reagent tube.

6 Slide the reagent rack into the reagent compartment, aligning the rack with the raised guide on the floor of the compartment.

7 Lower the sippers into the paired-end reagent tubes.

8 Close the reagent compartment door and select **Next** to resume the run.

Reagents for Nextera Libraries

Table 5 Paired-End Reagent Positions for Read 2 Resynthesis

Position	Reagent	Description
10	RMX**	Resynthesis Mix
11	LMX2	Linearization Mix 2
12	BMX	Blocking Mix
13	AMX2	Amplification Mix 2
14	APM2	AMX2 Premix
15	AT2	100% Formamide
16	HP11	Read 2 Sequencing Primer
18	HP3	Denaturation Solution
19	HT2	Wash Buffer

** For dual-indexed paired-end runs, RMX is already loaded in position 10.

Load ICB for Read 2

When prompted, load fresh ICB (Incorporation Mix) for Read 2.

1 Record the weight of the reagent on the lab tracking form.

2 Open the reagent compartment door.

3 Raise the sippers for the sequencing reagent rack.

4 Slide the reagent rack out of the reagent compartment using the rack handle.

5 Remove the existing ICB reagent bottle from position 1 of the reagent rack and remove the funnel cap from the bottle.

6 Place the funnel cap on the new bottle of ICB and load the bottle in position 1 on the reagent rack, making sure that the conical end of the bottle rests in the indentation on the base of the rack.

7 Slide the reagent rack into the reagent compartment, aligning the rack with the raised guide on the floor of the compartment.

8 Lower the sippers into the sequencing reagent bottles.

9 Close the reagent compartment door.

10 Select **Next** to resume the run.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 6 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 7 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at http://www.illumina.com/msds.

Product Documentation

You can obtain PDFs of additional product documentation from the Illumina website. Go to http://www.illumina.com/support and select a product. To download documentation, you will be asked to log in to MyIllumina. After you log in, you can view or save the PDF. To register for a MyIllumina account, please visit https://my.illumina.com/Account/Register.

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